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Development of Novel *N*-hydroxypyridone Derivatives as Potential Anti-Ischemic Stroke Agents

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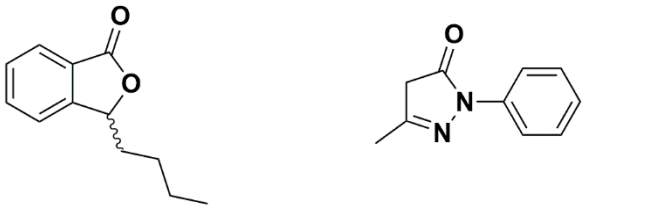
ABSTRACT

Our previous study had identified ciclopirox (CPX) as a promising lead compound for treatment of ischemic stroke. To find better neuroprotective agents, a series of *N*-hydroxypyridone derivatives based on CPX were designed, synthesized and evaluated in this study. Among these derivatives, compound **11** exhibits significant neuroprotection against oxygen glucose deprivation (OGD) and oxidative stress induced injuries in neuronal cells. Moreover, compound **11** possesses good blood-brain barrier (BBB) permeability and superior antioxidant capability. In addition, complex of compound **11** with olamine—**11·Ola** possesses good water solubility, negligible hERG inhibition and superior metabolic stability. In vivo experiment demonstrates that **11·Ola** significantly reduces brain infarction and alleviate neurological deficits in middle cerebral artery occlusion (MCAO) rats. Hence, compound **11·Ola** is identified in our research as a prospective prototype in the innovation of stroke treatment.

INTRODUCTION

Ischemic stroke, as the most common form of stroke, accounted for 2.57-2.82 million deaths per year, and it is a major cause of severe disability among adults worldwide.[1-3] Currently, US Food and Drug Administration (FDA) only approved recombinant tissue plasminogen activator (rtPA) for thrombolysis in the treatment of ischemic stroke. However, rtPA must be administered within 4.5 h after stroke, and because of its short therapeutic time window, only a limited number of ischemic stroke patients can receive thrombolysis.[4, 5] Furthermore, the subsequent ischemia-reperfusion (I/R) injury, which happens when returning blood flow to ischemic brain tissue after thrombolysis, can mitigate the benefits of thrombolysis. The clinical use of neuroprotective agents, such as edaravone and NBP (racemic 3-n-butylphthalide, Figure 1), exerted beneficial effects on outcome in stroke patients.[6,7] Moreover, neuroprotective agents can be administrated in a relatively wide therapeutic time window. This suggests that the neuroprotection strategy, namely protecting the penumbra from I/R injury after thrombolysis, is an effective therapy for stroke.[8] Hence, in order to protect ischemic neurons and minimize I/R injury, it is urgent to develop novel neuroprotective agents with wide therapeutic time window to provide better prognosis for ischemic stroke patients. The mechanisms and targets of reported neuroprotective agents include free radical scavenging, alleviating oxidative stress, ionotropic glutamate receptors, anti-inflammation and antiplatelet aggregation.[9-14] Among these neuroprotective agents, the well-known NXY-059, a spin-trapping free radical scavenging agent which was firstly reported in 1990s significantly improved the overall distribution of scores on the

modified Rankin scale (indicating its potential on reducing disability) in SAINT-I (a clinical trial involving 1722 patients).[15,16] Although NXY-059 had not been approved at last,[17] this work which demonstrated the ability of free radical scavenging agents to reduce oxidative stress-induced damage in I/R injury is still enlightening.



NBP **Edaravone**

Approved in China, 2002 **Approved in Japan, 2001**

Figure 1. Structures of NBP and Edaravone.

In order to find hit compound for ischemic stroke, an FDA-approved drugs library (778 drugs) was screened through the oxygen glucose deprivation (OGD)-stimulated ischemic cellular model in our previous study. Among them, the antifungal drug ciclopirox (CPX) conferred significant neuroprotective effects against the ischemic injury. CPX, which was first reported as an antifungal agent in 1970,[18] is used for the treatment of tinea pedis and tinea corporis caused by trichophyton rubrum. Later on, it was reported that CPX could promote the survival of neuronally differentiated PC12 cells in serum-free medium in 1998.[19] In our previous studies, in vivo & in vitro results had confirmed the effectiveness of CPX for ischemic brain injury. CPX treatment not only reduced the brain infarction and neurological deficits in middle cerebral artery occlusion (MCAO) rats significantly, but also ameliorated blood-brain barrier (BBB).[20] Moreover, previous study proved that the neuroprotective effects of CPX was associated with the enhancement of AKT and GSK3β phosphorylation.[20]

In this study, CPX was selected as a lead compound for anti-stroke drug discovery. As the course of NBP and edaravone treatment is typically 14 – 20 days, long-lasting anti-ischemic effect is vital to ischemic stroke treatment. Thus, besides neuroprotective activity, metabolic stability which contributes to long-lasting efficacy is also important for anti-stroke agents. CPX consists of the *N*-hydroxyl pyridone ring, methyl at position 4 and cyclohexyl at position 6 (Figure 2). Therefore, in order to elucidate the structure and activity relationship and improve neuroprotective activity and metabolic stability, *N*-hydroxyl pyridone derivatives were designed and synthesized by replacing the hydrogens or substituents at position 1, 3, 4, 5 and 6 with different substituents , and their neuroprotective activities were evaluated through in vitro and in vivo experiments.

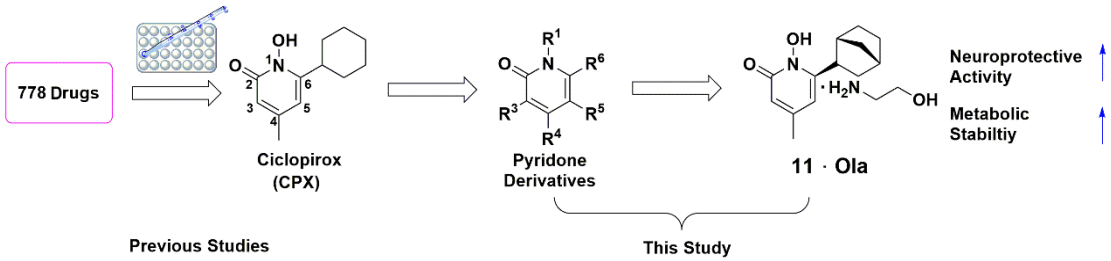


Figure 2. Structures of CPX and Pyridone Derivatives.

Among them, compound **11** showed better neuroprotective activity, good BBB permeability and superior antioxidant capacity. Moreover, **11·Ola** (complex of **11** and olamine) showed better metabolic stability, good water solubility, safety and better efficacy in vivo, indicating that it possesses development potential as a prospective anti-ischemic stroke agent.

RESULTS and DISCUSSION

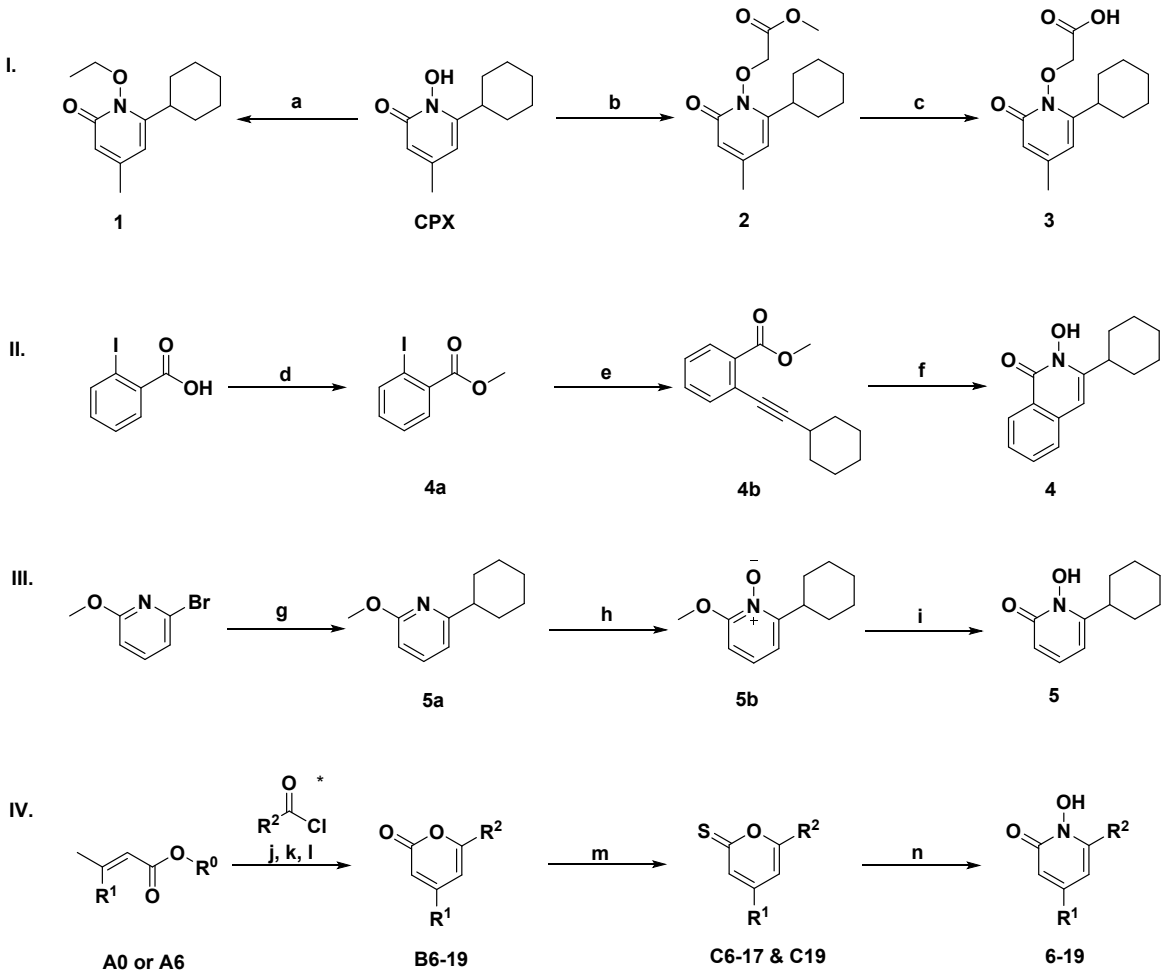
Design and Synthesis

To identify the pharmacophore of CPX, compounds **1-4** were designed and synthesized, firstly. The hydroxyl at position 1 was replaced by ethoxyl, 2-methoxy-2-oxoethoxy and carboxymethoxy to produce compounds **1–3** (ineffective, Table 1). Compound **4** was given by replacing the pyridone of CPX with isoquinoline. After *N*-hydroxypyridone was confirmed as the pharmacophore, the methyl at position 4 was replaced by hydrogen and ethyl to produce compound **5** and compound **6**. Because of the inferior activity of compounds **5** and **6** (Table 1), methyl was fixed as the substituent at position 4 for compounds **7-31**. Next, to explore the ideal substituent at position 6, compounds **7-19** were produced by replacing the cyclohexyl with alkyls, cycloalkyls, norbornane, phenyl and benzyl. Then, in order to elucidate the effects of substituents at position 3 and 5, halogen substituted derivatives (compounds **20-23**) and formyl or aminomethyl substituted derivatives (compounds **24-31**) were designed and synthesized.

The synthetic route for the preparation of compounds **1-19** is shown in Scheme 1. Reaction of CPX with iodoethane and methyl 2-bromoacetate produced compound **1** and **2**, respectively (reactions a-b, Scheme1-I). Compound **3** was obtained by hydrolyzing the methyl of compound **2** using lithium hydroxide (reaction c, Scheme1-I). Reaction of commercially available 2-iodobenzoic acid and methanol in the presence of sulfurous dichloride generated methyl 2-iodobenzoate (**4a**). Then Intermediate **4a** was used in the subsequent Sonogashira cross coupling with ethynylcyclohexane to generate **4b**, which was then refluxed in methanol with hydroxylamine hydrochloride and hydroxyl potassium to afford compound **4** (Scheme1-II). Intermediate **5a** was

obtained through Kumada cross coupling of 2-bromo-6-methoxypyridine and cyclohexylmagnesium bromide, which was oxidized by mCPBA to generate intermediate **5b.** Intermediate **5b** was reflux in acetyl chloride and then in methanol to afford compound **5**. (Scheme 1-III). Using ethyl (*Z*)-3-methylpent-2-enoate (**A6**) and cyclohexyl chloride as starting material, compound **6** was generated through reactions j, k, m and n, this synthesis method is similar to that of compounds **7-19** as following. Reaction of methyl 3-methylbut-2-enoate (**A0**, commercially available) with corresponding acyl chloride in presence of aluminum trichloride provided different substituted acyl *α, β*- unsaturated esters, which were then heated with a mixture of sulfuric acid and acetic acid to give intermediates **B7-B18** (Scheme 1-IV, reactions j, k). The synthesis of **B19** is an exception. The starting materials of **19** are bromoacetyl chloride and methyl 3-methylbut-2-enoate, and the corresponding product 6-(bromomethyl)-4-methyl-2H-pyran-2-one was then used in the Suzuki cross coupling with phenylboronic acid in the presence of $Pd(dppf)Cl_2$ to generate intermediate **B19** (Scheme 1-IV, reaction l). Intermediates **B7-B17**, **B19** spontaneously formed **C7-C17**, **C19** when heated with tetraphosphorus decasulfide in toluene (Scheme 1-IV, reaction m). Compounds **C7-C17**, **C19** and hydroxylamine hydrochloride were refluxed in pyridine to afford target compounds **7-17**, **19** (Scheme 1-IV, reaction n). Intermediate **B18** directly formed compound **18** through an adjusted reaction n, see experiment section.

Scheme 1. Synthesis of Compounds **1-19**.



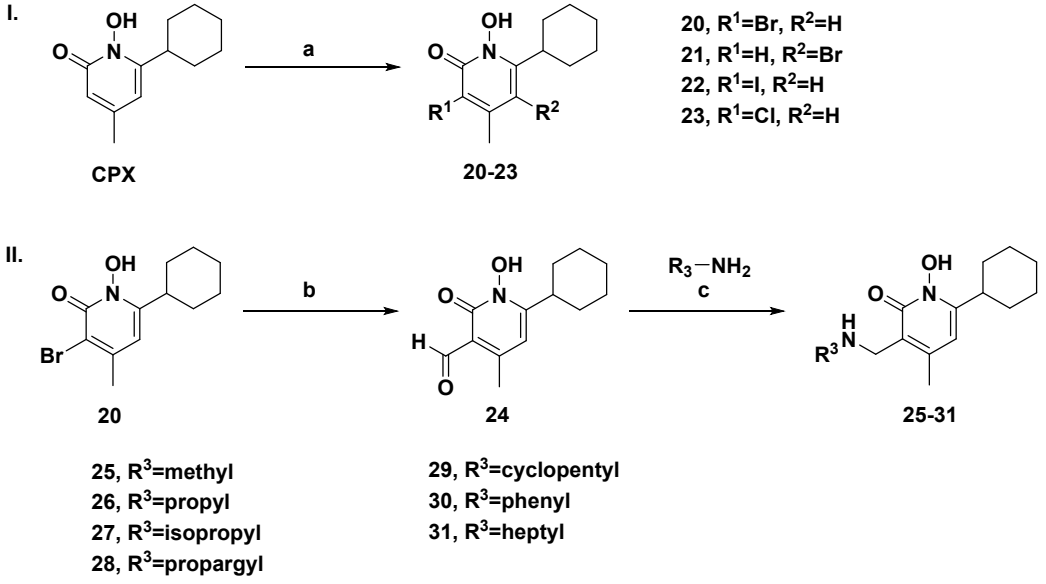
A0 = methyl 3-methylbut-2-enoate
A6, R^1=ethyl, R^0=ethyl
B6, C6, 6, R^1=ethyl, R^2=cyclohexyl
B7, C7, 7, R^1=methyl, R^2=cyclopropyl
B8, C8, 8, R^1=methyl, R^2=cyclobutyl
B9, C9, 9, R^1=methyl, R^2=cyclopentyl
B10, C10, 10, R^1=methyl, R^2=cycloheptyl
B11, C11, 11, R^1=methyl, R^2=endo-bicyclo[2.2.1]heptan-2-yl
B12, C12, 12, R^1=methyl, R^2=methyl
B13, C13, 13, R^1=methyl, R^2=propyl
B14, C14, 14, R^1=methyl, R^2=isobutyl
B15, C15, 15, R^1=methyl, R^2=pentyl
B16, C16, 16, R^1=methyl, R^2=heptyl
B17, C17, 17, R^1=methyl, R^2=nonyl
B18, 18, R^1=methyl, R^2=phenyl
B19, C19, 19, R^1=methyl, R^2=benzyl

*** The reactants of B19 are bromoacetyl chloride and A_0.**

Reagents and conditions: (a) iodoethane, K_2CO_3, DMF, 60 °C; (b) methyl 2-bromoacetate, K_2CO_3, acetonitrile, reflux; (c) lithium hydroxide, water/THF, 40 °C; (d) $SOCl_2$, then methanol and Et_3N; (e) ethynylcyclohexane, Et_3N, CuI, $PdCl_2(PPh_3)_2$, toluene, 4.5 h, 40 °C under Ar; (f) NH_2OH, KOH, CH_3OH, reflux; (g) cyclohexylmagnesium bromide, $Pd(dppf)Cl_2$; (h) mCPBA, DCM, reflux; (i) acetyl chloride, reflux, then MeOH; (j) $AlCl_3$, DCM, reflux; (k) H_2SO_4, acetic acid; (l) only for **B19**: phenylboronic acid, $Pd(dppf)Cl_2$, Cs_2CO_3, 1,4-dioxane, reflux; (m) P_4S_{10}, toluene, reflux; (n) $NH_2OH{\cdot}HCl$ pyridine, reflux.

The synthetic route for the preparation of compounds **20-31** is shown in Scheme 2. CPX and *N*-halogen butanimide were refluxed in carbon tetrachloride in the presence of initiator (AIBN) to afford halogen substituted (at position 3 or 5) pyridone derivatives **20–23** (Scheme 2-II, reaction a). The sodium salt of compound **20** (prepared by adding sodium hydride into the THF solution of compound **20**) and *tert*-butyl lithium were used in the halogen-lithium exchange reaction, then reacted with DMF to afford the aldehyde, namely compound **24** (Scheme 2-II, reaction b). Compound **25-31** were synthesized through reductive amination using compound **24** as reactant (Scheme 2-II, reaction c).

Scheme 2. Synthesis of Compounds **20-31**.



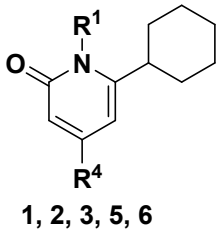
Reagents and conditions: (a) NBS/NIS/NCS, AIBN, CCl_4, reflux; (b) NaH, THF, 0 °C, then *tert*-BuLi, DMF, THF, -78 °C; (c) $Na(CH_3COO)_3BH$, acetic acid, $Cl(CH_2)_2Cl$, r. t.

Pyridone Analogues Protecting SH-SY5Y Cells against Oxygen-Glucose Deprivation (OGD) Damage

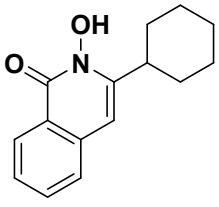
Firstly, the neuroprotective effect of the compounds **1-31** was evaluated by Oxygen-Glucose Deprivation (OGD) model, which is an in vitro model for ischemic stroke, and it was widely used in the study of anti-stroke agents[21-23]. The results are summarized in Tables 1-3 and Figure 3.

As shown in Table 1, compounds **1-3** with the substituents of ethoxyl, 2-methoxy-2-oxoethoxy and carboxymethoxy at position 1 (R^1) showed no neuroprotective activity. This results indicate that hydroxyl at position 1 is necessary for the neuroprotective activity of pyridone analogues. Compound **4** which was obtained by replacing pyridone ring of CPX with isoquinoline showed good neuroprotective activity (78.49%) at 1 μM, but no activity at 10 μM. Considered the activity of compounds **1-4**, the *N*-hydroxypyridone was fixed as the core of compounds **5-31**. Compound **5** with hydrogen at position 4 showed good neuroprotective activity (76.61%) at 10 μM, but no activity at 1 μM. Compound **6** with ethyl substituent at position 4 showed moderate neuroprotective activity at both 1 μM and 10 μM. The activities of compounds **5** and **6** is inferior to CPX. Therefore, methyl was fixed as the substituent at position 4 for compounds **7-31**.

Table 1. Effect of Compounds **1-6** on SH-SY5Y Cells Exposed to OGD[a].

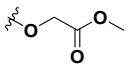
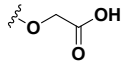


1, 2, 3, 5, 6



4

Substituents	Cell Viability (% of Control)

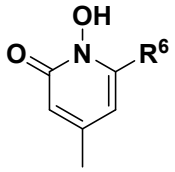
Compd.	R^1	R^4	1 μM	10 μM
CPX	-OH	$-CH_3$	$70.13 \pm 0.91^{***}$	$69.75 \pm 4.17^{**}$
1[b]	$-OC_2H_5$	$-CH_3$	46.33 ± 9.22	43.86 ± 6.76
2		$-CH_3$	49.94 ± 5.38	56.44 ± 0.61
3		$-CH_3$	56.05 ± 6.44	55.89 ± 3.71
4	----		$78.49 \pm 5.81^{**}$	61.62 ± 9.04
5	-OH	-H	51.75 ± 5.71	$76.61 \pm 1.64^{***}$
6	-OH	$-C_2H_5$	$69.81 \pm 4.08^{**}$	$67.34 \pm 5.25^{*}$
	OGD		$54.92 \pm 2.12\%^{###}$	

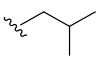
[a]Cell viability was measured by MTT assay at 24 h after OGD exposure. The compounds **1-6** at concentrations of 1 μM or 10 μM were added to the cells immediately before OGD stimulus. The data are presented as the percentage of surviving cells relative to control cells (100%) as the mean ±SD, n = 3. ###$p < 0.001$ vs control, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs OGD group, one-way ANOVA, Dunnett's multiple comparison test. [b]The data of compound **1** is gathered from another assay (n = 3) in which the cell viability of OGD group was 49.91% of control.

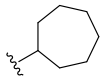
The cell viability of groups treated with compounds **7-11** demonstrated that the neuroprotective activity at 1 μM of compounds with large cycloalkyl substituents (**11**: *endo*-norbornane, **10**: cycloheptyl) at position 6 was better than compounds with smaller substituents (**7**: cyclopropyl, **8**: cyclobutyl). Compound **9** with cyclopentyl substituent at position 6 showed good neuroprotective activity (70.66% at 10 μM), but no activity at 1 μM. Compound **10** with cycloheptyl substituent at position 6) showed good neuroprotective activity (71.01% at 1 μM). It is noteworthy that compound **11**

(*endo*-norbornane at position 6) showed good neuroprotective activity at both 1 μM (70.62%) and 10 μM (64.46%). Compounds **12, 13, 15-17** showed no neuroprotective activity (Table 2), revealing unbranched alkyl substitution at position 6 was adverse to neuroprotective activity. Whereas compound **14** with branched alkyl substitution isobutyl (Table 2) showed slightly better activity at 10 μM (73.30%) compared with CPX, but no neuroprotective activity at 1 μM. So alkyl substitution at position 6 is not favored compared to cycloalkyl or bicycloalkyl substitution. Compound **18** bearing a phenyl substituent at position 6 showed no neuroprotective activity. Compound **19** bearing a benzyl substituent at position 6 showed comparable activity to that of CPX at 10 μM, but no neuroprotective activity at 1 μM. Generally speaking, large cycloalkyl or bicycloalkyl substitution at position 6 is better than small cycloalkyl, alkyl, phenyl and benzyl substitution.

Table 2. Effect of Compounds **7-19** on SH-SY5Y cells Subjected to OGD[a].



Compd.	R^6	Cell Viability (% of Control)		Compd.	R^6	Cell Viability (% of Control)	
		1 μM	10 μM			1 μM	10 μM
CPX		70.13 ± 0.91***	69.75 ± 4.17**	**13**		47.04 ± 2.90	64.04 ± 5.58*
7		58.77 ± 4.31	62.75 ± 15.02	**14**		47.67 ± 5.72	73.30 ± 3.55**
8		46.02 ± 7.0	71.15 ± 8.81*	**15**		63.55 ± 2.28	61.96 ± 13.60
9		52.36 ± 3.22	70.66 ± 6.15**	**16**		71.68 ± 7.74	58.52 ± 12.01

10		71.01 ± 2.40*	38.42 ± 7.86	**17**		52.98 ± 8.66	42.52 ± 8.19
11		70.62 ± 3.83**	64.46 ± 4.26*	**18**		56.96 ± 2.52	20.13 ± 1.76
12		55.81 ± 5.82	18.33 ± 4.84	**19**		46.46 ± 4.59	76.77 ± 5.72**

OGD = 54.92 ± 2.12%###

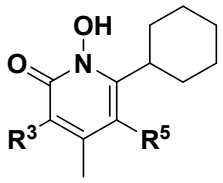
[a]The data are presented as the percentage of surviving cells relative to control cells and as the mean ±SD, n=3. ###$p < 0.001$ vs control, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs OGD group, one-way ANOVA, Dunnett's multiple comparison test.

As shown in Table 3, compounds **20-31** are series of *N*-hydroxypyridone derivatives with halogen, *N*-substituted aminomethyl or formyl substituents at position 3 or 5, and the substituents at position 4 and position 6 of compounds **20-31** are methyl and cyclohexyl respectively. The introduction of bromine at position 5 (**21**) or iodine at position 3 (**22**) produced a considerable activity improvement at 1 μM. Compounds **20** (bromine at position 3), **23** (chlorine at position 3) and **24** (3-formyl derivative) showed no activity at both 1 μM and 10 μM. Compounds **25-31** are a series of compounds bearing *N*-substituted aminomethyl substitution at position 3 (Table 3). Compound **26** (*N*-propyl aminomethyl), **27** (*N*-isopropyl aminomethyl) and **30** (*N*-phenyl aminomethyl) exhibited neuroprotective activity at 10 μM, and **29** (*N*-cyclopentyl aminomethyl) showed moderate activity at 1 μM. Compound **31** showed no activity at 1 μM, but good activity at 10 μM (87.18%) (Table 3). Compound **25** (*N*-methyl aminomethyl) showed moderate activity at 10 μM, but no activity at 1 μM. However,

compound **28** (*N*-propargyl aminomethyl) had no effects in OGD-exposed SH-SY5Y cell. In general, the neuroprotective activity of compounds with aminomethyl substitution at position 3 did not improve compared to CPX.

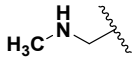
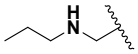
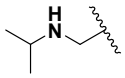
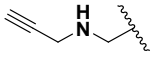
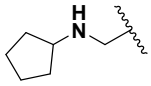
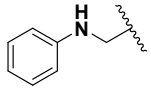
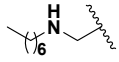
Among these 31 compounds, some compounds showed lower neuroprotective effects at 10 μM than that at 1 μM. To evaluate whether these compounds have toxic effect on normal cells at higher concentration, we tested the influence of the compounds (**1**, **3**, **4**, **5**, **6**, **10**, **11**, **12**, **15**, **16**, **17**, **18**, **20**, **21**, **22**, **23**, **28**, **29** and CPX) on the cell viability of SH-SY5Y cells without OGD insult. The results indicated CPX, **10**, **11** and **29** reduce the viability at the concentration of 10 μM (Table S7), which might be related with their lower neuroprotective activity at concentration of 10 μM.

Table 3. Effect of Compounds **20-31** on SH-SY5Y Cells Exposed to OGD[a].



20-31

Compd.	Substituents		Cell Viability (% of Control)	
	R^3	R^5	1 μM	10 μM
CPX	-H	-H	70.13 ± 0.91***	69.75 ± 4.17**
20	-Br	-H	62.88 ± 5.41	59.87 ± 7.08
21	-H	-Br	81.04 ± 6.97*	63.20 ± 11.04
22	-I	-H	76.35 ± 5.61**	55.12 ± 8.29
23	-Cl	-H	63.40 ± 4.55	57.08 ± 4.07

24	-CHO	-H	54.77 ± 6.49	56.52 ± 4.99
25		-H	60.90 ± 4.03	67.47 ± 7.40*
26		-H	61.01 ± 4.51	68.01 ± 2.47**
27		-H	60.95 ± 2.87	65.21 ± 4.25*
28		-H	63.62 ± 5.22	61.62 ± 8.41
29		-H	67.04 ± 5.96*	61.87 ± 5.02
30		-H	56.81 ± 3.69	75.47 ± 7.12**
31[b]		-H	60.00 ± 12.37	87.18 ± 2.07**[c]

OGD = 54.92 ± 2.12%###

[a]The data are presented as the percentage of surviving cells relative to control cells and as the mean ± SD, n=3. ###p < 0.001 vs control, *p < 0.05, **p < 0.01, ***p < 0.001 vs OGD group, one-way ANOVA, Dunnett's multiple comparison test. [b]The data of compound 31 is gathered from another assay of which the cell viability of OGD group was 54.23 ± 4.48% of control. [c]An exception, the concentration was 4 μM.

In the first round OGD experiment, the concentrations of the tested compounds are 1 μM and 10 μM. Among them, seven of the tested compounds (**4**, **6**, **10**, **11**, **21**, **22**, **29**) exerted neuroprotective effect at 1 μM, and they were selected for further studies. Although the cell viability of SH-SY5Y cells treated with 1 μM of compound**16** was beyond 70%, there was no statistical significance. An additional experiment confirmed that it is ineffective at 1 μM (Figure S6). Some of the derivatives showed

inferior neuroprotective effect at 10 μM compared to the effect at 1 μM, thus, in order to further evaluate the activity of these seven compounds, it was necessary to set more doses. Thus, we carried out another OGD experiment in which the doses of the compounds were ranged from 0.25 μM to 4 μM (0.25, 0.5, 1, 2, 4 μM, Figure 3). There were three compounds showed excellent neuroprotective activity (cell viability > 80%): **10** (at 2 μM), **11** (at 1, 2, 4 μM), **22** (at 2 μM). Among them, compound **11** provided excellent neuroprotective effect at 1, 2, 4 μM (cell viability of these 3 doses are all above 80%, $p < 0.001$).

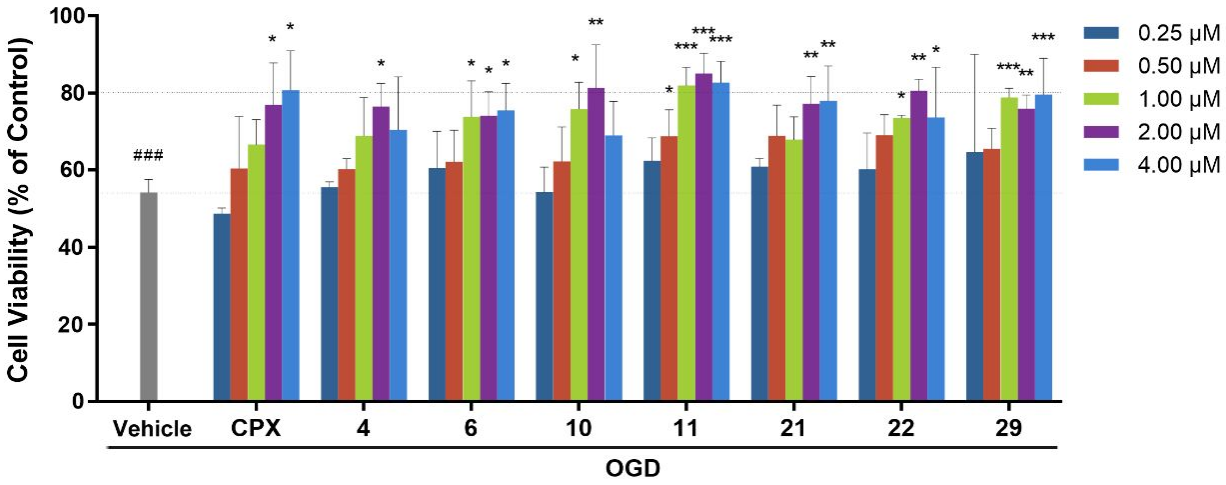


Figure 3. Neuroprotective Effect of Compounds **4**, **6**, **10**, **11**, **21**, **22**, **29** at Concentrations in the Range from 0.25 μM to 4 μM on SH-SY5Y Cells Exposed to OGD Injury. The data are presented as the percentage of surviving cells relative to control cells (100%) and as the mean ± SD, n=3, ###$p < 0.001$ vs control, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs OGD group, one-way ANOVA, Dunnett's multiple comparison test.

Structure and Activity Relationships

Analysis of the data revealed the structures and activities relationship (SAR) of these derivatives: (**I**). The *N*-hydroxyl is necessary for the neuroprotective activity. Replacing the *N*-hydroxyl with ethoxyl, 2-methoxy-2-oxoethoxy and carboxymethoxy (compounds **1-3**) leads to the loss of activity (Table 1). (**II**). Methyl is the optimal substituent at positon 4. Replacing methyl with hydrogen and ethyl reduced the neuroprotective activity of compounds **5-6** (Table 1). (**III**). Large cycloalkyl and bicycloalkyl substituents at position 6 are better than small cycloalkyl, alkyl, phenyl and benzyl substituents (compounds **10-11** vs **7-9, 12-19**, Table 2). (**IV**). Halogen substituents at position 3 or 5 has no obvious influence to the neuroprotective activity (compounds **20-23**, Table 3). (**V**). The *N*-substituted aminomethyl at position 3 could not improve the neuroprotective activity.

Antioxidant Ability of Pyridone Analogues.

In the pathological process of ischemia-reperfusion, there are excessive free radicals including ROS generated within the penumbra, causing oxidative damage.[21] Therefore the antioxidant property of compound is considered as an important indicator for its neuroprotective activity.[22, 23] The antioxidant activities of compounds with good neuroprotective activities in OGD model (exhibited significant improvement in cell viability compared with OGD group at 1 μM) were evaluated by the oxygen radical absorbance capacity assay (ORAC).[24] In this experiment, the fluorescent probe fluorescein can react with the peroxide radical induced by AAPH, which is widely used as the free radical reactions initiator, to generate a non-fluorescent substance, and the quenching of fluorescence can be slowed by antioxidants. This process closely mimics

the production and scavenging of in vivo peroxide radicals. Compared with other assays like DPPH assay, the ORAC method combines both inhibition time and degree of inhibition into a single quantity though measuring the kinetics of the decrease in fluorescence for each sample compared to a blank and building an antioxidant curves (fluorescence versus time, normalized to the curve of the blank).[25] In general, ORAC values were expressed as trolox equivalents which were calculated through plugging the area under the curve (AUC) into the regression equations between the net AUC and Trolox concentrations, the higher values indicated the better antioxidant activity. The results in Table 4 manifested that compound **11**, which exhibited the best neuroprotective effect in OGD model, also demonstrated superior antioxidant activity (1.13 ± 0.19) compared to CPX (0.52 ± 0.32).

BBB Permeability of Pyridone Analogues

Good BBB penetration ability is required for central nervous system drugs. To evaluate the BBB penetration ability of pyridone analogues, the permeability (P_e) values of compounds with good neuroprotective activities in OGD model (**4**, **6**, **10**, **11**, **21**, **22**, **29)** were determined through the parallel artificial membrane permeation assay (PAMPA, using lipid extract of porcine brain covered membrane). 13 marketed drugs were tested to build the standard that compounds with P_e values greater than 3.08×10^{-6} cm/s could cross the BBB by passive permeation (marked CNS+). Among them, compounds **11** (7.50×10^{-6} cm/s), **22** (7.65×10^{-6} cm/s) and CPX (8.17×10^{-6} cm/s) show good BBB permeation. In short, compound **11** demonstrated the best neuroprotective activity in OGD model, and it also has good BBB permeability ability

(7.50 × 10^{-6} cm/s) and better antioxidants capability (1.13 ± 0.19) compared to lead compound CPX (0.52 ± 0.32). Thus, compound **11** was selected for further studies.

Table 4. Oxygen Radical Absorbance Capacity (ORAC, Trolox Equivalents) and Permeability Results (P_e×10^{-6} cm s^{-1}) from the PAMPA-BBB Assay for Compounds **4**, **6**, **10**, **11**, **21**, **22**, **29**.

Compd.	ORAC[a]	P_e (10^{-6} cm s^{-1})[b]	Prediction
CPX	0.52 ± 0.32	8.17 ± 1.43	CNS+
4	NA	4.43 ± 0.72	CNS+
6	NA	4.53 ± 0.34	CNS+
10	NA	4.42 ± 0.57	CNS+
11	1.13 ± 0.19	7.50 ± 0.33	CNS+
21	NA	3.97 ± 0.25	CNS+
22	0.20 ± 0.17	7.65 ± 0.52	CNS+
29	NA	3.89 ± 2.01	CNS+

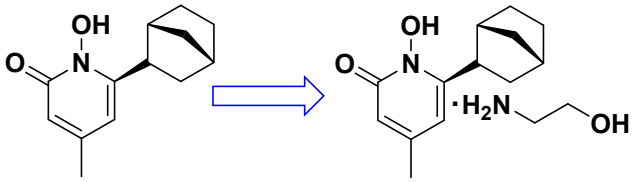
[a]Values are expressed as the mean ± SD of three independent experiments, NA means no activity. Data are expressed as μmol of Trolox equivalent/μmol of tested compounds. [b]Values are expressed as the mean ± SD of three independent experiments.

Water-Solubility of Compounds 11 and 11·Ola

Since marketed anti-stroke drugs include butylphthalide and edaravone are administered through injection, the water-solubility is important for anti-stroke agent. Therefore the solubility of **11** and its complex with olamine in normal saline (0.9 % for intravenous injection) was calculated by comparing the HPLC peak area to the calibration curves which were plotted as peak areas versus concentrations. However, compound **11** shows poor water-solubility (1.22 mg/mL). Ciclopirox olamine is the complex consist of ciclopirox and 2-aminoethan-1-ol (olamine is USAN-approved contraction for ethanolamine), it is water-soluble and widely used in external

medicament. Olamine is not only used in external medicament but also used in oral medicaments like eltrombopag olamine, and it is considered a low toxic compound. Thus, the complex of compound **11** and olamine (named **11·Ola** for short) was synthesized and it processed good water-solubility (10.90 mg/mL), as shown in Table 5. Compound **11·Ola** was an ideal form for intravenously administration in the in vivo experiment.

Table 5. Water-solubility of Compounds **11** and **11·Ola** (n = 3, mean ± SD).



Compd.	Water-solubility (mg/mL)
11	1.22 ± 0.01
11·Ola	10.90 ± 1.53
CPX	1.43 ± 0.03
CPX Olamine	6.82 ± 0.45

The Anti-apoptotic Effect of Compound 11·Ola on OGD-exposed SH-SY5Y Cells

Considering that inflammation and oxidative damage caused by the I/R injury can lead to neuronal apoptosis which results in neuron death in the penumbra, reduction of apoptotic rate is another important indicator of neuroprotective capability.[21, 26] Thus, Annexin-V-FITC and PI staining was performed to examine whether the neuroprotective effects of compound **11·Ola** on SH-SY5Y cells correlated with reduced apoptosis. The distribution of SH-SY5Y cells to the lower left quadrant

indicated that these cells were viable and without measurable apoptosis. OGD insults induced obvious increases in cell numbers in the lower and upper right quadrants, which indicated that these SH-SY5Y cells underwent early and late apoptosis. (Figure 4A) The percentage of SH-SY5Y cells at early and late stages of apoptosis increased from 15.88% to 40.98% in the OGD group compared to the control group (Figure 4B). Treatment with compound **11·Ola** at 4 μM significantly reduced the apoptotic ratio to 25.91% (Figure 4B). Moreover, the OGD treatment induced obvious morphological changes of SH-SY5Y cells, including neurite disappearance, bright and shrinking cell body, and reduced cell number. **11·Ola** treatment remarkably attenuated the cell morphological changes (Figure 4C). The results above indicated that treatment with **11·Ola** not only reduce the cell apoptosis but also protect the cell morphology against OGD injury.

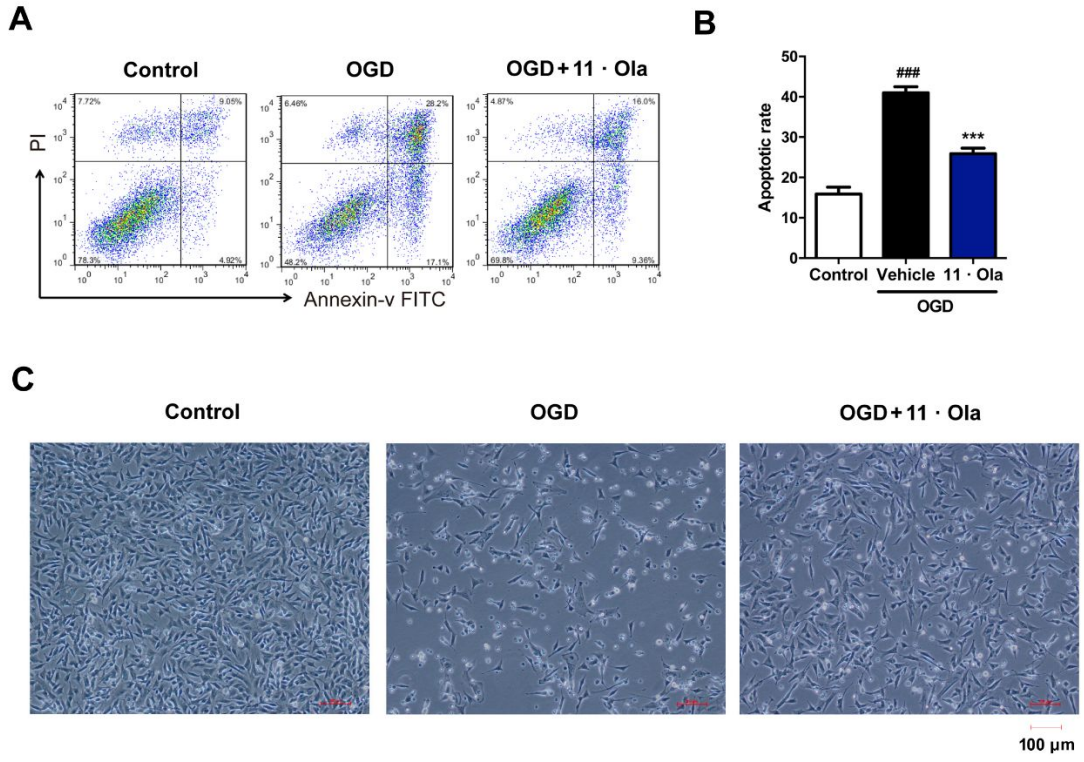


Figure 4. Compound **11·Ola** Decreases OGD-induced Apoptosis in SH-SY5Y Cells. (A) Representative Plot of Annexin V-FITC/PI Staining; (B) Statistical Results of Apoptotic rate (n = 4, error bars represent standard error of the mean (SEM), $^{###}p < 0.001$ vs control, $^{***}p < 0.001$ vs OGD group, t-test); (C) Photomicrographs of cell morphology (scale bar = 100 μm).

Neuroprotective Effect of Compound 11·Ola on Glutamate-exposed HT22 Cells and H_2O_2-exposed SH-SY5Y Cells.

In the process of I/R injury, the release of glutamate activates ionotropic glutamate receptors and causes excitotoxicity which can result in neuronal death. In addition, the consequent reactive oxygen species (ROS) are also account for neuronal death in the ischemic penumbra. Therefore, in vitro models using glutamate and H_2O_2 stimulus are widely used to mimic ischemic stroke injury.[27-30] In present study, we further evaluated the neuroprotection of optimal compound **11·Ola** on glutamate and H_2O_2 stimulated cellular models. As shown in Figure 5A, excessive glutamate exposure significantly reduced the cell viability of HT22 cells to 28.53% of the control, but incubation with **11·Ola** at concentrations of 0.5 μM, 1 μM, 2 μM and 4 μM significantly protected the cells against the glutamate insult, and the protective effect is similar to that of ciclopirox olamine. Moreover, as shown in Figure 5B, 100 μM H_2O_2 reduced cell viability to 45.73% of the control. Compound **11·Ola** at 1 μM, 2 μM and 4 μM promoted cell viability up to 80%, which is also similar to that of ciclopirox olamine. These results suggested that compound **11·Ola** could attenuate oxidative damage in SH-SY5Y cells and glutamate-induced toxicity in HT22 cells.

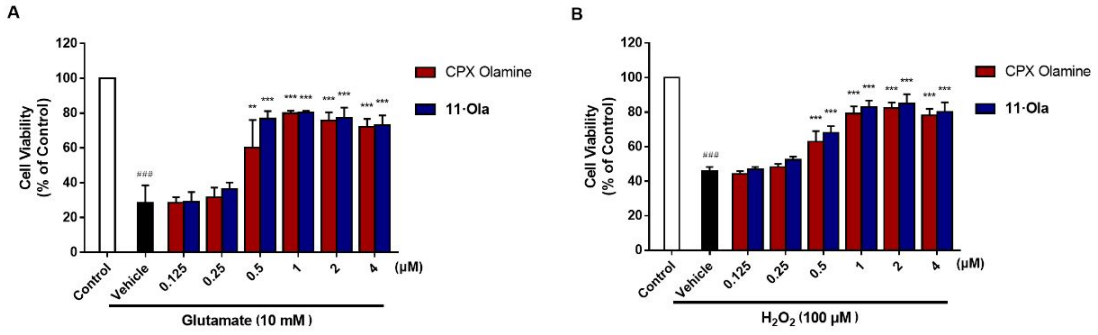


Figure 5. Neuroprotective Effect of Compound **11·Ola** on HT22 Cells Subjected to Glutamate Stimulus (A) and SH-SY5Y Cells Exposed to H_2O_2 Injury (B). The data are presented as the percentage of surviving cells relative to control cells (100%) and as the mean ± SD, n = 3 for (A), n = 4 for (B). ###$p < 0.001$ vs control, **$p < 0.01$, ***$p < 0.001$ vs glutamate group or H_2O_2 group, one-way ANOVA, Dunnett's multiple comparison test.

Iron Chelation and bSDTNBI Prediction Results

The hypoxia-inducible factor (HIF)-1α plays an important role in the regulation of gene expression in response to hypoxia and ischemia. It induces transcription of genes involved in energy metabolism, angiogenesis and erythropoiesis which promotes the neuronal survival under ischemic conditions.[31] HIF-1α is regulated by HIF-prolyl hydroxylases (PHDs), in other words, hydroxylation of the proline residue leads to the degradation of HIF-1α. Iron ion which is utilized as a cofactor in the hydroxylation process is necessary for the activity of PHDs. Moreover, it is reported that stabilizing HIF-1α through the inhibition of PHDs significantly reduced the infarct volumes and improved behavior after MCAO.[32] Previous study had confirmed that ciclopirox could induce HIF-1α stability through iron chelating.[33] In this study, in silico bSDTNBI

prediction also suggested the strong correlation between HIF1A gene (encoded HIF-1α) and the neuroprotective activity of CPX and compound **11** (CPX: HIF1A ranked 4th, **11**: HIF1A ranked 5th, Figure 6A).[34] The bSDTNBI (balanced substructure–drug–target network-based inference) is a network-based method which can identify new drug–target interactions (DTIs) for further understanding the mechanism of action (MoA). Derived from recommendation algorithms for networks, it uses resource diffusion processes to predict potential targets for both known drugs and new chemical entities and does not rely on either three-dimensional structures of target proteins or negative samples. Furthermore, it can predict potential ligands for targets for the development of potential targeted therapies (Figure S2 Schematic diagram of bSDTNBI). Here, iron chelating experiments was performed to clarify the relationship between the iron chelation and neuroprotective activity of **11**, CPX and **1**, and the ion chelating ability was determined by UV−visual spectroscopy. As shown in Table 1 & 2, both CPX olamine and **11·Ola** exerted good neuroprotective activity, and compound **1** showed no activity. As shown in Figure 6B & 6C, the absorption between 350 nm-500 nm increased after the addition of ammonium iron(III) citrate and $FeSO_4$ to both **11** solutions and the CPX solutions. The change of the absorption curves of **11** and CPX demonstrated the production of the iron chelating complex. In addition, the color charge of these solutions also corresponded with the curve change (Figure S3. photographs). The addition of the other three iron salts (ammonium iron(III) sulfate, ammonium iron(II) sulfate, $FeCl_3$) led to similar changes in the absorption curve of the solutions, which indicated that these changes result from iron ion, not anion. (Figure S4). On the

contrary, the curve of compound **1** was almost unchanged when $FeSO_4$ and ammonium iron(III) citrate were added into the solutions (Figure 6D), this indicated that compound **1** (no neuroprotection) did not chelate iron ion. These findings suggested that the neuroprotective activity of **11** and CPX might correlate with iron chelation and stabilization of HIF1-α.

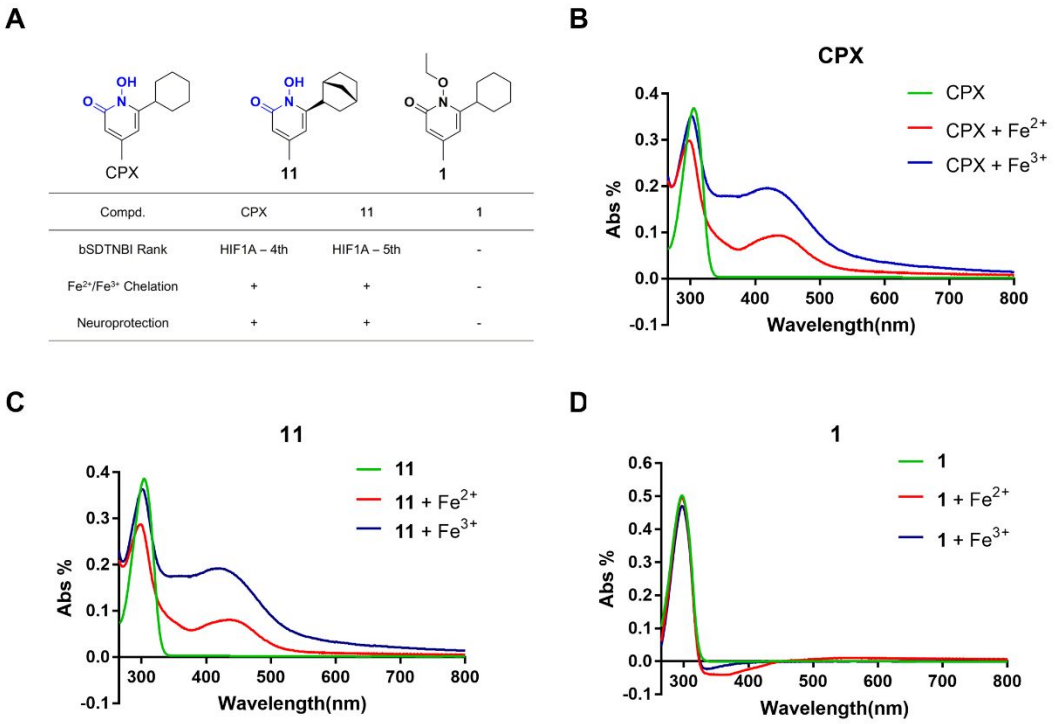


Figure 6. The bSDTNBI Prediction (A), Absorption Spectra of CPX, **11**, and **1** (60μM) Alone and in the Presence of $FeSO_4$, Ammonium Iron(III) Citrate (120 μM) (B, C, D).

Compound 11·Ola Attenuated Brain Injury of MCAO Rats.

In view of compound **11·Ola** possesses superior neuroprotective, good antioxidant ability, BBB permeability and water-solubility in vitro, its in vivo neuroprotective effect was further evaluated through a widely-used MCAO model for ischemic stroke study.[28, 35, 36] In our previous studies, the dose for CPX olamine in rat which afford the best efficacy was 3 mg/kg (i.v.), hence, this dose was used in our experiment for

comparison.[20] Due to the minimum effective dose of compound **11** is much lower than CPX (Figure 3), two experiment groups treated with 0.3 mg/kg (i.v.) and 1 mg/kg (i.v.) of compound **11·Ola** were set, respectively. As shown in Figure 7A, B, an obvious infarction (39%) was induced by MCAO insult, which displayed as the white region of brain sections. Treatment with 1 mg/kg of compound **11·Ola** significantly reduced the infarct volume to 23% (Figure 7A, B, $p < 0.01$ vs MCAO group) and decreased the neurological severity scores to 5 ($p < 0.01$ vs MCAO group, Figure 7C), and 0.3 mg/kg dose of compound **11·Ola** reduced the infarct volume to 25% ($p < 0.05$ vs MCAO group, Figure 7B). As a comparison, treatment with 3 mg/kg of CPX reduced the infarcted volume by 26% ($p < 0.01$ vs MCAO group) and decreased the neurological severity scores to 6 ($p < 0.05$ vs MCAO group). Above experiments indicated that compound **11·Ola** can significantly protected the brain and attenuated neurological deficits after I/R injury in vivo and its initial dose of efficacy is lower than that of CPX olamine. The protective effects of **11·Ola** at the dose of 1 mg/kg was comparable to that of CPX olamine at 3 mg/kg, and taken together the similar cytotoxicity of **11·Ola** and CPX olamine (see below Table 6), suggesting that **11·Ola** possesses superior safety window compared to CPX olamine.

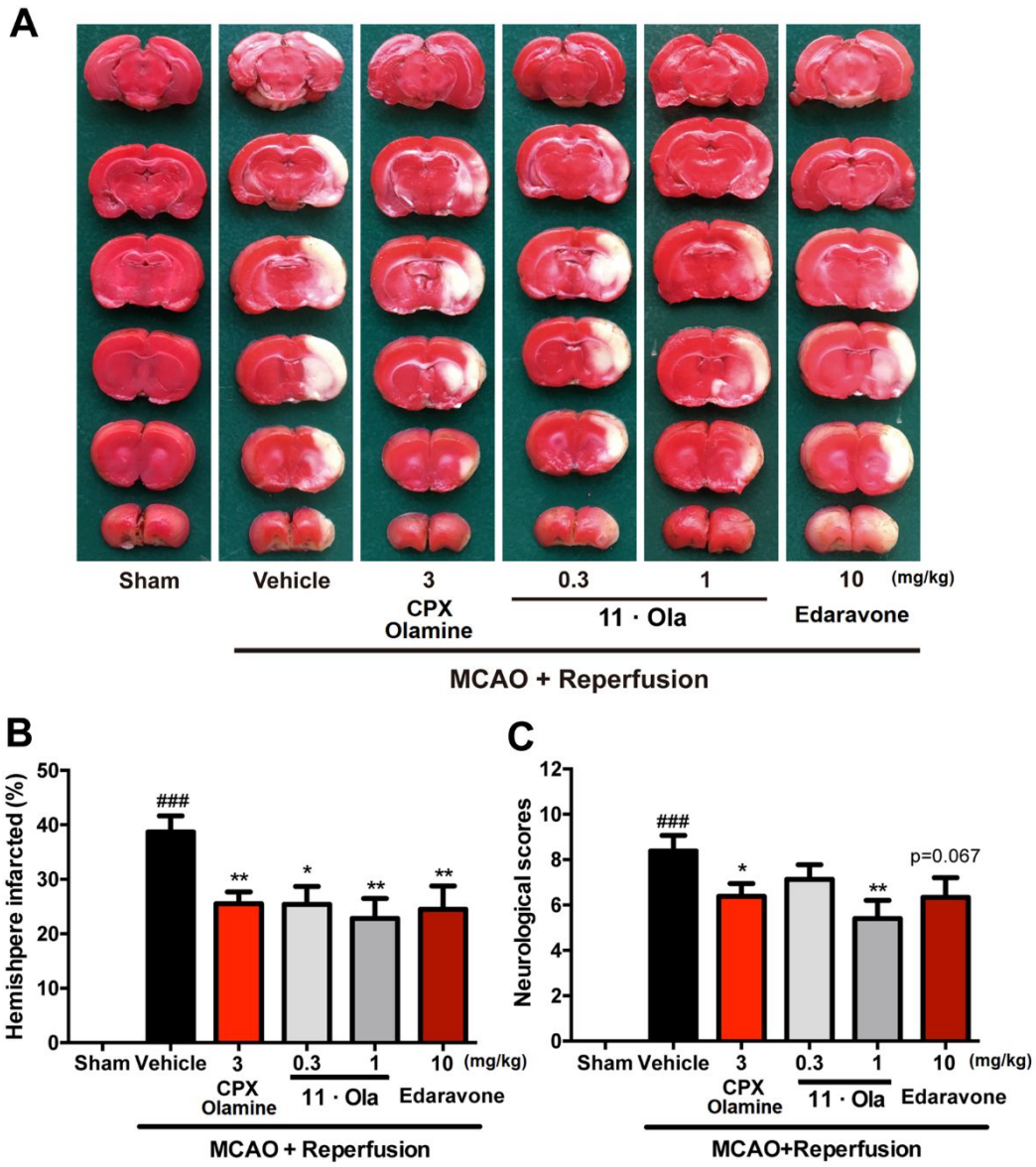


Figure 7. Effect of Compound **11·Ola** on Infarct Area and Neurological Function after 2 h of MCAO and 24 h of Reperfusion. (A) Representative Picture of TTC staining, (B) Quantification of Infarct Volumes, (C) Neurological Severity Scores. (n=15-18, data are presented as mean ± standard error of the mean (SEM), ###$p < 0.001$ vs sham group, *$p < 0.05$, **$p < 0.01$, vs vehicle-treated MCAO group, one-way ANOVA, Dunnett's multiple comparison test.).

The hERG Potassium Channels Inhibition, Cytotoxicity and Metabolic Stability of Compound 11·Ola and CPX Olamine

The safety is important for drug candidates, so we further evaluated the optimal compound **11·Ola** through hERG potassium channel inhibition assay and cytotoxicity assay. The hERG potassium channels inhibition can lead to the prolongation of Q-T interval, therefore, it is an important indicator of cardiotoxicity. The hERG IC_{50} value of compound **11·Ola** and CPX olamine is assessed through automated patch-clamp assay,[37] and the results in Table 6 shows that the hERG inhibition of compound **11·Ola** and CPX olamine is negligible (IC_{50} values of them are above 40 μM, Table 6). This suggests that these two compounds are unlikely to cause cardiovascular safety risk arising from hERG inhibition.

MRC-5 is a human cell line composed of fibroblasts which were derived from the embryonal lung tissue, and it is widely used in the evaluation of the toxicity in medicinal chemistry research.[38, 39] The IC_{50} values in SH-SY5Y cell line were assessed as the indicator of neurotoxicity. Therefore, we assessed the cytotoxicity of **11·Ola** and CPX olamine against MRC-5 and SH-SY5Y as indicators of the safety of these two compounds. As shown in Table 6, the IC_{50} of both **11·Ola** and CPX olamine are all above 100 μM. So these two compounds are safe for neurons at their therapeutic doses. Considering that neuroprotective agents are administered continuously during the middle and late stages, we prolonged the incubating time to 72 h for MRC-5, and the IC_{50} values of **11·Ola** and CPX olamine in MRC-5 cell line at 72 h are 23.60 ± 1.15 μM and 23.47 ± 4.19 μM respectively (Table 6). To compare the activity of **11·Ola** and

CPX olamine, we selected the dose which reduced the infarct volume to about 25% (control = 38.68%) in the MCAO experiments as an indicator to present their activity. The dose of **11·Ola** is 0.3 mg/kg, and that of CPX olamine is 3 mg/kg (Figure 7). Considering that the cytotoxicity of compound **11·Ola** and CPX olamine is at the same level, the treatment with lower doses of **11·Ola** possesses superior safety window compared to CPX olamine.

The metabolic stability of compound **11·Ola** and CPX olamine was evaluated through in vitro mouse liver microsome experiments. As shown in Table 6, compound **11·Ola** ($T_{1/2}$ = 91.63 min) showed superior metabolic stability compared to CPX olamine ($T_{1/2}$ = 53.14 min), which is conducive to the improvement of long-term efficacy. We further evaluated the in vivo blood plasma concentrations of **11·Ola** and CPX olamine after intravenous administration in rats. As shown in Figure S68, both of them were rapidly cleared in rats' plasma, but the group treated with **11·Ola** maintained higher plasma concentration compared with the group treated with CPX olamine. In general, **11·Ola** was metabolized more slowly compared with CPX olamine.

Table 6. The hERG Potassium Channels Inhibition, Cytotoxicity and Metabolic Stability of Compound **11·Ola** and CPX olamine

Compd.	hERG	IC_{50}(μM)		Dose in Rat (reduce infarct	$T_{1/2}$ in Mouse
	IC_{50} (μM)	SH-SY5Y, 24h	MRC-5,72h	volume to < 26%)	Microsomes(min)
11·Ola	> 40	>100	23.60 ± 1.15	0.3 mg/kg	91.63
CPX olamine	> 40	>100	23.47 ± 4.19	3 mg/kg	53.14

CONCLUSIONS

In order to find potential anti-ischemic stroke agent, we designed and synthesized 31 *N*-hydroxypyridone derivatives based on the structure of CPX which was identified as a lead compound in our previous study. Then the neuroprotective effect of these compounds at 1 μM and 10 μM was evaluated by OGD model. Among these derivatives, seven compounds (**4, 6, 10, 11, 21, 22, 29**) which exhibited potent neuroprotective effect at 1 μM were selected for the 2nd round screening with more concentrations ranging from 0.25 to 4 μM (0.25, 0.5, 1, 2, 4 μM, Figure 3). Among them, compound **11** exhibited significant neuroprotective effects (cell viability > 80% at 1, 2, 4 μM) against OGD-induced neuronal damage in SH-SY5Y cells compared to CPX (Figure 3). Moreover, optimal compound **11** possessed good BBB permeability (7.50×10^{-6} cm/s, Table 4) and better antioxidant capability (1.13 ± 0.19 vs CPX, 0.52 ± 0.32, Table 4). In addition, complex of compound **11** and olamine — **11·Ola** possessed good water solubility.

Treatment with compound **11·Ola** at 4 μM significantly suppressed the apoptotic ratio in SH-SY5Y cells after OGD exposure (Figure 4). Besides, treatment with compound **11·Ola** at 0.5, 1, 2, 4 μM significantly attenuated glutamate induced HT22 cell damage and H_2O_2 induced SH-SY5Y cell damage (Figure 5). These results further verify the neuroprotective activity of compound **11·Ola**, and also indicated that it might exhibit neuroprotective effects through anti-apoptosis and attenuating oxidative damage and oxidative glutamate toxicity. The safety of **11·Ola** was evaluated through hERG inhibition assay and cytotoxicity assay. As the hERG inhibition IC_{50} values of

both **11·Ola** and CPX olamine are all above 40 μM, these two compounds are unlikely to cause hERG inhibition at therapeutic dose. The IC_{50} values in MRC-5 cell line of **11·Ola** and CPX olamine are all around 23 μM (Table 6), and the IC_{50} values of them in SH-SY5Y cell line are above 100 μM (Table 6). In the in vivo experiment (MCAO rats, 24 h after the reperfusion), compound **11·Ola** exhibited marked neuroprotective effect at lower doses (0.3 mg/kg and 1 mg/kg for **11·Ola** vs 3 mg/kg for CPX olamine). Treatment with 0.3 mg/kg and 1 mg/kg of compound **11·Ola** significantly reduced brain infarct volume. Besides, the treatment with 1 mg/kg of compound **11·Ola** significantly reduced the neurological score (Figure 7). Considering that compound **11·Ola** and CPX olamine have similar cytotoxicity and **11·Ola** has lower effective doses, compound **11·Ola** possesses superior safety window compared with CPX olamine. Moreover, compound **11·Ola** ($T_{1/2}$ = 91.63 min) showed superior metabolic stability compared to CPX olamine ($T_{1/2}$ = 53.14 min) in the mouse liver microsomes experiments.

To conclude, among these derivatives investigated, the BBB-permeable and water-soluble compound **11·Ola** showed superior neuroprotective activity, good safety and metabolic stability in vitro and lower effective dose in vivo compared to CPX olamine. Compound **11·Ola** could be a promising drug candidate for the treatment of ischemic stroke.

EXPERIMEMTAL SECTION

Chemistry

Reagents and solvents were purchased from Adamas-beta, J&K, Energy Chemical, TCI, Alfa Aesar, and Bide Pharmatech and were used without further purification. Analytical thin-layer chromatography (TLC) was performed using HSGF 254 (0.2 mm thickness; Yantai Huiyou Co., China), and spots were visualized with UV light. Yields were not optimized. Melting points were measured in capillary tubes on a SGW X-4 melting point apparatus without correction. ^{1}H and ^{13}C Nuclear magnetic resonance (NMR) spectroscopy was performed on a Bruker AMX-400MHz NMR and Ascend 600MHz NMR (IS as TMS). Chemical shifts were reported in parts per million (ppm, δ) downfield from tetramethylsilane. Proton coupling patterns were described as singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m), and broad (br). High-resolution mass spectra (HRMS) were obtained by electric ionization (EI) and electrospray ionization (ESI) using a Waters GCT Premie and Waters LCT. HPLC data analysis of compounds **1-31** was performed on an Agilent 1100 with a quaternary pump and diode-array detector (DAD). The peak purity was verified by UV spectra. All analogs were confirmed to be ≥ 95% pure (Table S1, supporting information). The NMR spectroscopy and synthetic methods of intermediates are also contained in supporting information.

General Method A (Reactions j, k, m, n in Scheme 1) for Compounds 6-19:

This synthetic route was modified from several reported methods.[40, 41]

Reaction j in Scheme 1: To a vigorously stirred solution of $AlCl_3$ (3.99 g, 30 mmol) in dichloromethane (10 mL) at 0°C, a mix of methyl 3-methylbut-2-enoate (**A0** for compounds **7-19**, purchased from Energy Chemical) / **A6** (methyl (*Z*)-3-methylpent-2-

enoate, for compound 6) (10 mmol) and corresponding carboxyl acid chloride (10 mmol) in dichloromethane (5 mL) was added dropwise. The reaction was refluxed until the emission of HCl gas ceased (typically 3.5 h). The mixture was then cooled to room temperature, and quenched with 20ml 6N HCl (aq.). The organic phase was separated, and the aqueous phase was extracted with dichloromethane (20 mL × 3). The combined dichloromethane solution was washed with saturated $NaHCO_3$ water solution, dried by Mg_2SO_4, and concentrated under reduced pressure to provide the crude γ-acyl substituted α,β-unsaturated ester which was used in the next step without further purification. The yield for this step range from 55 - 85%.

Reaction k in Scheme 1: The crude γ-acyl substituted α, β-unsaturated ester of dissolved in acetic acid (1 mL/1 mmol), then concentrated H_2SO_4 (0.3 mL/1 mmol) was added. After 4.5h stirring at 105°C, the reaction mixture was cooled to room temperature and quenched with saturated $NaHCO_3$ water solution carefully. The resulting mixture was extracted with ethyl acetate. The ethyl acetate solution was washed by brine, dried by $NaSO_4$, and concentrated under reduced pressure to give crude product. The crude product was purified by column chromatography (silica gel, ethyl acetate/hexane, 1:10) to provide **B6-B18**. (**B19** was synthesized through an additional step reaction l using 6-(bromomethyl)-4-methyl-2H-pyran-2-one and phenylboronic acid as reactants). The yield for this step range from 15 - 35%.

Reaction m in scheme1: Compounds **C6-C17** and **C19** were synthesized through reaction m using compounds **B6-B17** and **B19** as reactants. The pyrone (referred to one of compounds **B6-B17** and **B19**) and P_4S_{10} (2.5 eq.) were refluxed in toluene (8 ml/1

mmol) for 6 h. The resulting mixture was filtered, and the filtrate was concentrated under reduced pressure to give the crude product. (The stinky filter cake was destroyed by NaClO (aq.)) The crude product was purified by column chromatography (silica gel, ethyl acetate/hexane, 1:10) to provide compounds **C6-17** and **C19**. The yield for this step range from 55 - 80%. To avoid the unpleasant smell, the instrument used in this reaction must be washed by NaClO (aq.).

Reaction n in scheme1: Compounds **C6-C17** and **C19** and hydroxyamine hydrochloride (5eq.) were refluxed in pyridine (10 mL/1 mmol) for 6 h. The reaction mixture was concentrated under reduced pressure, and the crude product was then purified by column chromatography (SiO_2, methanol: dichloromethane, from 1:40 to 1:15) to provide Compounds **6-19**. The yield for this step range from 30-50%. For the synthesis of compound 18, the method is adjusted: compound **B18** (372 mg, 2 mmol) and hydroxylamine hydrochloride (2.45 g, 35 eq.) was refluxed in pyridine (10 ml) for 18h, the reaction mixture was concentrated under reduced pressure, and purified by the same method (see above) to give compound 18 (150 mg, yield 37%).

General Method B (Reaction a in Scheme 2) for Compounds 20-23:

CPX (purchased from Bide pharmatech), AIBN (0.05eq.) and NBS (for 20, 21)/NIS (for 22)/NCS (for 23) (1.05eq.) was refluxed in CCl_4 for 6 h. The reaction mixture was cooled to 0 °C and the succinimide was filtered and removed. The filtrate was concentrated in reduced pressure, and the resulting crude product was washed by hexane, and purified by column chromatography (silica gel, methanol: dichloromethane, 1:20) to provide Compounds **20**, **22** and **23**. (yield: **20**: 90%, **22**: 63%, **23**: 70%) The

hexane solution which was used for washing crude compound **20** was collected. Then the solution was cooled to -20 °C, and the resulting suspension was filtered. The filtrate was concentrated under reduced pressure, and the crude product was purified by column chromatography (silica gel, methanol: dichloromethane, 1:30) to provide Compounds **21**. (yield = 5%)

General Method C (Reaction c in Scheme 2) for Compounds 25-31:

Compound **25** and the corresponding amine (2 eq.) was stirred at room temperature in 1, 2-dichloroethane (10 mL/1 mmol) for 30min. Then sodium triacetoxyborohydride (2 eq.) was added. After stirring overnight, the reaction mixture was quenched by water, and the organic phase was separated. The aqueous phase was extracted with dichloromethane for 2 times, and the combined organic phase was washed by brine and dried by Na_2SO_4. It was then concentrated under reduced pressure, and the crude product purified by column chromatography (silica gel, methanol: dichloromethane, 1:15) to provide Compounds **25-31**.

6-cyclohexyl-1-ethoxy-4-methylpyridin-2(1H)-one (1). 414 mg ciclopirox was dissolved in 5 mL DMF. Then 160 μL iodoethane and 300 mg K_2CO_3 was added. The mixture was heated to 60 degree Celsius. The reaction was quenched with 50 mL Li_2CO_3 water solution (4 mol/L) at 0 degree Celsius when TLC indicated the reaction to be completed. The resulting solution was extracted with DCM (50 mL × 3). At last, the combined organic phase was dried over $MgSO_4$, and concentrated in vacuo to afford 350 mg compound **1**, white solid, mp = 68 - 72 °C, yield = 74%. ^{1}H NMR (400 MHz,

Chloroform-*d*) δ 6.32 (s, 1H), 5.79 (s, 1H), 4.27 (q, *J* = 7.1 Hz, 2H), 2.82 – 2.72 (m, 1H), 2.15 (s, 3H), 2.02 – 1.92 (m, 2H), 1.91 – 1.83 (m, 2H), 1.82 – 1.72 (m, 1H), 1.51 – 1.14 (m, 8H). ^{13}C NMR (151 MHz, Chloroform-*d*) δ 159.53, 153.15, 149.76, 117.55, 104.48, 72.16, 38.43, 32.57, 26.57, 25.96, 21.46. HRMS (ES) m/z calcd for $C_{14}H_{21}NO_2$ $[M+H]^+$ 236.1650, found 236.1652.

Methyl 2-((6-cyclohexyl-4-methyl-2-oxopyridin-1(2H)-yl)oxy)acetate (2). 352 mg methyl 2-bromoacetate and 400 mg K_2CO_3 was added in a 8ml acetonitrile solution of 414 mg ciclopirox. The reaction was refluxed overnight. Then the mixture was diluted in 100 mL ethyl acetate, ethyl acetate solution was washed with saturated $NaHCO_3$ water solution. The organic phase was dried and concentrated to afford 468 mg compound 2, pale-yellow oil, yield = 80%. ^{1}H NMR (400 MHz, Chloroform-*d*) δ6.28 (s, 1H), 5.82 (s, 1H), 4.95 (s, 2H), 3.80 (s, 3H), 3.15 – 3.00 (m, 1H), 2.15 (s, 3H), 2.04 – 1.92 (m, 2H), 1.90 – 1.82 (m, 2H), 1.80 – 1.72 (m, 1H), 1.55 – 1.10 (m, 5H). ^{13}C NMR (151 MHz, Chloroform-*d*) δ 168.77, 160.26, 153.09, 152.72, 116.58, 107.28, 78.22, 38.50, 32.57, 26.36, 25.72, 21.71. HRMS (EI) m/z calcd for $C_{15}H_{21}NO_4$ M^+ 279.1471, found 279.1472.

2-((6-cyclohexyl-4-methyl-2-oxopyridin-1(2H)-yl)oxy)acetic acid (3). 200 compound 2 was dissolved in 6 mL THF, then 200 mg lithium hydroxide in 2 mL water was added. The reaction was stirred fiercely at 40 °C overnight. The resulting mixture was quenched with 20 mL 1M HCl solution at 0 degree Celsius, then the mixture was extracted with ethyl acetate (50 mL × 3). The organic phase was dried and concentrated

in vacuo to afford 120 mg Compound **3**, white powder, mp = 121 - 125 °C, yield=67%. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 6.47 (s, 1H), 6.05 (s, 1H), 4.70 (s, 2H), 2.85 – 2.72 (m, 1H), 2.24 (s, 3H), 2.05 – 1.93 (m, 2H), 1.96 – 1.86 (m, 2H), 1.86 – 1.77 (m, 1H), 1.52 – 1.18 (m, 5H). ^{13}C NMR (151 MHz, Chloroform-*d*) δ 168.98, 160.21, 153.10, 152.54, 116.64, 107.08, 77.95, 38.48, 32.57, 26.36, 21.70. HRMS (EI) m/z calcd for $C_{14}H_{19}NO_4$ M^+ 265.1314, found 265.1319.

3-cyclohexyl-2-hydroxyisoquinolin-1(2H)-one (4). 0.31 g (1.24 mmol) **4b** was added into a solution of 0.52 g (7.44 mmol, 6 eq.) hydroxylamine hydrochloride and 0.62 g (11.2 mmol, 9 eq.) KOH in 8ml methanol. The mixture was refluxed for 8h, and then quenched with 2N HCl at 0 °C. The resulting suspension was extracted with DCM (70 mL × 3). The combined organic phase was dried and concentrated in vacuo to afford the crude product. It was then purified through column chromatography (silica gel, methanol: DCM = 1:15) to afford 190 mg **4**, white solid, mp = 117 - 121 °C, yield = 65%. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.35 (d, *J* = 8.2 Hz, 1H), 7.63 (t, *J* = 7.2 Hz, 1H), 7.56 (d, *J* = 8.2 Hz, 1H), 7.47 (t, *J* = 7.2 Hz, 1H), 6.45 (s, 1H), 3.16 – 3.00 (m, 1H), 2.20 – 2.10 (m, 2H), 1.96 - 1.86 (m, 2H), 1.86 – 1.76 (m, 1H), 1.61 – 1.16 (m, 5H). ^{13}C NMR (151 MHz, Chloroform-*d*) δ 157.26, 144.38, 135.45, 131.73, 126.60, 126.11, 125.98, 101.39, 38.15, 31.73, 26.41, 26.13. HRMS (EI) m/z calcd for $C_{15}H_{17}NO_2$ M^+ 243.1259, found 243.1260.

6-cyclohexyl-1-hydroxypyridin-2(1H)-one (5). 121 mg **5b** was refluxed in 6ml acetyl chloride for 12 h. Then the acetyl chloride was removed under reduced pressure, 6ml methanol was added. The reaction mixture was reflux for another 12 h, and then

concentrated in vacuo. The resulting crude product was purified through column chromatography (silica gel, methanol: DCM=1:15) to afford 60 mg **5**, orange powder, mp = 142 - 146 °C, yield =50%. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.59 (t, *J* = 8.0 Hz, 1H), 7.14 (d, *J* = 8.0 Hz, 1H), 6.59 (d, *J* = 8.0 Hz, 1H), 5.98 - 5.78 (brs, 1H), 3.30 – 3.16 (m, 1H), 2.15 – 2.05 (m, 2H), 1.94 – 1.84 (m, 2H), 1.84 – 1.74 (m, 1H), 1.60 – 1.15 (m, 5H). ^{13}C NMR (151 MHz, Chloroform-*d*) δ 157.77, 149.81, 136.81, 112.41, 102.77, 38.25, 31.10, 26.23, 25.96. HRMS (EI) m/z calcd for $C_{11}H_{15}NO_2$ M^+ 193.1103, found 193.1096.

Ethyl(*Z*)-3-methylpent-2-enoate (A6). To a suspension of 950 mg CuI in 8 mL anhydrous THF at -10 °C, 6.5 mL CH_3Li (1.6 M in diethoxymethane, Adamas-beta) was added under nitrogen. After 20 min, orange $(CH_3)_2CuLi$ was formed. At this moment, 630 mg ethyl pent-2-ynoate in 5 mL THF was added dropwise. The reaction mixture was allowed to warm to room temperature, and stirred for 3 h. Then it was quenched with NH_4Cl (aq.), and extracted with ethyl acetate. The organic phase was dried with anhydrous Na_2SO_4 and concentrated in vacuo to give 430 mg ethyl (Z)-3-methylpent-2-enoate, colorless oil, yield = 60%. The NMR spectra of **A6** is consistent with the published data.[42]

6-cyclohexyl-4-ethyl-1-hydroxypyridin-2(1H)-one (6). Yellow solid, mp = 88 - 92 °C, yield = 42%. ^{1}H NMR (400 MHz, Methanol-*d*4) δ 6.37 (s, 1H), 6.22 (s, 1H), 3.02-3.12 (m, 1H), 2.55 (q, *J* = 7.6 Hz, 2H), 2.08 – 1.98 (m, 2H), 1.96 - 1.75 (m, 3H), 1.54 – 1.27 (m, 5H), 1.22 (t, *J* = 7.6 Hz, 3H). ^{13}C NMR (151 MHz, Methanol-d_4) δ 159.43, 151.42, 111.38, 104.49, 42.12, 38.17, 33.97, 31.16, 28.00, 26.08, 25.69, 13.01,

10.14. HRMS (EI) m/z calcd for $C_{13}H_{19}NO_2$ M^+ 221.1416, found 221.1417.

6-cyclopropyl-1-hydroxy-4-methylpyridin-2(1H)-one (7). Yellow powder, mp = 122 -125 °C, yield = 30%. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 6.42 (s, 1H), 5.99 (s, 1H), 4.15 (t, *J* = 6.3 Hz, 2H), 2.84 (t, *J* = 7.6 Hz, 2H), 2.22 (s, 3H), 2.15-2.20 (m, 1H). ^{13}C NMR (151 MHz, Chloroform-*d*) δ 171.16, 159.88, 146.53, 112.00, 110.55, 63.73, 27.58, 25.84, 20.98. HRMS (EI) m/z calcd for $C_9H_{11}NO_2$ M^+ 165.0790, found 165.0791.

6-cyclobutyl-1-hydroxy-4-methylpyridin-2(1H)-one (8). Pale yellow solid, mp = 100 -103 °C, yield = 35% ^{1}H NMR (400 MHz, Chloroform-*d*) δ 6.43 (s, 1H), 6.03 (s, 1H), 3.70-3.80 (m, 1H), , 2.35-2.47 (m, 2H), 2.25 (s, 3H), 2.00-2.20 (m, 3H), 1.89 (d, J = 9.4 Hz, 1H). ^{13}C NMR (151 MHz, Chloroform-*d*) δ 157.59, 148.48, 146.62, 112.19, 105.00, 35.04, 27.25, 21.59, 18.40 . HRMS (EI) m/z calcd for $C_{10}H_{13}NO_2$ M^+ 179.0946, found 179.0947.

6-cyclopentyl-1-hydroxy-4-methylpyridin-2(1H)-one (9). Yellow powder, mp = 85 -88 °C, yield = 45%. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 6.41 (s, 1H), 6.04 (s, 1H), 3.44 – 3.24 (m, 1H), 2.22 (s, 3H), 2.18 – 2.06 (m, 2H), 1.91 – 1.50 (m, 6H). ^{13}C NMR (151 MHz, Chloroform-*d*) δ 157.62, 148.41, 147.44, 111.81, 104.71, 39.74, 31.09, 25.13, 21.63. HRMS (EI) m/z calcd for $C_{11}H_{15}NO_2$ M^+ 193.1103, found 193.1104.

6-cycloheptyl-1-hydroxy-4-methylpyridin-2(1H)-one (10). White solid, mp = 94 – 97 °C, yield = 32%. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 6.36 (s, 1H), 5.98 (s, 1H), 3.08-3.18 (m, 1H), 2.21 (s, 3H), 1.95-2.06 (m, 2H), 1.80-1.90 (m, 2H), 1.75 – 1.64

(m, 2H), 1.50-1.65 (m, 6H). ^{13}C NMR (151 MHz, Chloroform-*d*) δ 159.75, 153.38, 144.11, 111.02, 107.50, 39.22, 33.50, 27.94, 26.89, 21.17. HRMS (EI) m/z calcd for $C_{13}H_{19}NO_2$ M^+ 221.1416, found 221.1417.

6-((1*R*,2*S*,4*S*)-bicyclo[2.2.1]heptan-2-yl)-1-hydroxy-4-methylpyridin-2(1H)-one (11). Pale yellow powder, mp = 138-141°C, yield = 50%. ^{1}H NMR (400 MHz, $CDCl_3$): *δ* 6.37 (s, 1H), 5.99 (s, 1H), 2.94-3.06 (m, 1H), 2.48(s,1H), 2.38 (s, 1H), 2.23(s,3H), 1.89(t, J=11.0Hz, 1H), 1.47-1.77(m, 3H), 1.20-1.46(m, 4H); ^{13}C NMR (151 MHz, Chloroform-*d*) δ 157.68, 148.28, 148.09, 111.63, 104.59, 41.51, 40.08, 37.10, 36.65, 36.50, 29.80, 28.66, 21.72. HRMS (EI) m/z calcd for $C_{13}H_{17}NO_2$ M^+ 219.1259, found 219.1265.

6-((1*R*,2*S*,4*S*)-bicyclo[2.2.1]heptan-2-yl)-1-hydroxy-4-methylpyridin-2(1H)-one·olamine (11·Ola). 900 mg 11 was dissolved in 3 mL hot ethyl acetate in 55 °C，then 0.3 mL olamine was added. After stirring for 10 minutes, the mixture was cooled to room temperature, and the suspension was filtered and washed with ethyl acetate to afford 1.05 g 11 olamine, white solid, mp = 120 °C(dec), yield = 91%. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 6.32 (s, 1H), 5.98 (s, 1H), 3.75-4.15 (brs, 4H), 3.63 (t, *J* = 5.1 Hz, 2H), 2.95-3.05 (m, 1H), 2.88 (s, 2H), 2.46 (s, 1H), 2.37 (s, 1H), 2.21 (s, 3H), 1.98 – 1.78 m, 1H), 1.76 – 1.17 (m, 7H). ^{13}C NMR (100 MHz, Chloroform-*d*) δ 160.68 , 152.21 , 144.56 , 111.86 , 105.50 , 60.71 , 43.20 , 42.00 , 40.23 , 37.83 , 36.66 , 30.01 , 28.76 , 21.30 . HRMS (EI) m/z calcd for $C_{13}H_{17}NO_2$ M^+ 219.1259, found 219.1262.

1-hydroxy-4,6-dimethylpyridin-2(1H)-one (12). Yellow solid, mp = 119-122°C, yield = 35%. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 6.45 (s, 1H), 6.05 (s, 1H), 2.45 (s,

3H), 2.22 (s, 3H). ^{13}C NMR (151 MHz, Chloroform-*d*) δ 157.90, 148.27, 140.89, 112.52, 108.34, 21.30, 16.83. HRMS (EI) m/z calcd for $C_7H_9NO_2$ M^+ 139.0633, found 139.0634.

1-hydroxy-4-methyl-6-propylpyridin-2(1H)-one (**13**). Orange solid, mp = 78 - 81 °C, yield = 50%. ^{1}H NMR (400 MHz, Chloroform-*d*) δ6.44 (s, 1H), 6.02 (s, 1H), 5.25 – 5.05 (brs, 1H), 2.74 (t, *J* = 7.7 Hz, 2H), 2.23 (s, 3H), 1.74 (q, *J* = 7.5 Hz, 2H), 1.01 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (151 MHz, Chloroform-*d*) δ 148.55, 144.34, 112.44, 107.57, 32.34, 21.44, 20.51, 13.71. HRMS (EI) m/z calcd for $C_9H_{13}NO_2$ M^+ 167.0946, found 167.0949.

1-hydroxy-6-isobutyl-4-methylpyridin-2(1H)-one (14). Yellow oil, yield = 32%. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 6.45 (s, 1H), 6.34 (s, 1H), 2.28 (s, 3H), 2.03-2.13 (m, 1H), 1.38 (s, 6H), 1.20-1.30 (m, 2H). ^{13}C NMR (151 MHz, Chloroform-*d*) δ 157.55, 148.25, 143.05, 112.18, 108.50, 39.52, 26.77, 22.37, 21.43. HRMS (EI) m/z calcd for $C_{10}H_{15}NO_2$ M^+ 181.1103, found 181.1104.

1-hydroxy-4-methyl-6-pentylpyridin-2(1H)-one (15). Orange solid, mp = 42 - 45 °C, yield = 49%. ^{1}H NMR (400 MHz, Chloroform-d) δ 6.40 (s, 1H), 5.98 (s, 1H), 2.74 (t, *J* = 7.8 Hz, 2H), 2.22 (s, 3H), 1.80 – 1.57 (m, 2H), 1.37 (q, *J* = 3.8 Hz, 4H), 1.01 – 0.77 (m, 3H). ^{13}C NMR (151 MHz, Chloroform-*d*) δ 157.67, 148.44, 144.37, 112.22, 107.24, 31.34, 30.34, 26.82, 22.36, 21.45, 13.94. HRMS (EI) m/z calcd for $C_{11}H_{19}NO_2$ M^+ 195.1259, found 195.1261.

6-heptyl-1-hydroxy-4-methylpyridin-2(1H)-one (16). Orange solid, mp = 53 –

56 °C, yield = 50%. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 6.38 (s, 1H), 5.98 (s, 1H), 2.89 – 2.66 (m, 2H), 2.20 (s, 3H), 1.69 (p, *J* = 7.5 Hz, 2H), 1.46 – 1.21 (m, 8H), 0.89 (t, J = 6.7 Hz, 3H). ^{13}C NMR (151 MHz, Chloroform-*d*) δ 157.71, 148.62, 144.63, 112.26, 107.52, 31.70, 30.40, 29.17, 28.96, 27.15, 22.61, 21.46, 14.08. HRMS (EI) m/z calcd for $C_{13}H_{21}NO_2$ M^+ 223.1572, found 223.1573.

1-hydroxy-4-methyl-6-nonylpyridin-2(1H)-one (17). Orange solid, mp = 59 – 62 °C, yield = 50%. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 6.40 (s, 1H), 5.98 (s, 1H), 2.80 – 2.65 (m, 2H), 2.21 (s, 3H), 1.69 (p, *J* = 7.7 Hz, 2H), 1.46 – 1.14 (m, 12H), 0.98 – 0.75 (m, 3H). ^{13}C NMR (151 MHz, Chloroform-*d*) δ 157.66, 148.51, 144.41, 112.20, 107.34, 31.87, 30.37, 29.45, 29.29, 29.20, 27.14, 22.67, 21.46, 14.11. HRMS (EI) m/z calcd for $C_{15}H_{25}NO_2$ M^+ 251.1885, found 251.1884.

1-hydroxy-4-methyl-6-phenylpyridin-2(1H)-one (18). Compound **B18** (372 mg, 2 mmol) and hydroxylamine hydrochloride (2.45 g, 35 eq.) was refluxed in pyridine (10ml) for 18h, the reaction mixture was concentrated under reduced pressure, and purified by the same method (see above) to give compound **18**, yellow powder, mp = 110-115 °C, yield = 37%. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.70 – 7.60 (m, 2H), 7.53 – 7.45 (m, 2H), 7.42 – 7.29 (m, 1H), 6.55 (s, 1H), 6.21 (s, 1H), 2.29 (s, 3H). ^{13}C NMR (151 MHz, Chloroform-*d*) δ 159.15, 143.97, 132.46, 129.50, 129.38, 129.26, 128.73, 128.01, 127.81, 126.64, 111.52, 21.25. HRMS (EI) m/z calcd for $C_{12}H_{11}NO_2$ M^+ 201.0790, found 201.0791.

6-benzyl-1-hydroxy-4-methylpyridin-2(1H)-one (19). Yellow sold, mp = 127 - 130 °C, yield = 35%. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.39 – 7.29 (m, 3H), 7.22-

7.28 (s, 2H), 6.43 (s, 1H), 5.81 (s, 1H), 4.10 (s, 2H), 2.17 (s, 3H). ^{13}C NMR (151 MHz, Chloroform-*d*) δ 157.51, 148.53, 143.03, 135.51, 128.80, 127.22, 112.73, 108.03, 36.12, 21.48. HRMS (EI) m/z calcd for $C_{13}H_{13}NO_2$ M^+ 215.0946, found 215.0947.

3-bromo-6-cyclohexyl-1-hydroxy-4-methylpyridin-2(1H)-one (20). White powder, mp = 174 - 177 °C, yield = 90%. ^{1}H NMR (400 MHz, $CDCl_3$): *δ* 6.09 (s, 1H), 3.03 – 2.93 (m, 1H), 2.34 (s, 3H), 1.95-2.15 (m, 2H), 1.83-1.94 (m, 2H), 1.75-1.93 (m, 1H), 1.18-1.55 (m, 5H). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 154.67 , 147.64 , 146.97 , 108.30 , 104.45 , 38.04 , 31.04 , 29.59 , 26.18 , 25.91 , 23.34. ^{13}C NMR (151 MHz, Chloroform-*d*, DEPT-135) δ CH104.60, CH38.02, $CH_2$31.10, $CH_2$26.18, $CH_3$23.35. HRMS (EI) m/z calcd for $C_{12}H_{16}BrNO_2$ M^+ 285.0364, found 285.0362.

5-bromo-6-cyclohexyl-1-hydroxy-4-methylpyridin-2(1H)-one (21). Yellow solid, mp = 126 – 130 °C, yield = 5%. ^{1}H NMR (400 MHz, $CDCl_3$): *δ* 6.54 (s, 1H), 3.65 - 3.46 (m, 1H), 2.33 (s, 3H), 2.30-2.21 (m, 2H), 1.85-1.95 (m, 2H), 1.60-1.82 (m, 2H), 1.18-1.55 (m, 4H). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 156.42 , 149.11 , 145.68 , 112.48 , 102.75 , 44.67 , 29.59 , 27.28 , 26.52 , 25.54 , 24.82 . ^{13}C NMR (151 MHz, Chloroform-*d*, DEPT-135) *δ* CH112.51, CH44.70, $CH_2$27.30, $CH_2$26.54, $CH_2$25.55, $CH_3$24.81. HRMS (EI) m/z calcd for $C_{12}H_{16}BrNO_2$ M^+ 285.0364, found 285.0360.

6-cyclohexyl-1-hydroxy-3-iodo-4-methylpyridin-2(1H)-one (22). Orange solid, mp = 161 – 165 °C, yield = 63%. ^{1}H NMR (400 MHz, $CDCl_3$): *δ* 6.10 (s, 1H), 3.07 – 2.87 (brs, 1H), 2.39 (s, 3H), 1.95-2.15 (m, 2H), 1.75-1.94 (m, 3H), 1.10-1.58 (m, 5H); ^{13}C NMR (101 MHz, Chloroform-d) δ 155.78 , 152.04 , 147.73 , 104.62 , 85.53 , 37.99 , 31.02 , 28.60 , 26.18 , 25.92 . ^{13}C NMR (151 MHz, Chloroform-*d*, DEPT-135) *δ*

CH104.63, CH37.99, $CH_2$31.02, $CH_3$28.59, $CH_2$26.17, $CH_2$25.90. HRMS (EI) m/z calcd for $C_{12}H_{16}INO_2$ M^+ 333.0226, found 333.0225.

3-chloro-6-cyclohexyl-1-hydroxy-4-methylpyridin-2(1H)-one (23). White solid, mp = 175 – 178 °C, yield = 70% ^{1}H NMR (400 MHz, Chloroform-*d*) δ 6.06 (s, 1H), 3.07 – 2.97 (m, 1H), 2.32 (s, 3H), 2.16 – 1.97 (m, 2H), 1.92 – 1.82 (m, 2H), 1.83 – 1.73 (m, 1H), 1.53 – 1.16 (m, 5H). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 154.70 , 147.00 , 145.35 , 117.83 , 104.30 , 37.98 , 31.16 , 26.19 , 25.92 , 20.38 . ^{13}C NMR (151 MHz, Chloroform-*d*, DEPT-135) *δ* CH104.21, $CH_2$38.00, CH31.15, $CH_3$26.06, $CH_2$20.37. HRMS (EI) m/z calcd for $C_{12}H_{16}ClNO_2$ M^+ 241.0870, found 241.0867.

The halogenated position of **20-23** was analyzed by comparing the ^{13}C and DEPT-135 ^{13}C NMR spectrogram of these compounds with spectrogram of CPX which was reported by Brandel (2015).[43]

6-cyclohexyl-1-hydroxy-4-methyl-2-oxo-1,2-dihydropyridine-3-carbaldehyde (24). Compound 24 is synthesized through a halogen−lithium exchange reaction using compound **20** as reactant. To a solution of compound **20** (1.99 g, 7 mmol) in dry THF (60 mL), 310 mg NaH (60%wt, 7.7mmol) was added at -10°C under nitrogen. After stirring for 30 min, the resulting suspension was cooled to -78 °C. Then, 14mL *tert*-BuLi solution (1.3 mol/L in pentane) was added dropwise. When the color of the reaction mixture changed to thick orange, 2.5 mL dry DMF was added. Next, shut down the cooling system, the stirring reaction mixture was allowed to warm to room temperature overnight. At last, the reaction was quenched by 1.5 N HCl at 0 °C. The organic phase was collected, and the aqueous phase was extracted with DCM for

two times. Combined the DCM solution and the organic phase, the combined mixture was washed by brine and dried by anhydrous Na_2SO_4. After removing the solvent under reduced pressure, the crude product was purified by column chromatography (methanol: DCM = 1:40(v/v)) to give 705 mg of compound **24** (pale-green solid), yield = 43%, mp = 106 – 109 °C. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 10.49 (s, 1H), 6.08 (s, 1H), 3.12-3.23 (m, 1H), 2.56 (s, 3H), 2.10 – 1.95 (m, 2H), 1.94 - 1.74 (m, 3H), 1.51 – 1.19 (m, 5H). ^{13}C NMR (151 MHz, Chloroform-*d*) δ 191.44, 157.13, 117.26, 115.79, 111.79, 108.73, 50.11, 38.37, 30.72, 26.16, 26.05. HRMS (ES+) m/z calcd for $C_{13}H_{17}NO_3$ 236.1286 $[M+H]^+$, found 236.1285.

6-cyclohexyl-1-hydroxy-4-methyl-3-((methylamino)methyl)pyridin-2(1H)-one (25). Yellow solid, mp = 103 – 107 °C, yield = 25%. ^{1}H NMR (400 MHz, Methanol-d_4) δ 6.42 (s, 1H), 4.20 (s, 2H), 3.49 – 3.34 (m, 1H), 2.76 (s, 3H), 2.35 (s, 3H), 2.10 – 1.98 (m, 2H), 1.92 – 1.75 (m, 3H), 1.55 – 1.26 (m, 5H). ^{13}C NMR (151 MHz, Chloroform-*d*) δ 132.46, 130.89, 129.93, 128.81, 108.45, 68.16, 65.58, 42.39, 38.74, 31.22, 29.70, 26.33, 22.70, 14.06, 11.16. HRMS (ES+) m/z calcd for $C_{14}H_{22}N_2O_2$ 250.1681 $[M+H]^+$, found 250.1758.

6-cyclohexyl-1-hydroxy-4-methyl-3-((propylamino)methyl)pyridin-2(1H)-one (26). Orange solid, mp = 79 – 83 °C, yield = 20%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.95-9.20 (br s, 1H), 6.18 (s, 1H), 4.22 (s, 1H), 3.49 (s, 1H), 2.68-2.86 (br s, 2H), 2.24 (s, 3H), 1.45-1.95 (m, 7H), 1.10-1.40 (m, 8H); ^{13}C NMR (151 MHz, Chloroform-*d*) δ 167.78, 153.53, 130.89, 128.81, 108.45, 68.16, 42.39, 38.74, 37.52, 31.22, 29.70, 26.33, 26.26. HRMS (ES+) m/z calcd for $C_{16}H_{26}N_2O_2$ 279.2072 $[M+H]^+$, found 279.2075.

6-cyclohexyl-1-hydroxy-3-((isopropylamino)methyl)-4-methylpyridin-2(1H)-one (27). Yellow solid, mp = 72 - 76 °C, yield = 35%. ^{1}H NMR (400 MHz, Methanol-d_4) δ 5.93 (s, 1H), 3.93 (s, 2H), 3.48 - 3.34 (m, 1H), 3.21 – 3.09 (m, 1H), 2.21 (s, 3H), 1.96 – 1.72 (m, 5H), 1.47 (d, J = 6.5 Hz, 6H), 1.41 – 1.15 (m, 5H). ; ^{13}C NMR (151 MHz, Methanol-d_4) δ 160.75, 154.09, 141.18, 106.61, 105.83, 49.45, 42.21, 37.46, 31.67, 31.31, 29.38, 26.31, 25.92, 18.53. HRMS (ES+) m/z calcd for $C_{16}H_{26}N_2O_2$ 279.2072 $[M+H]^+$, found 279.2074.

6-cyclohexyl-1-hydroxy-4-methyl-3-((prop-2-yn-1-ylamino)methyl)pyridin-2(1H)-one (28). White solid, mp = 155 – 159 °C, yield = 30%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.98 (s, 1H), 3.87 (s, 2H), 3.47 (d, J = 2.5 Hz, 2H), 3.05 – 2.86 (m, 1H), 2.30 (s, 3H), 2.26 (s, 1H), 2.07 – 1.96 (m, 2H), 1.74-1.94 (m, 3H), 1.56 – 1.17 (m, 5H); ^{13}C NMR (151 MHz, Methanol-d_4) δ 152.94, 129.48, 106.63, 78.17, 77.95, 77.73, 53.47, 41.50, 31.69, 31.05, 26.21, 25.92, 25.80, 25.40. HRMS (ES+) m/z calcd for $C_{16}H_{22}N_2O_2$ 275.1759 $[M+H]^+$, found 275.1761.

6-cyclohexyl-3-((cyclopentylamino)methyl)-1-hydroxy-4-methylpyridin-2(1H)-one (29). Orange solid, mp = 58 – 62 °C, yield = 27%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.93(s, 1H), 5.00 – 4.65 (brs, 1H), 3.99 (s, 2H), 3.60 - 3.40 (m, 1H), 2.90 - 3.10 (m, 1H), 2.29 (s, 3H), 1.55 - 2.05 (m, 9H), 1.15-1.47 (m, 9H); ^{13}C NMR (151 MHz, Methanol-d_4) δ 158.70, 152.25, 148.95, 114.43, 105.71, 59.46, 43.34, 38.11, 31.04, 29.40, 26.06, 25.65, 23.69, 18.02. HRMS (ES+) m/z calcd for $C_{18}H_{28}N_2O_2$ 305.2229 $[M+H]^+$, found 305.2224.

6-cyclohexyl-1-hydroxy-4-methyl-3-((phenylamino)methyl)pyridin-2(1H)-

one (30). Orange solid, mp = 40 – 44 °C, yield = 17%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.19 (t, *J* = 7.5 Hz, 2H), 7.11 (t, *J* = 7.5 Hz, 1H), 6.76 (d, *J* = 7.5 Hz, 2H), 6.01 (s, 1H), 4.29 (s, 2H), 2.90-3.10 (m, 1H), 2.10 (s, 3H), 2.06 – 1.98 (m, 2H), 1.72-1.92 (m, 3H), 1.15 – 1.55 (m, 5H); ^{13}C NMR (151 MHz, Chloroform-*d*) δ 157.36, 148.13, 147.20, 146.16, 129.17, 119.99, 117.93, 113.69, 105.38, 40.93, 38.04, 31.10, 26.23, 25.97, 19.49 . HRMS (ES+) m/z calcd for $C_{19}H_{24}N_2O_2$ 313.1916 $[M+H]^+$, found 313.1917.

6-cyclohexyl-3-((heptylamino)methyl)-1-hydroxy-4-methylpyridin-2(1H)-one (31). Orange solid, mp = 135 - 138 °C, yield = 20%. ^{1}H NMR (400 MHz, $CDCl_3$): *δ* 8.40 - 8.10 (brs, 2H), 6.15 (s, 1H), 4.07 (s, 2H) 3.00 - 3.10 (brs, 1H), 2.97 (t, *J* =7.7Hz, 2H), 2.38 (s, 3H), 1.65 - 1.95 (m, 7H), 1.10 - 1.45 (m, 18H); ^{13}C NMR (151 MHz, Chloroform-*d*) δ 158.16, 151.85, 148.46, 113.02, 106.48, 46.73, 44.58, 37.98, 31.55, 31.33, 28.73, 26.64, 26.21, 26.18, 25.94, 22.50, 19.73, 14.01. HRMS (ES+) m/z calcd for $C_{20}H_{34}N_2O_2$ 335.2698 $[M+H]^+$, found 335.2700.

Cell Culture

Human neuroblastoma SH-SY5Y cells (ATCC, 2266) were cultured in a 1:1 mixture of Eagle's minimum essential medium and F12 medium (Gibco, USA), supplemented with 10% fetal bovine serum (FBS; Gibco, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin, in a humidified atmosphere of 5% CO_2 at 37 °C. HT22 mouse hippocampal neuronal cells were purchased from Jennio Biotechnology Co., Ltd. and cultured in DMEM (Dulbecco's modified Eagle's medium) medium with 10% FBS and 100 U/ml penicillin and 100 μg/mL streptomycin.

Drug treatment: For the in vitro study, all the derivatives including the ciclopirox and ciclopirox olamine (purchased from Sigma-Aldrich) were prepared at 10 mM with 100% DMSO as stock solution, and freshly diluted with cell culture medium before use. Cells were incubated with the indicated concentrations of compounds immediately before OGD exposure, or 2 h before H_2O_2 and glutamate stimulus. For the in vivo study, the **11·Ola** and CPX olamine were dissolved in saline and administered intravenously after the monofilament was withdrawn for reperfusion.

OGD, H_2O_2 and Glutamate Injury

SH-SY5Y cells were seeded into 96-well plates at a density of 3×10^5 cells/mL. To mimic ischemia/reperfusion injury, oxygen glucose deprivation injury was induced in SH-SY5Y cells. Briefly, the cells were washed with glucose-free Earle's Balanced Salt Solution (EBSS) buffer (117.2 mM NaCl, 5.3 mM KCl, 0.8 mM $MgSO_4$, 26.2 mM $NaHCO_3$, 1.8 mM $CaCl_2$, 1.0 mM NaH_2PO_4•$2H_2O$), and maintained in glucose and serum free DMEM (Gibco, Grand Island, NY) with/without indicated concentrations of compounds in a hypoxia chamber (H35 hypoxystation, Don Whitley Scientific) containing a mixture of 85%N_2/10%H_2/5%CO_2 at 37°C for 2 h. Then, cells were supplemented with glucose and FBS, and cultured in the incubator for another 24 h. Control group without OGD were cultured in complete medium. For the H_2O_2 injury, cells were changed to fresh media and pre-incubated with compounds for 2 h, then H_2O_2 freshly prepared from 8.8 M stock solution was added to the cells to the final concentration of 100 μM. Glutamate stimulus was conducted in HT22 cell. Cells were seeded into 96-well plates at a density of 5×10^4 cells/ml and cultured for 12 h. Then,

cells were treated with different concentrations of compounds for 2 h, followed by exposure to 10 mM L-glutamic acid hydrochloride for 24 h.

Morphological Changes and Cell Viability Assay

Morphological changes of cells were observed under microscope (Nikon, TE200, Melville, NY, USA) after different toxic stimulus for 24 h. Cell viability was measured by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, BioDee Biotech, Beijing, China) assay. MTT (5 mg/mL) was added to each well and incubated away from light at 37 °C for 3 h. Then, the culture medium of each well was replaced with 100 μL of DMSO to fully dissolve the formazan and absorbance was recorded by DTX 800 multimode detector (Beckman Coulter, Fullerton, CA, USA) at 490 nm. The results were shown as percentage of control group.

Water-solubility Experiment

The water solubility of compounds **11**, **11·Ola**, CPX & CPX olamine were determined by HPLC (Agilent 1100 with quaternary pump, diode-array detector). Stock solutions of these samples were prepared in methanol. Then, 10 μL dilute methanol solutions with concentrations of 0.1, 0.5, 0.8, 1, 1.6, 2, and 4 mg/mL were injected into the HPLC system to assess linearity. Calibration curves were plotted as the peak area versus concentration of sample. To prepare the saturated water solution, 10 mg compound was added to a 2 mL EP tube, and 0.5 mL 0.9% saline was pipetted into the tube. If the solution was unsaturated and remained clear and transparent, additional test compound was added. After stirring for 24 h, the solution was filtered with a syringe filter, and 10 μL was injected into the HPLC system. Water solubility was calculated by comparing the peak area of the tested compound to the calibration curves.

Analysis of Apoptosis

Apoptotic cells was identified through Annexin V-FITC and propidium iodide (PI) staining kit (BD Biosciences, San Jose, CA, USA). Annexin V-FITC stained phosphatidylserine before the loss of membrane integrity and detected the early apoptotic cells, membrane damage accompanies the later stages of cell death as a result of apoptotic or necrotic processes, which were permeable to PI identified late apoptotic cells. SH-SY5Y cells were collected via centrifugation at 300×*g* for 5 min. After washing twice with cold PBS, the cells were resuspended in 100 μL (1×10^5 cells) of binding buffer. Annexin V-FITC (5 μL) and PI (5 μL) were added to the binding buffer, and the resulting mixture was incubated for 15 min at room temperature in the dark. Then 400 μL binding buffer was added, and cells were measured through flow cytometry (BD Biosciences, San Jose, CA, USA) within 1 h.

MCAO Model in Rats

Focal cerebral ischemia was performed in male Sprague−Dawley rats (Shanghai Laboratory Animal Center, Chinese Academy of Sciences, China) through occlusion of the intraluminal middle cerebral artery using a reported method.[44] All experimental procedures followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals, as well as the guidelines of the Animal Care and Use Committee of Shanghai Institute of Materia Medica, Chinese Academy of Sciences. First, anesthetized rats with chloral hydrate (400 mg/kg, i.p.), then a 40 monofilament nylon suture (Beijing Sunbio Biotech Co., China) was introduced into the left internal carotid artery and positioned to occlude the MCA origin through the proximal external carotid artery. The suture was withdrawn to restore blood flow (reperfusion) after 2 h of

occlusion. Saline (0.9%) solutions of edaravone (10 mg/kg), CPX olamine (3 mg/kg) and compound **11·Ola** (0.3 mg/kg & 1 mg/kg) were injected through tail vein immediately after reperfusion. The animal's body temperature was maintained at 37.0 ± 0.5 °C through the surgical procedure.

Neurological Deficit Evaluation.

The neurological function was evaluated at 24h after the reperfusion, according to the modified Neurological Severity Scores (mNSS).[45] The neurological deficits including motor, visual and tactile responses, proprioception, performance on a balance beam, reflex responses, and abnormal movements (scale: 0–18; normal score, 0; maximal deficits core, 18) were graded by the mNSS. The experimenters blinded to the experimental groups performed the neurological scores assessment.

Measurements of Infarct Volume

Infarct volumes were measured by triphenyltetrazolium chloride (TTC; Sinophar Chemical Reagent Co. Ltd., Shanghai, China) staining. After mNSS evaluation, rat brain coronal sections (equally 2 mm thick) were prepared and stained with 1% TTC in a 37 °C chamber away from light for 15 min followed by fixation in paraformaldehyde solution (4%) for 24 h. Digital images were taken after the fixation and analyzed with the image analysis system (Image-ProPlus). The infarct area was calculated by the formula: (area of normal hemisphere－area of non-infarcted ischemic hemisphere)/area of normal hemisphere× 100%.

SH-SY5Y Cytotoxicity Assay

SH-SY5Y cells were seeded into 96-well plates at a density of 3×10^4 cells/well and cultured for 24 h. For cytotoxicity assay, cells were treated with CPX olamine and compound **11·Ola** at the concentrations of 1.56 μM, 3.13 μM, 6.25 μM, 12.5 μM, 25 μM, 50 μM & 100 μM and incubated for 24 h. For the other 19 compounds, two concentrations – 1 μM and 10 μM were set. Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sangon Biotech, China) analysis and processed as described above. The cell viability was calculated and presented by the percentage of no-treated control group.

MRC-5 Cytotoxicity Assay

MRC-5 (Medical Research Council cell strain 5) were cultured in the media which contained MEM + 10% FBS + 1% P/S + 1% NEAA + 1% SP in 5% carbon dioxygen atmosphere 37 °C. Cells were seeded into 96-well plates (Corning) at a density of 7000 cells per well for the following experiments. For cytotoxicity test, cells were treated with CPX olamine and compound **11·Ola** at several concentrations for 72 h. Cell viability was measured by cell counting kit (CCK-8), determined by measuring the absorbance at 450 nm. The cell viability was calculated and presented by the percentage of vehicle-treated group.

Statistical Analysis

All data were reported only if at least three independent experiments showed consistent results. The data were analyzed by one-way ANOVA followed by Dunnett's

test for multiple comparisons or by Student's t test for single comparison. Statistical significance was established at p value <0.05.

SUPPORTING INFORMATION

Supporting information is available free of charge on the ACS publications website at DOI: .

- NMR multiplet reports and synthetic methods of the intermediates, HPLC reports for the purity check of compounds **1-31**, in vitro blood-brain barrier permeation assay, oxygen radical absorbance capacity (ORAC-FL) assay, hERG cardiac toxicity assay, determination of metabolic stability in liver microsomes, bSDTNBI in silico prediction results, Fe Ion chelation experiment, the effects of compounds on the viability of cells without insult, the comparison of protective effects between compounds **11** and **16**, $^{1}H/^{13}C$ NMR, MASS spectra and HPLC traces of compounds **1-31** and the intermediates, determination of the blood plasma concentrations of **11·Ola** and CPX Olamine after intravenous administration in rats.(PDF)
- Molecular formula strings and some data (CSV).

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Notes

The authors declare no competing financial interests.

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ABBREVIATIONS

Ola; olamine, recombinant tissue plasminogen activator; rtPA, racemic 3-n-butylphthalide; NBP, ischemia-reperfusion; I/R, reactive oxygen species; ROS, azodiisobutyronitrile; AIBN, 3-chloroperoxybenzoic acid; mCPBA, *N*-bromosuccinimide; NBS, *N*-iodosuccinimide; NIS, *N*-chlorosuccinimide; NCS, oxygen glucose deprivation; OGD, ciclopirox; CPX, middle cerebral artery occlusion; MCAO, oxygen radical absorbance capacity; ORAC, parallel artificial membrane permeability assay; PAMPA, blood-brain barrier; BBB, bSDTNBI; balanced substructure–drug–target network-based inference, hypoxia-inducible factor; HIF, prolyl hydroxylases, PHDs.

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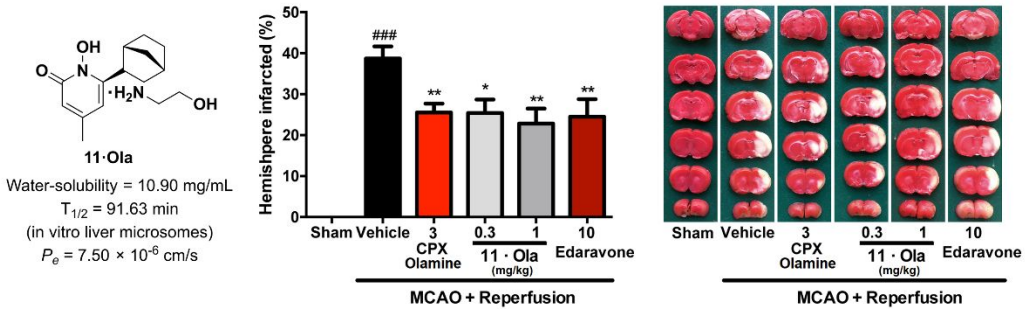

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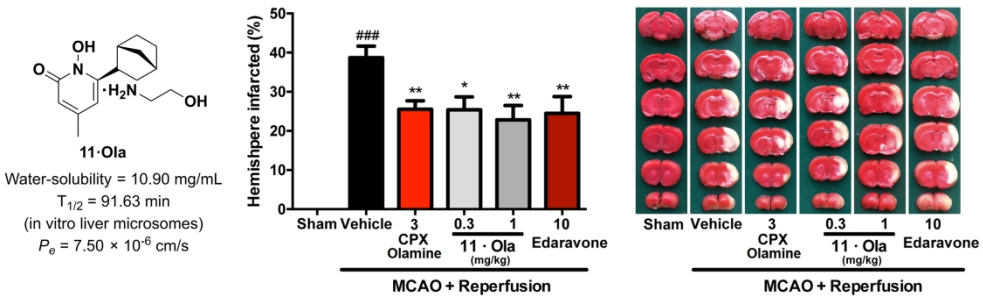

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