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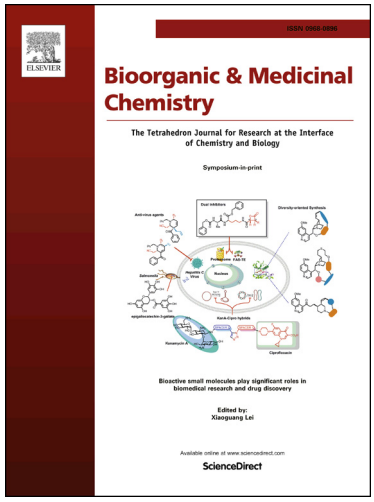

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Development of WNK Signaling Inhibitors as a New Class of Antihypertensive Drugs

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Abstract

Pseudohypoaldosteronism type II (PHAII) is characterized by hyperkalemia and hypertension despite a normal glomerular filtration rate. Abnormal activation of the signal cascade of with-no-lysine kinase (WNK) with OSR1 (oxidative stress-responsive kinase 1)/SPAK (STE20/SPS1-related proline/alanine-rich kinase) and NCC (NaCl cotransporter) results in characteristic salt-sensitive hypertension. Thus, inhibitors of the WNK-OSR1/SPAK-NCC cascade are candidates for a new class of antihypertensive drugs. In this study, we developed novel inhibitors of this signal cascade from the 9-aminoacridine lead compound **1**, one of the hit compounds obtained by screening our chemical library for WNK-SPAK binding inhibitors. Among the synthesized acridine derivatives, several acridine-3-amide and 3-urea derivatives, such as **10** (IC_{50}: 6.9 μM), **13** (IC_{50}: 2.6 μM), and **20** (IC_{50}: 4.8 μM), showed more potent inhibitory activity than the lead compound **1** (IC_{50}: 15.4 μM). Compounds **10** and **20** were confirmed to inhibit phosphorylation of NCC *in vivo*.

Keywords: antihypertensive drug, with-no-lysine kinase (WNK), acridine

1. Introduction

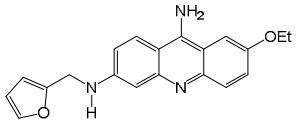
Hypertension is one of the most common diseases, affecting more than one billion people worldwide. Most are categorized as essential hypertension, which means that the origin of the disease is unknown. Increased blood pressure induces a remarkable increase in the risk of fatal illnesses such as heart attack, aneurysm, and stroke owing to the increased pressure on the arterial wall, which accelerates atherosclerosis. On the other hand, the renin-angiotensin-aldosterone system (RAAS) is a hormone system that regulates blood pressure and fluid balance. Under normal conditions, aldosterone production is regulated by the renin-angiotensin system and potassium balance. Renin is released from the renal juxtaglomerular apparatus (JGA) and its production is regulated by three major mechanisms: intrarenal baroreceptors of the afferent arterioles, solute delivery to the macula densa cells, and the effect of sympathetic nerves on the arterioles of the JGA.[1]

Pseudohypoaldosteronism type II (PHAII; also known as Gordon's disease or familial hyperkalemic hypertension) was first reported in 1964.[2] Patients with PHAII exhibit hypertension, severe hyperkalemia, and mild metabolic acidosis. This phenotype is Cl^--dependent and is corrected with low doses of thiazide-type diuretics.[3,4] Subsequent studies showed that PHAII is transmitted in an autosomal-dominant manner.[5] The understanding of PHAII was greatly advanced with the identification of causal mutations in the *WNK1* and *WNK4* genes in some patients with PHAII.[6] With no lysine (WNK) kinases are so named because of the lack of lysine in the ATP-binding cassette of the catalytic region. Specifically, patients with *WNK1* mutations have large intronic deletions that increase the abundance of *WNK1*, and patients with *WNK4* mutations have missense mutations that localize to a short region near putative coiled-coil domains in a highly conserved sequence of WNK.[6]

The role of WNK proteins in the regulation of transporters and channels has been studied. The WNK-oxidative stress-responsive 1 (OSR1) and STE20/SPS1-related proline-alanine-rich protein kinase (SPAK)-NaCl cotransporter (NCC) signal cascade is constitutively activated in PHAII.[7,8] Increased phosphorylation and activation of NCC cause excessive sodium reabsorption in the distal convoluted tubules in the kidneys, resulting in salt-sensitive hypertension. Previously, the Na/K/Cl cotransporter 1 (NKCC1) phosphorylation cascade in vascular smooth muscle cells was also found to be an important regulator of vascular tonus.[9,10] Furthermore, WNK signaling is positively controlled by aldosterone, angiotensin II, and insulin, all of which may contribute to hypertension in patients with hyperaldosteronism and hyperinsulinemia.[11-15] Therefore, drugs that inhibit this signal cascade are expected to help treat hypertension through dual actions (i.e., NaCl diuresis and vasodilation) and may be particularly effective in patients with hyperaldosteronism or hyperinsulinemia.

Both OSR1 and SPAK possess a CCT domain in their C-terminus, which forms a protein fold interacting with the Arg-Phe-Xaa-Val/Ile (RFxV/I) motif through a surface-exposed groove. WNK kinases and SLC12A transporters (NCC/NKCCs) harbor the RFxV/I motif and directly interact with OSR1/SPAK via the CCT domain. WNK signaling activity is known to be dependent on the binding,[16] suggesting that inhibitors of WNK binding to OSR1/SPAK would act as WNK signaling inhibitors. Based on this principle, we previously developed a new high-throughput screening system

that can detect the binding by means of fluorescence correlation spectroscopy (FCS), and identified several hit compounds among a 17,000-compound library.[17] Among them, compound **1** showed the highest inhibitory activity in the FCS assay (Figure 1). On the other hand, compound **2** bearing a 9-aminoacridine skeleton did not show inhibitory activity, which suggested that an appropriate substituent at the 3-position of the acridine ring is necessary for inhibitory activity. Compound **1** effectively inhibited phosphorylation of SPAK and NCC in mpkDCT and that of SPAK and NKCC1 in MOVAS cells, which indicates that it can inhibit the WNK signal cascade *in vivo*. In this study, therefore, we synthesized several acridine derivatives based on **1** as a lead compound in order to clarify the structure-activity relationships of WNK-SPAK-binding inhibitors and to develop specific inhibitors with high potency *in vivo*.



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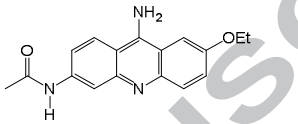


2

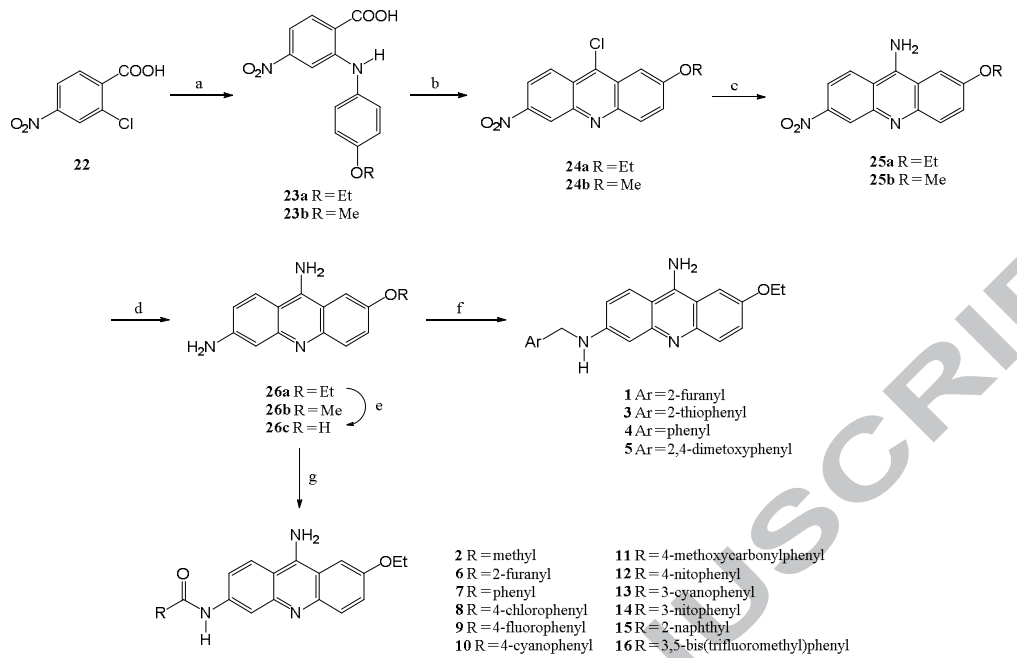
Figure 1. Structures of WNK-SPAK-binding inhibitor hit (**1**) and its derivative (**2**).

2. Results and Discussion

2.1. Synthesis of acridine derivatives

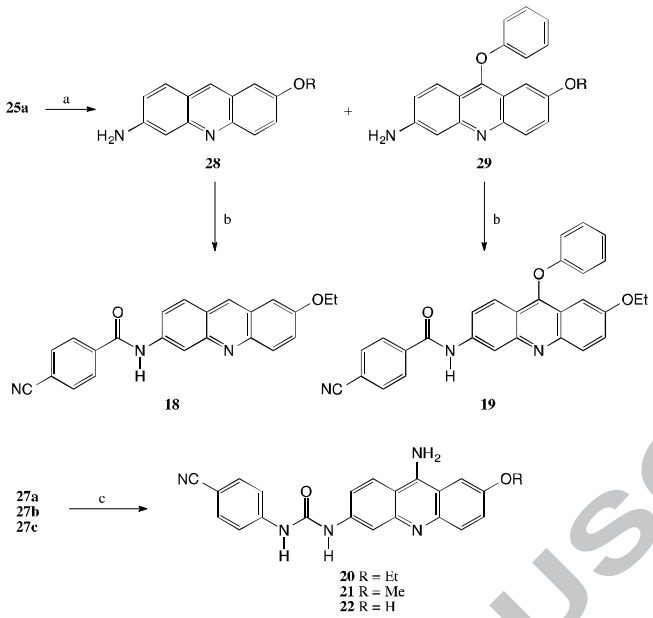
Our previous study showed that the 3-substituent of compound **1** is significant, as the corresponding 3-acetamide derivative **2** did not inhibit WNK-SPAK-binding. In order to clarify the structural requirements of the 3-substituent for WNK-SPAK-binding inhibition, we designed compounds **3** – **5** with a different aromatic group. Further, we considered that the methyleneamino linker at 3-position would be not stable both chemically and metabolically, we designed compounds **6** – **16** with the stable amide bond as the linking group (Scheme 1). Further, several derivatives such as compounds **17** and **18** without the 9-amino group and 3-urea derivatives **19** – **21** with different 7-substituents were synthesized (Scheme 2). Compounds **1** and **2** were also synthesized in order to confirm their structure and activity.

Syntheses of acridine derivatives are illustrated in Schemes 1 and 2. Ullmann reaction of 4-chloro-2-nitrobenzoic acid (**22**) with *p*-alkoxyanilines afforded *N*-phenyl-anthranilic acid derivatives **23**.[18] Cyclization of **23** was carried out using phosphorus oxychloride to give 9-chloroacridine derivatives **24**,[19] which were converted to 9-aminoacridine derivatives **25** by heating to 200°C with ammonium sulfate.[20] Catalytic hydrogenation of the 3-nitro group of **25** in DMF afforded key intermediates **26**. Reductive amination of **26a** with several arylaldehydes afforded the 3-arylmethylaminoasridine derivatives **1** – **4**. Acylation of **26a** with various aromatic acid chlorides afforded amide derivatives **6** – **16**.



Scheme 1. Synthesis of acridine derivertives **1**-**16**. Reagents and conditions: (a) *p*-ethoxyaniline, K_2CO_3, Cu_2O, Cu powder, 2-ethoxyethanol, reflux; (b) $POCl_3$, reflux; (c) $(NH_4)_2SO_4$, phenol, reflux; (d) H_2, Pd/C, DMF; (e) $AlCl_3$, xylene, reflux; (f) ArCHO, AcOH, $NaCNBH_3$, MeOH for **26a**; (g) RCOCl, pyridine, DMF for **26a**.

Phenol was an effective solvent for catalytic hydrogenation of **25**. Treatment of 9-chloro-7-ethoxy-3-nitoroacridine (**24a**) under the same reaction conditions afforded 9-unsubstituted compound **27** (5%) and 9-phenoxy compound **28** (85%). Compounds **27** and **28** were reacted with *p*-cyanobenzoyl chloride to give **17** and **18**, respectively. Derivatives **19** and **20** bearing a urea linkage at the 3-position of the acridine ring were obtained by reaction of **26a** or **26b** with *p*-cyanophenyl isocyanate, respectively. Finally, 7-hydroxy derivative **21** was prepared from **26c**, which was synthesized from **26a** by treatment with aluminum chloride in dry xylene at 100°C.



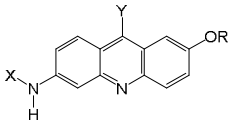
Scheme 2. Synthesis of acridine derivertives **17** - **21**. Reagents and conditions: (a) H_2, Pd/C, phenol; (b) *p*-cyanobenzoyl chloride, pyridine, DMF; (c) *p*-cyanophenyl isocyanate, DMSO.

2.2. Biological activity

The inhibitory activity of the synthesized compounds on WNK-OSR1/SPAK binding was evaluated using the FCS assay that we reported previously (Table 1).[17] The lead compound **1** exhibited moderate inhibitory activity with an IC_{50} value of 15.4 μM, while compound **2** did not show inhibitory activity, as reported previously.[17] Among 3-arylmethylamino derivatives, compound **1** was most potent inhibitor under our assay conditions. Since the arylmethylamino group on the acridine ring is expected to be rather unstable chemically and metabolically, we examined the activity of 3-amide derivatives. Most of the 3-amide derivatives inhibited WNK-OSR1/SPAK binding. This result indicates that the absence of inhibitory activity in compound **2** is due to the lack of the aromatic ring at the amide carbonyl group. Compound **6** with the same aromatic ring (2-furanyl moiety) as lead compound **1** showed more potent activity than **1** (its IC_{50} was 8.2 μM). Replacement of the furan ring with a phenyl ring, yielding compound **7**, decreased the activity, while introduction of a 4-cyano group on the phenyl ring increased the inhibitory activity. Among the 4-substituted benzamide series **8** – **12**, only the cyano group was effective, though a nitro group slightly increased the potency. On the other hand, all 3-monosubstituted benzamide derivatives **13** and **14** showed potent inhibitory activity with IC_{50} values of 2.6, 3.9 and 11.8 μM, respectively. Interestingly, 3,5-disubstituted benzamide derivative **16** showed inhibitory activity only at higher concentrations. Replacement of the 9-amino group of **10** with a hydrogen atom or phenoxy group resulted in loss of activity, indicating that the 9-amino group is important for the activity. Since the amide linkage in the 3-substituent was effective, we examined derivatives with a 3-ureido moiety. Compound **19** with a 4-cyanophenyl group on the ureido bond was insoluble under the assay conditions, so we synthesized compounds **20** and **21** bearing a 7-methoxy or 7-hydroxy group,

respectively. These urea derivatives also showed potent inhibitory activity, with IC_{50} values of 4.8 and 2.6 μM, respectively.

Table 1. Inhibitory activity of acridine derivatives on WNK-OSR1/SPAK-binding



Compound	3-substituent (X-NH-)	7-substituent (R)	9-substituent (Y)	IC_{50} (μM)
1	2-furanylmethylamino	Et	NH_2	15.4
2	acetamido	Et	NH_2	Inactive[a]
3	2-thiophenylmethylamino	Et	NH_2	53.5
4	benzylamino	Et	NH_2	35.6
5	2,4-dimethoxyphenylamino	Et	NH_2	21.1
6	2-furanylcarboxamido	Et	NH_2	8.2
7	benzamido	Et	NH_2	28.2
8	4-chlorobenzamido	Et	NH_2	49
9	4-fluorobenzamido	Et	NH_2	36.9
10	4-cyanobenzamido	Et	NH_2	6.9
11	4-methoxycarbonylbenzamido	Et	NH_2	Inactive[a]
12	4-nitrobenzamido	Et	NH_2	20.9
13	3-cyanobenzamido	Et	NH_2	2.6
14	3-nitrobenzamido	Et	NH_2	3.9
15	2-naphthylcarboxamido	Et	NH_2	7.6
16	3,5-bis(trifluoromethyl)benzamido	Et	NH_2	>100
17	3-cyanobenzamido	Et	H	Inactive[a]
18	3-cyanobenzamido	Et	PhO	Inactive[a]
19	*N*'-(3-cyanophenyl)ureido	Et	NH_2	ND[b]
20	*N*'-(3-cyanophenyl)ureido	Me	NH_2	4.8
21	*N*'-(3-cyanophenyl)ureido	H	NH_2	2.6

[a]Inactive: inactive above the concentration of 10^{-5} M
[b]ND: not determined.

We next examined the cytotoxicity of the selected compounds towards the human embryonic kidney cell line, 293T. The IC_{50} values of the lead compounds **1** and **10** were 4.2 μM and 25 μM, respectively. Mitomycin C was used as a positive control, and its IC_{50} value was in accordance with previously reported values. Compound **20** did not inhibit the growth of 293T even at 100 μM. Compounds **10** and **20** showed significantly lower cytotoxicity than lead compound **1** (Figure 2), indicating that these may be good candidates for the development of specific WNK-SPAK-binding

inhibitors without side effects.

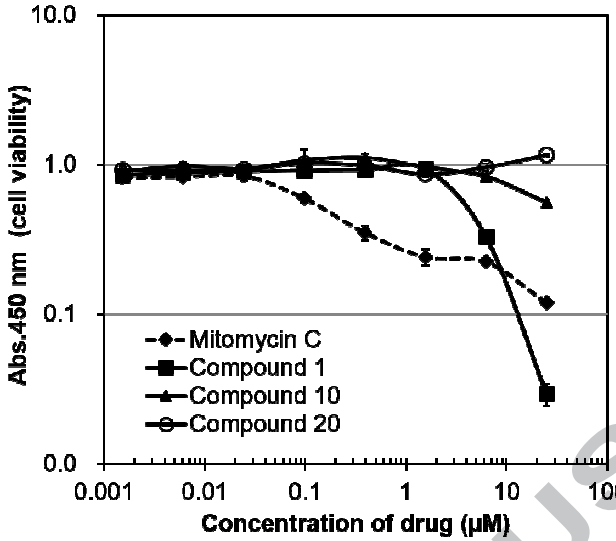


Figure 2. Evaluation of cytotoxicity toward 293-T cells of selected compounds **1** (square), **10** (triangle), **20** (open circle), and the positive control, mitomycin C (diamond and dashed line). All fitted curves were generated by non-linear regression. Bars represent the standard deviation of the mean.

Then, we selected compounds **10** and **20**, taking into account both inhibitory activity and solubility, for examination of the inhibitory activity toward phosphorylation in the WNK signal cascade *in vivo*. Test compounds were intraperitoneally injected into C57BL/6 mice. Ninety minutes after injection of either compound, the level of phosphorylated NCC was markedly decreased in a dose-dependent manner. In addition, phosphorylation is inhibited similarly for NKCC1 / NKCC2 belonging to the same SLC12A3 family and being regulated by the WNK-OSR1 / SPAK signaling. This suggests that these compounds indeed show inhibitory activity *in vivo* as well as *in vitro*. Interestingly, phosphorylation of NCC tends to be inhibited at a lower concentration than NKCC1/2. There are differences in the binding motifs to CCT domain (OSR1 / SPAK) among the SLC12A transporters. NKCC 1/2 possesses RFQV (Arg-Phe-Gln-Val) motif, but NCC has RFTI (Arg-Phe-Thr -Ile) motif. It has been confirmed that the affinity of the RFTI motif to the CCT domain is about 1/10 as compared with that of the RFQV,[17] and thus it is reasonable to be inhibited at a lower concentration. Taken together, this inhibitory effects on the phosphorylation is not due to toxic or non-specific ones, but supports the fact that phosphorylation of SLC12A transporters is actually inhibited via the binding inhibition.

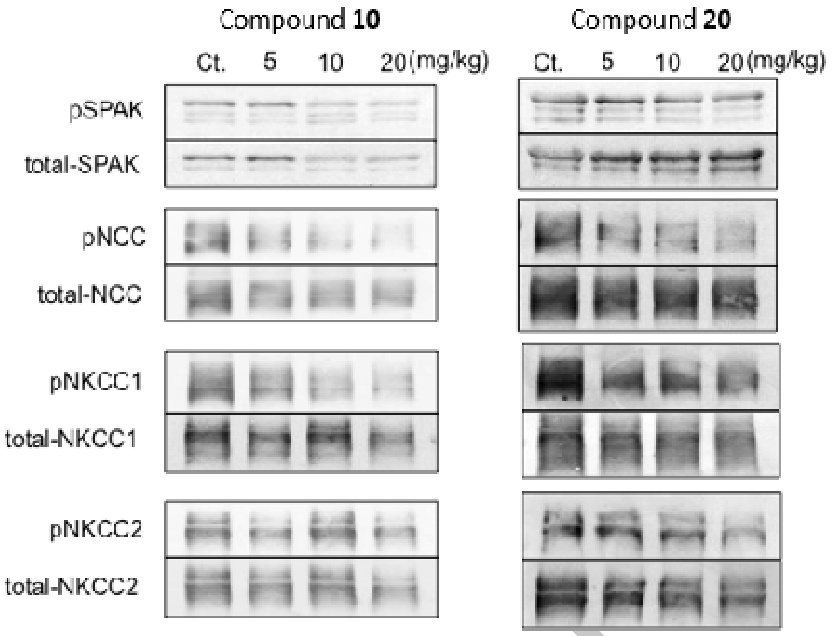


Figure 3. Effect of **10** and **20** on phosphorylation in the WNK signal pathway *in vivo*.

3. Conclusion

In this study, we carried out structural development of our hit compound **1** previously identified from a chemical library by high-throughput screening using fluorescence correlation spectroscopy (FCS), in order to find novel inhibitors of WNK-OSR1/SPAK binding. Several acridine-3-amide and 3-urea derivatives, such as **10**, **13**, and **20**, showed more potent inhibitory activity than the lead compound **1**. Although many of the acridine derivatives showed low solubility in various solvents, we found that compounds **10** and **20** markedly decreased the level of phosphorylated NCC *in vivo* in a dose-dependent manner. NCC plays a critical role in renal NaCl transport and blood pressure maintenance. Increased phosphorylation and activation of NCC causes excessive sodium reabsorption in the distal convoluted tubules in the kidney, which contributes to salt-sensitive hypertension. Thus, compounds **10** and **20** are promising candidates for a new class of antihypertensive drugs. Further studies on the structural optimization, specificity as WNK signaling inhibitor, and *in vivo* activities of these acridine derivatives are on-going.

4. Experimental

4.1. Chemistry

All reagents were purchased from Sigma–Aldrich Chemical Co., Tokyo Kasei Kogyo Co., Wako Pure Chemical Industries, and Kanto Chemical Co., Inc. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AVANCE 400 spectrometer or a Bruker AVANCE 500 spectrometer. Mass spectra were obtained with a Daltonics microTOF-2focus. Column chromatography was done using silica gel 60 spherical or silica gel 60 N spherical (Kanto Chemical Co., Inc.).

4.2. Synthesis

4.2.1. Synthesis of 2-[(4-ethoxyphenyl)amino]-4-nitrobenzoic acid (23a)

Potassium carbonate (2.06 g, 14.9 mmol), copper (I) oxide (85.0 mg, 0.596 mmol), and copper powder (83.0 mg, 1.31 mmol) were added to a solution of 2-chloro-4-nitrobenzoic acid (**22**, 3.00 g, 14.9 mmol) and 4-ethoxyaniline (2.29 ml, 15.6 mmol) in 2-ethoxyethanol under an Ar atmosphere. The mixture was heated at reflux overnight, then cooled to room temperature, and 1 M hydrochloric acid was added dropwise to the mixture. The resulting precipitate was collected, washed with 1 M hydrochloric acid and water, and dried *in vacuo* to yield **23a** (2.80 g, 62%) as an orange powder. ^{1}H-NMR (500 MHz, $CDCl_3$) δ 9.37 (1 H, s), 8.13 (1 H, d, *J* = 8.8 Hz), 7.70 (1 H, d, *J* = 2.2 Hz), 7.40 (1 H, dd, *J* = 2.2, 8.8 Hz), 7.17 (2 H, m), 6.96 (2 H, m), 4.07 (2 H, q, *J* = 7.0 Hz), 1.45 (3 H, t, *J* = 7.0 Hz).

2-[(4-Methoxyphenyl)amino]-4-nitrobenzoic acid (**23b**) was prepared similarly from **22** and 4-methoxyaniline (78%). ^{1}H-NMR (500 MHz, CD_3OD) δ 8.14 (1 H, d, *J* = 8.7 Hz), 7.66 (1 H, d, *J* = 2.2 Hz), 7.41 (1 H, d, *J* = 2.3, 8.7 Hz), 7.23-7.21 (2 H, m), 7.04-7.01 (2 H, m), 3.84 (3 H, s).

4.2.2. Synthesis of 9-chloro-2-ethoxy-6-nitroacridine (24a)

23a (2.00 g, 6.62 mmol) was added to phosphoryl chloride (15 mL) under an Ar atmosphere. The reaction mixture was heated at reflux overnight, then cooled to room temperature, poured into a mixture of aqueous ammonia (100 mL), crushed ice (200 g), and methylene chloride (200 mL), and extracted with methylene chloride. The organic layer was washed with water, dried over sodium sulfate, and concentrated *in vacuo*. The residue was purified by recrystallization from chloroform to yield **24a** (1.76 g, 88%) as yellow needles. ^{1}H-NMR (500 MHz, $CDCl_3$) δ 9.15 (1 H, d, *J* = 2.2 Hz), 8.53 (1 H, d. *J* = 9.5 Hz), 8.35 (1 H, dd, *J* = 2.2, 9.5 Hz), 8.17 (1 H, d, *J* = 9.4 Hz), 7.58 (1 H, dd, *J* = 2.6, 9.4 Hz), 7.53 (1 H, d, *J* = 2.6 Hz), 4.30 (2 H, q, *J* = 7.0 Hz), 1.57 (3 H, t, *J* = 7.0 Hz).

9-Chloro-2-methoxy-6-nitroacridine (**24b**) was prepared similarly from **23b** (53%). ^{1}H-NMR (400 MHz, CD_3OD) δ 9.06 (1 H, d, *J* = 2.3 Hz), 8.66 (1 H, dd, *J* = 0.3, 9.3 Hz), 8.43 (1 H, dd, *J* = 2.3, 9.5 Hz), 8.18 (1 H, dd, *J* = 0.9, 9.1 Hz), 7.70-7.69 (2 H, m), 4.10 (3 H, s).

4.2.3. Synthesis of 9-amino-2-ethoxy-6-nitroacridine (25a)

Ammonium sulfate (0.990 g, 7.55 mmol) was added to a solution of **24b** (1.14 g, 3.77 mmol) in phenol (38 mL) under an Ar atmosphere. The reaction mixture was heated at reflux for 4 h, then cooled to room temperature, and methylene chloride (150 mL) and 1 M sodium hydroxide (50 mL) were added to the mixture. The precipitate was collected by filtration, washed with 1 M sodium hydroxide and water, and dried *in vacuo* to yield **25a** (1.02 g, 95%) as a red powder. ^{1}H-NMR (400 MHz, DMSO-d_6) δ 8.62 (1 H, d, J = 2.3 Hz), 8.59 (1 H, d, J = 9.4 Hz), 7.96 (1 H, dd, J = 2.4, 9.5 Hz), 7.89 (2 H, s), 7.86 (1 H, d, J = 9.4 Hz), 7.72 (1 H, d, J = 2.4 Hz), 7.45 (1 H, dd, J = 2.6, 9.3 Hz), 4.22 (2 H, q, J = 7.0 Hz), 1.44 (3 H, t, J = 7.0 Hz).

9-Amino-2-ethoxy-6-nitroacridine (**25b**) was prepared similarly from **24b** (quant). ^{1}H-NMR (400 MHz, CD_3OD) δ 8.75 (1 H, d, J = 2.3 Hz), 8.47 (1 H, d, J = 9.8 Hz), 8.03 (1 H, dd, J = 2.5, 9.5 Hz), 7.88 (1 H, d, J = 9.4 Hz), 7.60 (1 H, d, J = 2.6 Hz), 7.48 (1 H, dd, J = 2.8, 9.4 Hz), 4.00 (3 H, s).

4.2.4. Synthesis of 6,9-diamino-2-ethoxyacridine (26a)

25a (1.05 g, 3.72 mmol) was hydrogenated in DMF (18.5 mL) with 10% palladium on carbon (100 mg) under an H_2 atmosphere overnight. Water was added to the mixture and the solution was filtered through Celite. Brine was added to the filtrate. The resulting yellow precipitate was collected by filtration, and dried *in vacuo* to yield **26a** (726 mg, 77%) as a yellow powder, which was purified by recrystallization from DMF. ^{1}H-NMR (400 MHz, DMSO-d_6) δ 8.15 (1 H, d, J = 9.3 Hz), 7.74 (1 H, d, J = 2.5 Hz), 7.62 (1 H, d, J = 9.2 Hz), 7.53 (1 H, dd, J = 2.6, 9.2 Hz), 6.95 (1 H, dd, J = 2.2, 9.3 Hz), 6.66 (1 H, d, J = 2.1 Hz), 4.21 (2 H, q, J = 7.0 Hz), 1.50 (3 H, t, J = 7.0 Hz).

6,9-Diamino-2-methoxyacridine (**26b**) was prepared similarly from **25b** (quant). ^{1}H-NMR (400 MHz, CD_3OD) δ 8.14 (1 H, d, J = 9.3 Hz), 7.74 (1 H, d, J = 2.5 Hz), 7.60 (1 H, d, J = 9.3 Hz), 7.52 (1 H, dd, J = 2.6, 9.2 Hz), 6.94 (1 H, dd, J = 2.2, 9.3 Hz), 6.63 (1 H, d, J = 2.2 Hz), 3.96 (1 H, s).

4.2.5. Synthesis of 6,9-diamino-2-hydroxyacridine (26c)

Aluminum chloride (341 mg, 2.56 mmol) was added to a solution of **26a** (50.0 mg, 0.198 mmol) in dry xylene (4 mL), and the mixture was heated under reflux. The yellow mixture turned red, then black. After 1 h, the solution was diluted with cold water (2 mL). The yellow precipitate was collected by filtration, and washed with water and ether to yield **26c** (27.0 mg, 60%) as a yellow powder. ^{1}H-NMR (400 MHz, CD_3OD) δ 8.10 (1 H, d, J = 9.3 Hz), 7.58 (1 H, d, J = 2.2 Hz), 7.44 (1 H, dd, J = 0.3, 9.0 Hz), 7.43 (1 H, dd, J = 2.6, 9.2 Hz), 6.90 (1 H, dd, J = 2.2, 9.3 Hz), 6.61 (1 H, d, J = 2.2 Hz).

4.2.6. Typical procedure for 3-arylmethylamino derivatives: Synthesis of 9-amino-2-ethoxy-6-(2-furanylmethylamino)acridine (1)

Acetic acid (1.32 mL), 2-furaldehyde (0.260 mL, 3.19 mmol) and sodium cyanoborohydride (252 mg, 4.01 mmol) were added to a solution of **26a** (200 mg, 0.790 mmol) in methanol (4 mL) under an Ar atmosphere. The mixture was stirred at 50°C overnight, then poured into cold water, and extracted with ethyl acetate. The organic layer was washed with water and brine, dried over sodium sulfate, and evaporated. Water was added to the residue, and the resulting yellow precipitate was collected by filtration, washed with water and dried *in vacuo* to yield **1** (202 mg, 77%) as a yellow powder, which was purified by recrystallization from hexane / ethanol. Mp 210°C (dec); ^{1}H-NMR (500 MHz, CD_3OD) δ 8.09 (1 H, d, *J* = 9.4 Hz), 7.65 (1 H, d, *J* = 2.5 Hz), 7.57 (1 H, d, *J* = 9.2 Hz), 7.48 (1 H, dd, *J* = 2.6, 9.2 Hz), 7.47 (1 H, m), 6.94 (1 H, dd, *J* = 2.3, 9.4 Hz), 6.57 (1 H, d, *J* = 2.2 Hz), 6.38 (2 H, m), 4.47 (2 H, s), 4.17 (2 H, q, *J* =7.0 Hz), 1.47 (3 H, t, *J* = 7.0 Hz); ^{13}C-NMR (125 MHz, CD_3OD) δ 156.8, 156.2, 155.2, 153.0, 143.7, 143.7, 136.0, 127.9, 126.1, 120.7, 117.7, 113.8, 111.6, 108.8, 105.1, 104.3, 93.4, 65.5, 41.0, 15.2; HRMS (ESI^+) calcd for $C_{20}H_{20}N_3O_2$ $(M+H)^+$ 334.1550. found 334.1541.

Compounds **3** – **5** were prepared similarly from **26a**.

9-Amino-2-ethoxy-6-(2-thiophenylmethylamino)acridine (**3**): 68%; ^{1}H-NMR (400 MHz, CD_3OD) δ 8.13 (1 H, d, *J* = 9.4 Hz), 7.71 (1 H, d, *J* = 2.4 Hz), 7.58 (1 H, d, *J* = 9.2 Hz), 7.51 (1 H, dd, *J* = 2.4, 9.2 Hz), 7.31 (1 H, dd, *J* = 1.2, 5.1 Hz), 7.13 (1 H, dd, *J* = 1.0, 3.4 Hz), 7.00 (2 H, m), 6.57 (1 H, d, *J* = 2.4 Hz), 4.67 (2 H, s), 4.13 (2 H, q, *J* = 7.0 Hz), 1.46 (3 H, t, *J* = 7.0 Hz); ^{13}C-NMR (125 MHz, CD_3OD) δ 156.7, 156.0, 155.0, 143.5, 143.0, 135.9, 128.1, 127.8, 126.8, 126.1, 126.0, 120.6, 117.7, 113.7, 105.1, 104.2, 93.6, 65.5, 43.1, 15.2; HRMS (ESI^+) calcd for $C_{20}H_{20}N_3OS$ $(M+H)^+$ 350.1322. found 350.1317.

9-Amino-2-ethoxy-6-(2-phenylmethylamino)acridine (**4**): 44%; Mp 230°C (dec); ^{1}H-NMR (400 MHz, CD_3OD) δ 8.12 (1 H, d, *J* = 9.4 Hz), 7.70 (1 H, d, *J* = 2.4 Hz), 7.54 (1 H, d, *J* = 10.2 Hz), 7.49 (1 H, dd, *J* = 2.4, 10.2 Hz), 7.41 (2 H, d, *J* = 7.2 Hz), 7.37 (2 H, t, *J* = 7.2 Hz), 7.27 (1 H, t, *J* = 7.2 Hz), 7.00 (1 H, dd, *J* = 2.4, 10.2 Hz), 6.44 (1 H, d, *J* = 2.4 Hz), 4.45 (2 H, s), 4.07 (2 H, q, *J* = 7.0 Hz), 1.44 (3 H, t, *J* = 7.0 Hz); ^{13}C-NMR (125 MHz, CD_3OD) δ 156.8, 156.1, 155.6, 143.8, 139.5, 136.0, 129.9, 128.6, 127.8, 126.1, 120.6, 117.8, 113.9, 105.0, 104.4, 93.4, 65.6, 47.9, 15.2; HRMS (ESI^+) calcd for $C_{22}H_{22}N_3O$ $(M+H)^+$ 344.1757. found 344.1748.

9-Amino-6-[(2,4-dimethoxyphenyl)methylamino]-2-ethoxyacridine (**5**): 30%; ^{1}H-NMR (500 MHz, CD_3OD) δ 8.05 (1 H, d, *J* = 9.4 Hz), 7.63 (1 H, d, *J* = 2.5 Hz), 7.51 (1 H, d, *J* = 9.2 Hz), 7.44 (1 H, dd, *J* = 2.5, 9.2 Hz), 7.18 (1 H, d, *J* = 8.4 Hz), 6.95 (1 H, dd, *J* = 2.2, 9.4 Hz), 6.58 (1 H, d, *J* = 2.4 Hz), 6.47 (1 H, dd, *J* = 2.4, 8.3 Hz), 6.39 (1 H, d, *J* = 2.0 Hz), 4.38 (2 H, s), 4.15 (2 H, q, *J* = 7.0 Hz), 3.87 (3 H, s), 3.77 (3 H, s), 1.47 (3 H, t, *J* = 7.0 Hz); ^{13}C-NMR (125 MHz, CD_3OD) δ 162.3, 160.0, 156.7, 155.9, 155.7, 143.9, 135.9, 130.3, 127.7, 125.9, 120.6, 118.9, 117.9, 113.8, 105.5, 104.8, 104.4, 99.6, 93.0, 65.5, 56.1, 56.0, 42.9, 15.2; HRMS (ESI^+) calcd for $C_{24}H_{26}N_3O_3$ $(M+H)^+$ 404.1969.

found 404.1959.

4.2.7. Typical procedure for 3-amide derivatives: Synthesis of *N*-(9-amino-2-ethoxyacridin-6-yl)benzamide (7)

Benzoyl chloride (50.0 μL, 0.594 mmol) was added to a mixture of **26a** (100 mg, 0.396 mmol) and pyridine (3 drops) in DMF (4 mL) under an Ar atmosphere. The mixture was stirred at room temperature overnight, and then poured into water (20 mL). The resulting yellow precipitate was collected by filtration, washed with hot water and ethyl acetate, and dried *in vacuo* to yield **7** (62.2 mg, 42%) as a yellow powder, which was purified by recrystallization from DMSO / methylene chloride. Mp 279°C (dec); ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.5 (1 H, s), 8.37 (1 H, s), 8.33 (1 H, d, J = 9.3 Hz), 8.01 (2 H, m), 7.73 (1 H, d, J = 9.3 Hz), 7.68-7.55 (7 H, m), 7.35 (1 H, 9.2 Hz), 4.18 (2 H, q, J = 7.0 Hz), 1.43 (3H, t, J = 7.0 Hz); ^{13}C-NMR (125 MHz, DMSO-d_6) δ 166.0, 153.4, 148.5, 147.5, 144.8, 139.8, 135.0, 131.7, 129.4, 128.5, 127.8, 124.0, 123.4, 117.3, 115.5, 112.9, 109.6, 101.3, 63.6, 14.7; HRMS (ESI$^+$) calcd for $C_{22}H_{20}N_3O_2$ (M+H)$^+$ 358.1550. found 358.1552.

Compounds **2**, **6**, **8** – **16** were prepared similarly from **26a**.

N-(9-Amino-2-ethoxyacridin-6-yl)acetamide (**2**): ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.8 (1 H, s), 9.64 (2 H, br d), 8.58 (1 H, d, J = 9.4 Hz), 8.50 (1 H, d, J = 1.8 Hz), 8.01 (1 H, d, J = 2.5 Hz), 7.80 (1 H, d, J = 9.3 Hz), 7.62 (1 H, dd, J = 2.5, 9.3 Hz), 7.48 (1 H, dd, J = 2.0, 9.3 Hz), 4.19 (2 H, q, J = 7.0 Hz), 1.48 (3 H, t, J = 7.0 Hz); ^{13}C-NMR (125 MHz, DMSO-d_6) δ 170.0, 155.4, 154.8, 144.5, 140.1, 134.8, 127.4, 125.5, 120.4, 116.9, 112.3, 107.0, 104.2, 103.8, 64.2, 24.4, 14.5; HRMS (ESI$^+$) calcd for $C_{17}H_{18}N_3O_2$ (M+H)$^+$ 296.1394. found 296.1389.

N-(9-Amino-2-ethoxyacridin-6-yl)furan-2-carboxamide (**6**): ^{1}H-NMR (500 MHz, DMSO-d_6) δ10.9 (1 H, s), 9.63 (2 H, s), 8.63 (1 H, s), 8.62 (1 H, d, J = 1.7 Hz), 8.03 (1 H, dd, J = 1.6, 6.7 Hz), 8.02 (1 H, d, J = 2.0 Hz), 7.83 (1 H, d, J = 9.2 Hz), 7.81 (1 H, dd, J = 2.0, 9.4 Hz), 7.64 (1 H, dd, J = 2.3, 9.3 Hz), 7.60 (1 H, d, J = 3.5 Hz), 6.77 (1 H, dd, J = 1.7, 3.6 Hz), 4.21 (2 H, q, J = 7.0 Hz), 1.42 (3 H, t, J = 7.0 Hz); ^{13}C-NMR (125 MHz, DMSO-d_6) δ 156.9, 155.2, 154.8, 146.8, 144.1, 140.2, 140.1, 135.2, 127.4, 125.3, 120.7, 117.7, 116.3, 112.5, 112.4, 107.4, 105.9, 103.7, 64.2, 14.5; HRMS (ESI$^+$) calcd for $C_{20}H_{18}N_3O_3$ (M+H)$^+$ 348.1343. found 348.1347.

N-(9-Amino-2-ethoxyacridin-6-yl)-4-chlorobenzamide (**8**): Mp >300°C; ^{1}H-NMR (400 MHz, DMSO-d_6) δ11.0 (1 H, s), 9.55 (2 H, s), 8.64 (1 H, s), 8.61 (1 H, d, J = 9.4 Hz), 8.08 (2 H, d, J = 8.6 Hz), 8.00 (1 H, d, J = 2.1 Hz), 7.84 (1 H, d, J = 8.1 Hz), 7.78 (1 H, d, J = 9.1 Hz), 7.66 (2 H, d, J = 8.6 z), 7.61 (1 H, dd, J = 2.2, 9.3 Hz), 4.20 (2 H, q, J = 7.0 Hz), 1.42 (3 H, t, J = 7.0 Hz); ^{13}C-NMR (125 MHz, DMSO-d_6) δ 165.6, 155.0, 154.7, 143.8, 140.8, 137.1, 133.0, 131.4, 130.0, 128.6, 127.1, 125.1, 122.8, 117.7, 112.4, 107.7, 103.4, 69.8, 14.5; HRMS (ESI$^+$) calcd for $C_{22}H_{19}ClN_3O_2$ (M+H)$^+$ 392.1160. found 392.1160.

N-(9-Amino-2-ethoxyacridin-6-yl)-4-fluorobenzamide (**9**): Mp >300°C; ^{1}H-NMR (500 MHz, DMSO-d_6) δ 10.8 (1 H, s), 9.05 (2 H, s), 8.59 (1 H, s), 8.52 (1 H, d, *J* = 9.2 Hz), 8.11 (2 H, m), 7.90 (1 H, s), 7.80 (1 H, d, *J* = 9.2 Hz), 7.73 (1 H, dd, *J* = 1.6, 9.3 Hz), 7.57 (1 H, d, *J* = 8.7 Hz), 7.43 (2 H, m), 4.20 (2 H, q, *J* = 7.0 Hz), 1.43 (3 H, t, *J* = 7.0 Hz); ^{13}C-NMR (125 MHz, DMSO-d_6) δ 165.5, 164.3 (d, J_{CF} = 274 Hz), 163.4, 154.6, 130.9, 130.8 (d, J_{CF} = 9.6 Hz), 124.8, 117.7, 115.6 (d, J_{CF} = 21.9 Hz), 112.5, 109.3, 107.9, 103.1, 64.1, 14.5; HRMS (ESI$^+$) calcd for $C_{22}H_{19}FN_3O_2$ $(M+H)^+$ 376.1456. found 376.1453.

N-(9-Amino-2-ethoxyacridin-6-yl)-4-cyanobenzamide (**10**): ^{1}H-NMR (400 MHz, CD_3OD) δ 8.73 (1 H , d, *J* = 1.9 Hz), 8.46 (1 H, d, *J* = 9.4 Hz), 8.15 (2 H, m), 7.95 (2 H, m), 7.81 (1 H, d, 2.5 Hz), 7.78 (1 H, d, *J* = 9.3 Hz), 7.70 (1 H, dd, *J* = 2.0, 9.3 Hz), 7.64 (1 H, dd, *J* = 2.6, 9.3 Hz), 4.24 (2 H, q, *J* = 7.0 Hz), 1.51 (3 H, t, *J* = 7.0 Hz) ; ^{13}C-NMR (125 MHz, DMSO-d_6) δ 165.4, 155.2, 154.8, 143.8, 138.3, 132.6, 128.8, 127.5, 125.3, 120.4, 118.2, 117.7, 114.4, 112.4, 107.7, 106.2, 103.5, 64.2, 14.5; HRMS (ESI$^+$) calcd for $C_{23}H_{19}N_4O_2$ $(M+H)^+$ 383.1503. found 383.1496.

N-(9-Amino-2-ethoxyacridin-6-yl)-4-(methoxycarbobyl)benzamide (**11**): ^{1}H-NMR (500 MHz, DMSO-d_6) δ 8.71 (1 H, d, *J* = 1.3 Hz), 8.60 (1 H, d, *J* = 9.4 Hz), 8.15 (4 H, br s), 7.99 (1 H, d, *J* = 1.8 Hz), 7.82 (1 H, d, *J* = 9.2 Hz), 7.76 (1 H, dd, *J* = 1.9, 9.3 Hz), 7.68 (1 H, dd, *J* = 2.3, 9.1 Hz), 4.22 (2 H, q, *J* = 7.0 Hz), 3.97 (3 H, s), 1.44 (3 H, t, *J* = 7.0 Hz); ^{13}C-NMR (125 MHz, DMSO-d_6) δ 166.0, 165.6, 155.4, 154.9, 144.2, 140.0, 138.3, 134.8, 132.6, 129.3, 128.4, 127.6, 125.3, 120.6, 117.8, 112.4, 107.5, 105.9, 103.6, 64.2, 52.6, 14.5; HRMS (ESI$^+$) calcd for $C_{24}H_{22}N_3O_4$ $(M+H)^+$ 416.1605. found 416.1617.

N-(9-Amino-2-ethoxyacridin-6-yl)-4-nitorobenzamide (**12**): Mp 187°C (dec); ^{1}H-NMR (500 MHz, DMSO-d_6) δ 10.8 (1 H, s), 8.41 (2 H, d, *J* = 7.2 Hz), 8.37 (1 H, d, *J* = 1.7 Hz), 8.34 (1 H, d, *J* = 9.3 Hz), 8.25 (2 H, d, *J* = 7.2 Hz), 7.74 (1 H, d, *J* = 9.3 Hz), 7.64 (2 H, m), 7.56 (2 H, br s), 7.34 (1 H, dd, *J* = 2.6, 9.3 Hz), 4.18 (2 H, q, *J* = 7.0 Hz), 1.43 (3 H, t, *J* = 7.0 Hz); ^{13}C-NMR (125 MHz, DMSO-d_6) δ 163.3, 154.4, 149.2, 148.4, 147.6, 145.3, 140.7, 139.2, 129.9, 129.4, 123.9, 123.6, 123.6, 117.2, 117.1, 116.4, 112.9, 109.9, 101.1, 63.6, 14.7; HRMS (ESI$^+$) calcd for $C_{22}H_{19}N_4O_4$ $(M+H)^+$ 403.1401. found 403.1393.

N-(9-Amino-2-ethoxyacridin-6-yl)-3-cyanobenzamide (**13**): ^{1}H-NMR (500 MHz, DMSO-d_6) δ 11.1 (1 H, s), 9.58 (2 H, s), 8.64 (1 H, d, *J* = 1.5 Hz), 8.61 (1 H, d, *J* = 9.3 Hz), 8.49 (1 H, s), 8.32 (1 H, d, *J* = 8.0 Hz), 8.12 (1 H, d, 7.7 Hz), 7.98 (1 H, d, *J* = 2.2 Hz), 7.83 (1 H, d, *J* = 9.4 Hz), 7.79 (1 H, d, *J* = 7.8 Hz), 7.75 (1 H, dd, *J* = 1.8, 9.3 Hz), 7.63 (1 H, dd, *J* = 2.4, 9.3 Hz), 4.20 (2 H, q, *J* = 7.0Hz), 1.42 (3 H, t, *J* = 7.0 Hz); ^{13}C-NMR (125 MHz, DMSO-d_6) δ 164.9, 155.2, 154.8, 143.9, 140.1, 135.6,

135.3, 132.9, 131.7, 130.0, 127.4, 125.3, 120.9, 118.3, 117.7, 112.4, 111.7, 107.6, 106.4, 103.7, 103.6, 64.2, 14.5; HRMS (ESI^+) calcd for $C_{23}H_{19}N_4O_2$ $(M+H)^+$ 383.1503. found 383.1492.

N-(9-Amino-2-ethoxyacridin-6-yl)-3-nitrobenzamide (**14**): Mp >300°C; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 11.2 (1 H, s), 9.61 (2 H, s), 8.82 (1 H, t, *J* = 1.9, 2.0 Hz), 8.62 (2 H, m), 8.47 (2 H, m), 7.97 (1 H, d, *J* = 2.4 Hz), 7.88 (1 H, d, *J* = 8.0, 8.0 Hz), 7.83 (1 H, d, *J* = 9.3 Hz), 7.78 (1 H, dd, *J* = 1.9, 9.3 Hz), 7.61 (1 H, dd, *J* = 2.5, 9.2 Hz), 4.19 (2 H, q, *J* = 7.0 Hz), 1.41 (3 H, t, *J* = 7.0 Hz); ^{13}C-NMR (125 MHz, DMSO-d_6) δ 164.7, 155.4, 155.0, 147.9, 144.0, 140.1, 135.7, 135.2, 134.6, 130.5, 127.6, 126.9, 125.5, 122.9, 120.8, 117.9, 112.5, 107.7, 106.4, 103.7, 64.4, 14.6; HRMS (ESI^+) calcd for $C_{22}H_{19}N_4O_4$ $(M+H)^+$ 403.1401. found 403.1398.

N-(9-Amino-2-ethoxyacridin-6-yl)naphthalene-2-carboxamide (**15**): Mp >300°C; ^{1}H-NMR (500 MHz, DMSO-d_6) δ 11.1 (1 H, s), 9.48 (2 H, s), 8.73 (1 H, s), 8.70 (1 H, s), 8.61 (1 H, d, *J* = 9.2 Hz), 8.16-8.03 (4 H, m), 7.99 (1 H, s), 7.83 (2 H, m), 7.71-7.63 (3 H, m), 4.21 (2 H, q, *J* = 7.0 Hz), 1.43 (3 H, t, *J* = 7.0 Hz); ^{13}C-NMR (125 MHz, DMSO-d_6) δ 166.8, 154.9, 154.7, 144.2, 140.8, 134.5, 132.0, 131.6, 129.3, 129.1, 128.6, 128.3, 127.8, 127.2, 127.1, 125.2, 125.2, 124.5, 121.2, 117.8, 112.4, 107.6, 106.4, 103.5, 64.2, 14.5; HRMS (ESI^+) calcd for $C_{26}H_{22}N_3O_2$ $(M+H)^+$ 408.1707. found 408.1696.

N-(9-Amino-2-ethoxyacridin-6-yl)-3,5-bis(trifluoromethyl)benzamide (**16**): Mp >300°C; ^{1}H-NMR (500 MHz, DMSO-d_6) δ 8.67 (3 H, m), 8.63 (1 H, d, *J* = 9.3 Hz), 8.45 (1 H, s), 8.00 (1 H, d, *J* = 2.5 Hz), 7.84 (1 H, d, *J* = 9.3 Hz), 7.75 (1 H, dd, *J* = 1.8, 9.3 Hz), 7.68 (1 H, dd, *J* = 2.5, 8.8 Hz), 4.21 (2 H, q, *J* = 7.0 Hz), 1.43 (3 H, t, *J* = 7.0 Hz); ^{13}C-NMR (125 MHz, DMSO-d_6) δ 163.9, 155.5, 154.9, 143.8, 139.9, 136.6, 135.0, 130.6 (q, *J* = 32.5 Hz), 129.0, 127.7, 125.8, 125.4, 125.0 (q, *J* = 271 Hz), 120.6, 117.8, 112.4, 107.7, 106.3, 103.6, 64.2, 14.5; HRMS (ESI^+) calcd for $C_{24}H_{18}F_6N_3O_2$ $(M+H)^+$ 494.1298. found 494.1300.

4.2.8. Synthesis of 6-amino-2-ethoxyacridine (27) and 6-amino-2-ethoxy-9-phenoxyacridine (28)

24a (100 mg, 0.331 mmol) was hydrogenated in phenol (4 mL) with 10% palladium on carbon (10.0 mg) under an H_2 atmosphere overnight. Then, 1 M sodium hydroxide was added, and the mixture was extracted with ethyl acetate. The organic layer was washed with water, dried over sodium sulfate, and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (eluent; methylene chloride: methanol 7 : 1 to methylene chloride : methanol : aqueous ammonia 175 : 25 : 1) to give **27** (4.0 mg, 5%) as a brown powder and **28** (95 mg, 85%) as a yellow powder.

27: ^{1}H-NMR (500 MHz, CD_3OD) δ 8.78 (1 H, s), 7.88 (1 H, d, *J* = 9.5 Hz), 7.85 (1 H, d, *J* = 9.4Hz), 7.52 (1 H, d, *J* = 9.5 Hz), 7.39 (1 H, s), 7.18 (1 H, dd, *J* = 1.8, 9.1 Hz), 6.95 (1 H, s), 4.20 (2 H, q, *J* = 7.0 Hz), 1.49 (3 H, t, *J* = 7.0 Hz).

28: ^{1}H-NMR (500 MHz, CD_3OD) δ 7.86 (1 H, d, *J* = 9.2 Hz), 7.75 (1 H, d, *J* = 9.2 Hz), 7.38 (1 H, dd,

J = 2.8, 9.4 Hz), 7.26 (2 H, m), 7.08-6.98 (4 H, m), 6.87 (2 H, d, *J* = 8.8 Hz), 3.89 (2 H, q, *J* = 7.2 Hz), 1.31 (3 H, t, *J* = 7.2 Hz); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 159.2, 155.3, 153.3, 150.7, 148.1, 147.4, 130.4, 129.8, 125.5, 123.6, 122.3, 120.2, 119.3, 115.5, 115.4, 107.0, 98.9, 63.6, 14.5; HRMS (ESI$^+$) calcd for $C_{21}H_{19}N_2O_2$ $(M+H)^+$ 331.1441. found 331.1442.

4.2.9. 4-Cyano-*N*-(2-ethoxyacridin-6-yl)benzamide (17)

4-Cyanobenzoyl chloride (33.4 mg, 0.202 mmol) was added to a mixture of **27** (24.0 mg, 0.101 mmol) and pyridine (3 drops) in DMF (4 mL) under an Ar atmosphere. The mixture was stirred at room temperature overnight, and then poured into water (10 mL). The resulting yellow precipitate was collected by filtration, washed with hot water and ethyl acetate, and dried *in vacuo* to yield **17** (5.5 mg, 81%) as a brown powder, which was purified by recrystallization from methanol / ethyl acetate. ^{1}H-NMR (400 MHz, CD_3OD) δ 8.79 (1 H, s), 8.69 (1 H, d, *J* = 1.9 Hz), 8.14 (2 H, d, *J* = 8.2 Hz), 8.05 (1 H, d, *J* = 9.1 Hz), 8.02 (1 H, d, *J* = 9.4 Hz), 7.92 (2 H, d, *J* = 8.2 Hz), 7.85 (1 H, dd, *J* = 2.1, 9.1 Hz), 7.50 (1 H, dd, *J* = 2.8, 9.4 Hz), 7.35 (1 H, d, *J* = 2.7 Hz), 4.23 (2 H, q, *J* = 7.0 Hz), 1.50 (3 H, t, *J* = 7.0 Hz); ^{13}C-NMR (125 MHz, DMSO-d_6) δ 164.8, 155.4, 147.4, 145.9, 139.2, 138.8, 133.4, 132.5, 130.3, 128.7, 128.4, 126.6, 125.5, 123.8, 121.6, 118.3, 116.3, 114.0, 104.5, 63.5, 14.6; HRMS (ESI$^+$) calcd for $C_{23}H_{18}N_3O_2$ $(M+H)^+$ 368.1394. found 368.1392.

4-Cyano-*N*-(2-ethoxy-9-phenoxyacridin-6-yl)benzamide (**18**) was prepared similarly from **28** in 39%. ^{1}H-NMR (500 MHz, DMSO-d_6) δ 10.90 (2 H, s), 8.80, (1 H, d, *J* = 1.9 Hz), 8.17 (2 H, d, *J* = 8.5 Hz), 8.11 (1 H, d, *J* = 9.5 Hz), 8.07 (2 H, d, *J* = 8.0 Hz), 7.99 (1 H, d, *J* = 9.3 Hz), 7.85 (1 H, dd, *J* = 2.0, 9.3 Hz), 7.53 (1 H, dd, *J* = 2.8, 9.5 Hz), 7.33 (2 H, t, *J* = 8.0 Hz), 7.12 (1 H, d, *J* = 2.8 Hz), 7.09 (1 H, t, *J* = 7.4, 7.5 Hz), 6.92-6.90 (2 H, d, *J* = 8.0 Hz), 4.03 (2 H, q, *J* = 7.0 Hz), 1.31 (3 H, t, *J* = 7.0 Hz); ^{13}C-NMR (125 MHz, DMSO-d_6) δ 164.9, 158.7, 155.8, 152.2, 148.8, 147.6, 139.6, 138.7, 132.6, 131.1, 130.2, 128.7, 125.7, 122.8, 122.3, 119.9, 118.3, 117.1, 116.6, 115.5, 114.1, 98.2, 63.6, 14.2; HRMS (ESI$^+$) calcd for $C_{29}H_{22}N_3O_3$ $(M+H)^+$ 460.1656. found 460.1652.

4.2.10. Synthesis of *N*-(9-Amino-2-ethoxyacridin-6-yl)-*N'*-(4-cyanophenyl)urea (19)

4-Cyanophenyl isocyanate (60mg, 0.451 mmol) was added to a solution of **26a** (52 mg, 0.218 mmol) in DMSO (1 mL) under an Ar atmosphere. The reaction mixture was stirred at 100°C overnight, and then poured into water (20 mL) and ethyl acetate (20 mL). The resulting yellow precipitate was collected by filtration, washed with hot water, and dried *in vacuo* to yield **19** (18.0 mg, 20%) as a yellow powder. Mp >300°C; ^{1}H-NMR (500 MHz, DMSO-d_6) δ11.20 (1 H, s), 11.02 (1 H, s), 10.23 (1H, s), 8.95 (2H, br s), 8.38 (1H, d, *J* = 9.0 Hz), 8.08 (1H, s), 7.72-7.61 (4 H, m), 7.56 (2 H, d, *J* = 8.6 Hz), 7.50 (2 H, m), 4.11 (2H, q, *J* = 7.0 Hz), 1.41 (3H, t, *J* = 7.0 Hz); ^{13}C-NMR (125 MHz, DMSO-d_6) δ 155.2, 153.7, 152.0, 144.8, 143.7, 140.3, 133.8, 133.5, 133.4, 127.0, 125.7, 120.1, 119.3, 118.2, 118.1, 116.4, 112.9, 106.2, 106.1, 103.9, 103.9, 102.6, HRMS (ESI$^+$) calcd for $C_{23}H_{20}N_5O_2$ $(M+H)^+$ 398.1612. found 398.1610.

N-(9-Amino-2-methoxyacridin-6-yl)-*N'*-(4-cyanophenyl)urea (**20**) and *N*-(9-amino-2-hydroxyacridin-6-yl)-*N'*-(4-cyanophenyl)urea (**21**) were prepared similarly from **26b** and **26c**, respectively.

20: 27%; Mp >300°C; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 11.22 (1 H, s), 11.04 (1 H, s), 9.17 (1 H, s), 8.39 (1 H, d, *J* = 9.1 Hz), 8.08 (1 H, s), 7.71-7.47 (8 H, m), 3.87 (3 H, s); ^{13}C-NMR (125 MHz, DMSO-d_6) δ 155.0, 154.3, 152.3, 152.1, 145.2, 144.5, 144.2, 140.5, 135.2, 133.3, 132.9, 126.5, 124.9, 120.7, 119.4, 119.3, 118.3, 118.2, 117.2, 112.0, 106.2, 103.4, 103.0, 102.6, 55.9; HRMS (ESI$^+$) calcd for $C_{22}H_{18}N_5O_2$ $(M+H)^+$ 384.1455. found 384.1448.

21: 28%; Mp >300°C; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.42 (1 H, s), 10.24 (1 H, s), 10.20 (1 H, s), 9.32 (2 H, s), 8.50 (1 H, d, *J* = 9.4 Hz), 8.22 (1 H, d, *J* = 2.0 Hz), 7.80-7.67 (6 H, m), 7.58 (1 H, dd, *J* = 2.4, 9.1 Hz), 7.37 (1 H, dd, *J* = 2.0, 9.3 Hz); ^{13}C-NMR (125 MHz, DMSO-d_6) δ 155.5, 154.1, 152.4, 145.2, 140.7, 134.2, 133.9, 133.8, 127.4, 126.1, 120.5, 119.6, 118.6, 118.5, 113.3, 106.6, 106.5, 104.3, 104.3, 103.0; HRMS (ESI$^+$) calcd for $C_{21}H_{14}N_5O_2$ $(M+H)^+$ 368.1153. found 368.1159.

4.3. Biology

4.3.1. Expression and purification of GST–SPAK-CCT in *Escherichia coli*.[17]

DNA encoding residues 452–553 of rat SPAK (CCT, almost identical to residues 442–545 of human SPAK) was cloned into pGEX6P-1 vector (Promega). *E. coli* BL21 cells were transformed with recombinant GST-fusion SPAK (GST–SPAK-CCT) protein expression vector, and seeded into 1 liter of 2YT broth [1.6% (w/v) tryptone/1% (w/v) yeast extract/0.5% NaCl] containing 100 μg/ml ampicillin. Culture was continued at 37°C until the absorbance at 600 nm reached 0.6. Then IPTG (1 mM) was added, and the cells were cultured for an additional 16 h at 28°C. Cells were isolated by centrifugation and resuspended in 40 ml of ice-cold PBS, followed by sonication (Tomy Ultrasonic Disrupter UD-201). Lysates were centrifuged at 4°C for 5 min at 10000 x g. The GST-fusion protein was affinity-purified on 1.2 ml of glutathione–Sepharose beads and eluted in elution buffer containing 83 mM Tris/HCl, 150 mM KOH and 30 mM glutathione.

4.3.2. Cytotoxicity assay

HEK293T cells were seeded in a 96-well plate at a density of 1 x 10^5 cells/ml in a volume of 200 μL per well, then various concentrations of compounds were added and the plate was incubated for 40 hours. Cell Counting Kit-8 reagent (10 μL) (Dojindo, CK04, Japan) was added to each well and incubation was continued for 3.5 hours at 37℃ under 5% CO_2. Absorbance was measured at 450 nm using a microplate reader (ARVO MX, PerkinElmer Inc.). Dose–response curves were fitted by non-linear regression to a sigmoidal dose–response model, and the value of IC_{50} was calculated with KaleidaGraph 4.0J.

4.3.3. FCS assay.[17]

Fluorescent TAMRA (6-carboxytetramethylrhodamine)-labeled WNK4 peptide covering the RFQV motif [WNK4-RFQV (TAMRA-SEEGKPQLVGRFQVTSSK)] and TAMRA-labeled WNK4 peptide in which the RFXV motif was mutated [WNK4-AFQV (TAMRA-SEEGKPQLVGAFQVTSSK)] as a negative control were prepared (Hokkaido System Science). The TAMRA-labeled peptides were incubated with various concentrations of GST–SPAK-CCT (1–65 μM) in reaction buffer consisting of 1×PBS with 0.05% Tween 20 at room temperature (25°C) for 30 min, and FCS measurements of single-molecule fluorescence were performed using a FluoroPoint-light analytical system (Olympus).[21] The confocal volume for detection of fluorescence signals in this equipment is in the range of a single femtolitre. Therefore, TAMRA-labeled peptide was diluted so that a single fluorophore was likely to be present in the confocal space. The final concentration of each peptide in the FCS assays was 2.7 nM. The concentration of GST–SPAK-CCT used for screening was 0.5 μM, since at this concentration the binding reaction was not saturated and was reproducibly detected. The concentration of DMSO (used as a solvent of compounds) was 1% in the final assay solutions. The assay was performed in a 384-well plate. All experiments were performed with a data acquisition time of 15 s, and the measurements were repeated five times per sample. Kd and IC_{50} values were calculated by Origin 8.1 data analysis and graphing software (OriginLab).

4.3.4. Animal Studies

C57BL/6 mice (10 weeks old) were maintained with standard laboratory chow and water. The Animal Care and Use Committee of Tokyo Medical and Dental University approved the experimental protocol. In an acute treatment study, compounds were administered intraperitoneally at a dose of 5 mg/kg, 10 mg/kg or 20 mg/kg in 100 μL of DMSO; the control mice were administered 100 μL of DMSO alone. The mice were euthanized 90 minutes after injection. The kidneys were removed and rapidly homogenized in a 10-fold mass excess of ice-cold 1% (w/v) Triton X-100 lysis buffer. Whole homogenates without the nuclear fraction (600 x g) were collected and denatured at 60°C for 20 minutes as previously reported,[3] then sonicated, and centrifuged at 6000 x g for 5 minutes at 4°C. A 120 μl aliquot of the supernatant was denatured at 60°C for 20 minutes. The total protein concentration was determined by the Bradford method using BSA as a standard, and lysates were stored at -80°C.

4.3.5. Immunoblotting

Kidneys were dissected from the mice. Quantitative immunoblotting was performed as described previously.[8] Membranes were blocked with blocking reagent and reacted with the following primary antibodies: anti-total SPAK (Cell Signaling Technology), anti-phosphorylated SPAK[22] anti-(total NCC),[23] anti-phospho-NCC (pSer71)[12], anti-total NKCC1 (T4) (Hybridoma Bank, University of Iowa, Iowa City, IA), anti-phosphorylated NKCC1 (pThr206 in mouse NKCC1),[24] anti-total NKCC2 (Alpha Diagnostic, San Antonio, TX), and anti-phosphorylated NKCC2 (termed pT2 antibody;

kindly provided by K. Mutig).[25] Alkaline phosphatase-conjugated anti-mouse IgG antibodies (Promega) were used as secondary antibodies for colorimetric detection. The intensities of the bands were analysed and quantified by ImageJ (NIH) software.

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Graphical Abstract

Development of WNK Signaling Inhibitors as a New Class of Antihypertensive Drugs

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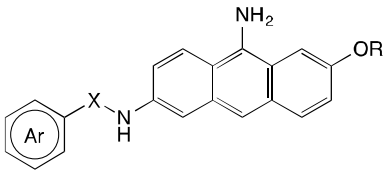

1 X = CH_2, Ar = 2-furanyl, R = Et
10 X = C=O, Ar = 4-cyanophenyl, R = Et
21 X = NHCO, Ar = 4-cyanophenyl, R = Me