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N-(4-(2-chloro-4-(trifluoromethyl)phenoxy)phenyl)picolinamide as a new inhibitor of mitochondrial complex III: Synthesis, biological evaluation and computational simulations

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ABSTRACT

Mitochondrial complex III is one of the most promising targets for a number of pharmaceuticals and fungicides. Due to the wide-spread use of complex III-inhibiting fungicides, a considerable increase of resistance has occurred worldwide. Therefore, inhibitors with novel scaffolds and potent activity against complex III are still in great demand. In this article, a new series of amide compounds bearing the diaryl ether scaffold were designed and prepared, followed by the biological evaluation. Gratifyingly, several compounds demonstrated potent activity against succinate-cytochrome *c* reductase (SCR, a mixture of mitochondrial complex II and complex III), with compound **3w** possessing the best inhibitory activity (IC_{50} = 0.91 ± 0.09 μmol/L). Additional studies verified that **3w** was a new inhibitor of complex III. Moreover, computational simulations elucidated that **3w** should bind to the Q_o site of complex III. We believe this work will be valuable for the preparation and discovery of more complex III inhibitors.

The mitochondrial electron transport chain (ETC), which is composed of oxidative phosphorylation and Krebs cycle, creates an electrochemical proton gradient across the membrane via several redox reactions. It has been well documented that the electron transfer is from NADH to O_2, with the production of ATP simultaneously.[1] In particular, oxidative phosphorylation contains five different transmembrane proteins/complexes (namely complexes **I-V**) and two mobile electron carriers (ubiquinone and cytochrome *c*). Among them, complex III (cytochrome bc_1 complex) is of vital significance in the life cycle of eukaryotes. In general, complex III is responsible for the electron transfer from ubiquinol to cytochrome *c*. It is also associated with hydroquinone oxidation and cytochrome *c* reduction.[2–5] If its activity is inhibited, the synthesis of ATP in the mitochondria will be blocked, which is lethal to eukaryotes.[2,3,6,7]. Most complex III-inhibiting fungicides could inhibit the activity of complex III in eukaryotes at the enzyme level, while these fungicides mainly inhibit the activity of complex III during the germination of fungal spores at the physiological level. Among eukaryotes, spore germination occurs in fungi (not in plants), so these inhibitors largely prohibited the growth of fungi. With the above considerations, numerous complex III-inhibiting fungicides have been discovered and commercialized, which have ranked first for disease control in worldwide plant protection during the past several decades.[8–10] Meanwhile, significant advances have also been achieved for complex III inhibitors with different modes of action.[8,11–35] However, most of the existing fungicides suffer from serious resistance problems.[9,36,37] Therefore, more complex III inhibitors with new chemical structures and high potency are still in high demand.

In our previous work, a series of 4-aryloxy-*N*-arylanilines (**Series A**) were designed and synthesized via Cu-promoted Chan-Lam-Evans reactions.[38–41] Afterward, their activity against succinate-cytochrome *c* reductase (SCR) was evaluated.[42] The results implied that several compounds showed excellent activity against SCR with their IC_{50} values in the nanomolar level. In practice, SCR has been reported as a mixture of complex II and complex III.[43] However, further experiments revealed that these molecules were neither complex II nor complex III inhibitors (as depicted in Fig. 1, left column). Considering the potential usefulness of these molecules and our continuous interest in the development of potent inhibitors with novel scaffolds, we carried out further structural modifications from **Series A**. Due to the significant role of the amide bond in numerous bioactive molecules,[44–46] we attempted to replace

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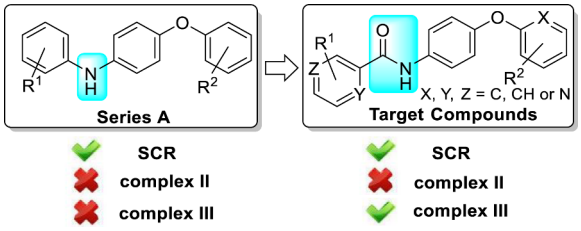
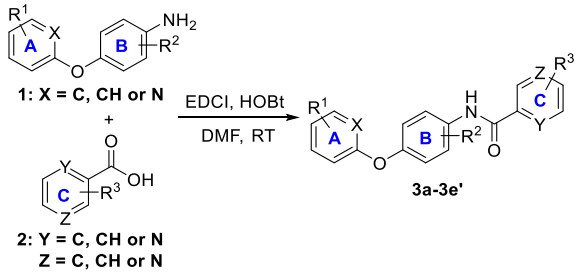


Fig. 1. The design strategy.

the amino group of **Series A** with an amide functionality (as shown in Fig. 1, right column). The resulting target molecules, namely *N*-(4-phenoxyphenyl) benzamides, could give rise to potent activity against SCR as well as complex II and/or complex III. To our delight, *N*-(4-(2-chloro-4-(trifluoromethyl)phenoxy)phenyl)picolinamide (**3w**), one of these target compounds, displayed good activity against both SCR and complex III, leading to an active inhibitor of complex III which has never been reported before. Furthermore, computational simulations provided more information about its possible binding mode.

First of all, the designed target compounds were synthesized using the traditional amide synthesis from activated carboxylic acids and amines. In detail, the amide bond was constructed from aromatic carboxylic acids and diphenyl ether-containing anilines using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI) combined with 1-hydroxybenzotriazole (HOBt) as the activating reagents (as depicted in Scheme 1). Using this procedure, total 31 target compounds were prepared and characterized by NMR and HRMS. To better present our experimental results, the three phenyl rings were assigned as Ring A, Ring B and Ring C, respectively (Scheme 1).

The inhibitory activity of our target compounds against porcine SCR was then assayed at a concentration of 10 μmol/L, and those with the inhibitory rates of above 50% were further selected to determine their IC_{50} values (as listed in Table 1). Two commercial SCR-inhibiting fungicides (penthiopyrad and azoxystrobin) were chosen as positive controls. Initially, various aromatic groups were attempted while retaining the optimized diphenyl ether fragment (2,4-Cl_2 as R^1, X = CH, R^2 = H) from our previous work (entries 1–15).[33] If an unsubstituted phenyl ring (R^3 = H) was introduced as Ring C, a high inhibition rate and an IC_{50} value of 1.52 ± 0.11 μmol/L were obtained (entry 1). It seemed that the substituent positions on Ring C had a significant impact on the inhibitory activity (entries 2–7). A methyl group employed at different positions triggered varied inhibition potency, with 3-Me as R^3 exhibiting higher performance than the 2-Me and 4-Me counterparts (entry 3 vs. entries 2 and 4). If 2-Cl, 3-Cl or 4-Cl was introduced as R^3, a similar trend was observed (entries 5–7). Polysubstituted groups were also employed as R^3, but only moderate inhibition rates of 31–46% were obtained (entries 8–9). Other 3-substituted groups were



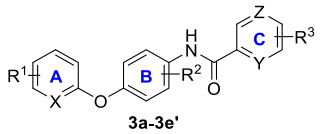
Scheme 1. The synthesis of target compounds **3a–3e′**.

introduced as R^3, but only low to moderate inhibition potency was obtained (entries 10–15). Among all the tested groups, the H atom demonstrated the best activity and thus was identified as the optimal group for R^3 (entries 2–15 *vs.* entry 1). If other diphenyl ether structures were employed, comparable or reduced activity was attained (entries 16–20 *vs.* entry 2). Interestingly, a pyridine (Y = N) instead of a phenyl group (Y = CH) led to improved activity (IC_{50} = 1.21 ± 0.10 μmol/L for entry 21 *vs.* IC_{50} = 1.52 ± 0.11 μmol/L for entry 1). However, employment of both Ring A and Ring C with pyridine-derived groups (X = Y = N) resulted in an inhibition ratio of only 14% (entry 22). From the above results, X and Y were optimized as CH and N, respectively. Furthermore, replacement of the 2,4-Cl_2 group with 2-Cl-4-CF_3 at the R^1 position gave rise to slightly higher activity (entry 23 *vs.* entry 21). Unfortunately, poor levels of inhibition were produced when substituents other than H were introduced into both Ring A and Ring B (entries 24–28). Moreover, a pyrazine-containing target compound (**3c'**) was also synthesized, but it merely exhibited moderate inhibitory activity against SCR (entry 29, IC_{50} = 8.72 ± 0.16 μmol/L). Replacing the electron-withdrawing group (CF_3) of compound **3w** with an electron-donating group (CH_3) resulted in compound **3d'**, which showed slightly reduced potency than **3w** (entry 30 *vs.* entry 23). Besides, changing the Cl group with a bulkier Br group gave rise to compound **3e'**, which also exhibited marginally lower activity than **3w** (entry 31 *vs.* entry 23). It appeared that both electronic and steric properties affected the inhibitory activity of the resulting target compounds against SCR, with the Cl and CF_3 groups as the optimal groups on the diphenyl ether skeleton. Finally, compound **3w** was identified as the most active SCR inhibitor, which manifested comparable potency with the two commercial fungicides (entry 23 *vs.* entries 32–33).

Aiming to confirm the inhibiting target of **3w** (complex II, complex III or dual–target), we further tested its activity against SCR, complex II and complex III (as listed in Table 2). As expected, penthiopyrad selectively inhibited the activity of complex II, while azoxystrobin demonstrated outstanding inhibitory activity exclusively against complex III (entries 2–3). Similar with azoxystrobin, **3w** showed an inhibition rate of only 31% against complex II at the concentration of 10 μmol/L, but considerably higher activity against complex III (entry 1). Therefore, compound **3w** was identified as a potent inhibitor of complex III.

With the discovery of **3w** as an active complex III inhibitor, its possible binding mode was then elucidated using computational simulations (shown in Fig. 2). Complex III is composed of two binding sites (Q_o site and Q_i site), so it's of great significance to dock **3w** into both sites. It was worth noting that the calculation of the binding free energies was based on the docking scores in AutoDock 4.2. The docking of **3w** into the Q_o site imparted the calculated binding energy of −7.7 kcal/mol for the best binding conformation, while the binding energy was calculated as −6.7 kcal/mol when it was docked into the Q_i site. These results indicated that **3w** would presumably bind to the Q_o site, which inspired us to investigate the binding mode of **3w** into the Q_o site of complex III (shown in Fig. 2A). It appeared that crucial non-polar interactions were formed between this inhibitor and the target enzyme (PDB ID: 1SQB). The pyridine ring of **3w** could enter the hydrophobic pocket surrounded by residues P270, F274, Y131 and F128. Additionally, it could also form π-π stacking interactions with residues Y131 and F274. Meanwhile, hydrophobic interactions were observed between the diaryl ether scaffold and residues L294, I298, L121, I146, M124, F274. Notably, the Cl and CF_3 groups on the diphenyl ether skeleton favored the enhancement of the hydrophobic interactions. As we know, amide-π stacking interaction is one of the most frequently observed interactions between proteins and ligands, which can largely affect the biological activity.[47] The amide bond in **3w** showed good amide-π stacking interactions with residues F274 and Y278. In terms of

Table 1
Inhibitory activity of the synthesized compounds against porcine SCR.

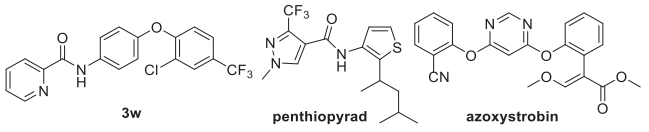


3a-3e'

Entry	Compound	X	Y	Z	R^1	R^2	R^3	Yield (%)[a]	I (%)[b] IC_{50} (μmol/L)[c]
1	**3a**	CH	CH	CH	2,4-Cl_2	H	H	33	75[b], 1.52 ± 0.11[c]
2	**3b**	CH	CH	CH	2,4-Cl_2	H	2-Me	68	46[b]
3	**3c**	CH	CH	C	2,4-Cl_2	H	3-Me	83	65[b], 1.65 ± 0.15[c]
4	**3d**	CH	CH	CH	2,4-Cl_2	H	4-Me	49	34[b]
5	**3e**	CH	CH	CH	2,4-Cl_2	H	2-Cl	70	41[b]
6	**3f**	CH	CH	C	2,4-Cl_2	H	3-Cl	65	57[b], 2.86 ± 0.27[c]
7	**3g**	CH	CH	CH	2,4-Cl_2	H	4-Cl	53	11[b]
8	**3h**	CH	C	CH	2,4-Cl_2	H	2,4,6-Cl_3	13	31[b]
9	**3i**	CH	CH	CH	2,4-Cl_2	H	2-Cl-4-CF_3	76	46[b]
10	**3j**	CH	CH	C	2,4-Cl_2	H	3-OMe	74	49[b]
11	**3k**	CH	CH	C	2,4-Cl_2	H	3-*t*Bu	64	20[b]
12	**3l**	CH	CH	C	2,4-Cl_2	H	3-F	73	45[b]
13	**3m**	CH	CH	C	2,4-Cl_2	H	3-Br	73	49[b]
14	**3n**	CH	CH	C	2,4-Cl_2	H	3-OCF_3	64	41[b]
15	**3o**	CH	CH	C	2,4-Cl_2	H	3-CF_3	50	48[b]
16	**3p**	CH	CH	CH	H	H	H	90	22[b]
17	**3q**	CH	CH	CH	2,4-Cl_2	3,5-Cl_2	H	23	63[b], 1.59 ± 0.12[c]
18	**3r**	C	CH	CH	2,4,6-Cl_3	3-Cl	H	32	42[b]
19	**3s**	C	CH	CH	2,4,6-Cl_3	3,5-Cl_2	H	54	36[b]
20	**3t**	C	CH	CH	2,4,6-Me_3	3,5-Cl_2	H	46	27[b]
21	**3u**	CH	N	CH	2,4-Cl_2	H	H	70	71[b], 1.21 ± 0.10[c]
22	**3v**	N	N	CH	2,4-Cl_2	H	H	22	14[b]
23	**3w**	CH	N	CH	2-Cl-4-CF_3	H	H	39	75[b], 0.91 ± 0.09[c]
24	**3x**	CH	N	CH	2-Cl-4-CF_3	3-Cl	H	51	34[b]
25	**3y**	CH	N	CH	2,4-Cl_2	3,5-Cl_2	H	55	26[b]
26	**3z**	C	N	CH	2,4,6-Cl_3	3,5-Cl_2	H	62	21[b]
27	**3a′**	CH	N	CH	2-naphthyl[d]	3-Cl	H	55	25[b]
28	**3b′**	CH	N	CH	2-naphthyl[d]	3,5-Cl_2	H	54	21[b]
29	**3c′**	CH	N	N	2-Cl-4-CF_3	H	H	42	52[b], 8.72 ± 0.16[c]
30	**3d′**	CH	N	CH	2-Cl-4-Me	H	H	63	67[b], 2.36 ± 0.19[c]
31	**3e′**	CH	N	CH	2-Br-4-CF_3	H	H	38	65[b], 2.57 ± 0.23[c]
32	penthiopyrad								70[b], 2.01 ± 0.12[c]
33	azoxystrobin								96[b], 0.31 ± 0.03[c]

[a] Isolated yields.
[b] Inhibition rates against SCR at a concentration of 10 μmol/L.
[c] IC_{50} values against SCR.
[d] The whole ring A was substituted with a 2-naphthyl group.

Table 2
The inhibition effect of the select inhibitors against SCR, complex II and complex III.



3w penthiopyrad azoxystrobin

Entry	Compound	IC_{50} (μmol/L)		
		SCR	complex II	complex III
1	**3w**	0.91 ± 0.09	> 10 (31%[a])	6.03 ± 0.20
2	penthiopyrad	2.01 ± 0.12	0.95 ± 0.07	> 10 (11%[a])
3	azoxystrobin	0.31 ± 0.03	> 10 (23%[a])	0.85 ± 0.05

[a] Inhibition rates against SCR at a concentration of 10 μmol/L.

azoxystrobin, its docking result suggested an analogous binding mode with **3w** (as shown in Fig. 2B). From the above results, it was concluded that compound **3w** could bind well into the Q_o pocket of complex III. Notably, the amide linkage is pivotal for the potent inhibition of **3w** against complex III.

In summary, a new series of amides comprising the diaryl ether scaffold were designed and synthesized based on our previously reported 4-aryloxy-*N*-arylanilines. With these molecules in hand, their inhibitory activity was examined against SCR, with several compounds exhibiting potent activity against SCR. Additional experiments were carried out to determine the target of representative inhibitor **3w** and its possible binding mode in the target enzyme. The results indicated that **3w** was a potent inhibitor of complex III. Computational simulations showed that **3w** could bind into the Q_o pocket well and form strong interactions with the target enzyme. Notably, the evolvement of an amide bond in **3w** induced good amide-π stacking interactions with residues F274 and Y278. This work can be beneficial for the further development of other complex III inhibitors.

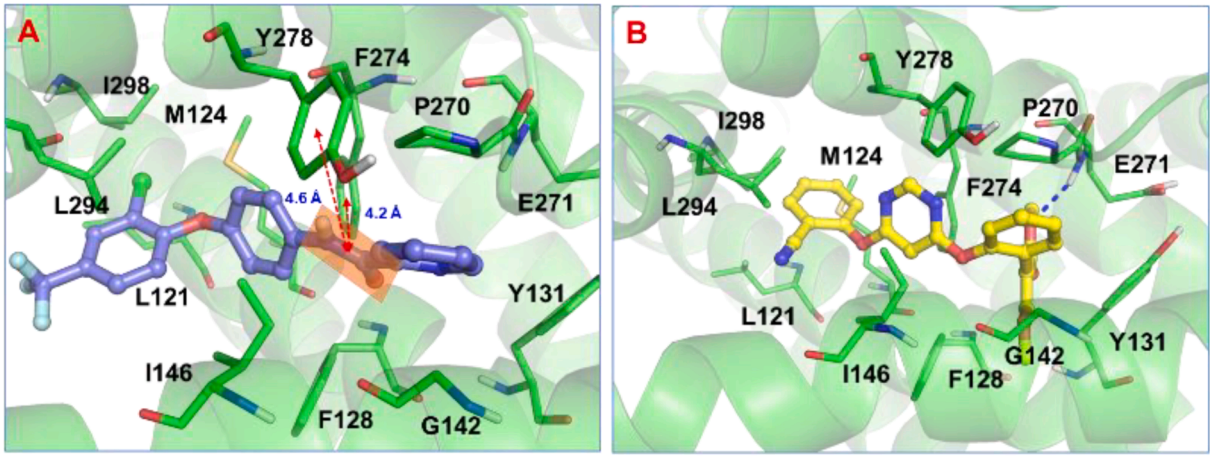


Fig. 2. (A) The binding mode of **3w** in the Q_o site of complex III (PDB ID: 1SQB); (B) the binding mode of azoxystrobin in the Q_o site of complex III (PDB ID: 1SQB).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2020.127302.

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