European Journal of Medicinal Chemistry 117 (2016) 19–32

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Research paper

Discovery and analgesic evaluation of 8-chloro-1,4-dihydropyrido[2,3-*b*]pyrazine-2,3-dione as a novel potent D-amino acid oxidase inhibitor

Dongsheng Xie [1], Jun Lu [1], Jin Xie, Junjun Cui, Teng-Fei Li, Yan-Chao Wang, Yuan Chen, Nian Gong, Xin-Yan Li, Lei Fu*, Yong-Xiang Wang**

School of Pharmacy, Shanghai Jiao Tong University, 800 Dongchuan Road, Shanghai 200240, China

ARTICLE INFO

Article history:
Received 3 September 2014
Received in revised form
5 April 2016
Accepted 6 April 2016
Available online 9 April 2016

Keywords:
D-amino acid oxidase
DAAO inhibitors
5-Azaquinoxaline-2,3-diones
Analgesic effects
8-Chloro-1,4-dihydropyrido[2,3-*b*]pyrazine-2,3-dione

ABSTRACT

A series of 5-azaquinoxaline-2,3-dione derivatives were synthesized and evaluated on D-amino acid oxidase (DAAO) inhibition as potential α-hydroxylactam-based inhibitors. The potent inhibitory activities *in vitro* suggested that 5-nitrogen could significantly enhance the binding affinity by strengthening relevant hydrogen bond interactions. The analgesic effects of intrathecal and systemic injection of 8-chloro-1,4-dihydropyrido[2,3-*b*]pyrazine-2,3-dione, a representative molecule of 5-azaquinoxaline-2,3-dione, were investigated in rodents. This research not only confirmed the analgesic effect of the DAAO inhibitors but provided a new class of chemical entities with oral application potential for the treatment of chronic pain and morphine analgesic tolerance.

1. Introduction

D-serine has been demonstrated to be antipsychotic by activating the glycine site of the N-methyl-D-aspartic acid (NMDA) receptor in the central nervous system [1–4]. However, high doses of D-serine required for clinical efficacy may cause nephrotoxicity, which is generated by D-amino acid oxidase (DAAO)-mediated metabolism of D-serine in kidneys [5–7]. Inhibition of peripheral DAAO may be an effective means of delivering D-serine to the brain [8–10]. Due to the promising therapeutic application in schizophrenia, great interest has been aroused in discovery of novel and potent DAAO inhibitor molecules [8,11–17]. In addition, researchers have also investigated the roles of D-serine and DAAO in painful responses [18–22]. In recent years, the analgesic effects of the structurally different DAAO inhibitors have been reported in animal models such as formalin-induced tonic pain [21–26], neuropathic pain [27,28] and bone cancer pain [29], with a proposed mechanism related to the inhibition of spinal hydrogen peroxide [24,25]. Furthermore, DAAO inhibition has also been implicated with morphine analgesic tolerance [25,26] and hyperalgesia [30]. DAAO inhibitors thus represent a new drug discovery opportunity for the treatment of chronic pain and morphine analgesic tolerance. In search of potent DAAO inhibitors with novel structures for pharmacological research on pains, herein we reported the synthesis and evaluation of a series of 5-azaquinoxaline-2,3-dione derivatives as a new family of noncarboxylic DAAO inhibitors.

As potential α-hydroxylactam-based inhibitors, 5-azaquinoxaline-2,3-diones exhibited significantly improved potency (IC_{50} = 100–500 nM for 10 derivatives) over our previously reported quinoxaline-2,3-diones [31]. The newly synthesized compound **16** (8-chloro-1,4-dihydropyrido[2,3-*b*]pyrazine-2,3-dione) with higher potency (IC_{50} = 100–200 nM against porcine, rat and human DAAO) dose-dependently relieved formalin-induced tonic pain, effectively blocked spinal nerve ligation induced mechanical allodynia and heat hyperalgesia, and significantly prevented and reversed morphine analgesic tolerance in rodents.

2. Chemistry

5-Azaquinoxaline-2,3-dione derivatives were prepared from appropriate 2,3-diaminopyridines by condensation with diethyl

* Corresponding author.
** Corresponding author.
E-mail addresses: leifu@sjtu.edu.cn (L. Fu), yxwang@sjtu.edu.cn (Y.-X. Wang).
[1] The two authors contributed equally to this work.

http://dx.doi.org/10.1016/j.ejmech.2016.04.017
0223-5234/

oxalate [32]. However, most 2,3-diaminopyridines needed to be synthesized due to their commercial limitation. The general synthesis procedure for 5-azaquinoxaline-2,3-diones was outlined in Scheme 1. 2-Aminopyridines (**a**) without protection of the amino could be nitrified, and rearrange in nitro-sulfuric acid to give 2-amino-3-nitropyridines (**c**) and 5-nitroisomers at room temperature or above. Nevertheless, in most cases, the intermediates (**b**) which is readily isolated in early stage of the nitration at low temperature (−5 to 0 °C), could be more effectively and completely converted to nitropyridines just in concentrated sulfuric acid. When 5-position of 2-aminopyridines was occupied, the 3-nitroisomer products were exclusively obtained. Otherwise, 3-nitroisomers had to be separated from mixed isomers in various yields from 2% to 50%. 2-Amino-3-nitropyridines (**c**) were chemically reduced with stannous chloride or zinc powder/acetic acid, or hydrogenated with Pd/C to give 40–100% yield of 2,3-diaminopyridines (**d**), which are unstable during long exposure to air and light. They should be taken into next reaction without purification or immediately once purified after column chromatography. The cyclization of 2,3-diaminopyridines were carried out in excess of diethyl oxalate under reflux to afford 40–70% yield of 5-azaquinoxaline-2,3-diones (**e**) as grey to brown solid. To avoid the data deviation in enzyme assay, the colored solid products must be strictly purified by first decolourization with activated charcoal and then recrystallization in water and DMF or DMSO.

Six and 8-alkoxyl-5-azaquinoxaline-2,3-dione derivatives' preparation was illustrated in Scheme 2. 2-Amino-3-nitro-6-chloropyridine, an intermediate for compound **8**, was first converted to 6-alkyloxy-2-amino-3-nitropyridines by treatment in DMF with corresponding sodium alkoxides, which could also be produced *in situ* using corresponding alcohols and sodium hydride. Next steps followed the general procedure in Scheme 1 to give 6-alkoxyl-5-azaquinoxaline-2,3-dione derivatives. In the same way, 8-alkoxyl derivatives were prepared from 2-amino-3-nitro-4-chloropyridine, an intermediate in preparation of compound **16**.

7,8-Dihalogen-5-azaquinoxaline-2,3-dione derivatives were prepared from 2-amino-4-halogenpyridine (Scheme 3). Halogenations were carried out in CH_3CN or DMF using NCS or NBS, and 60–80% yield of 2-amino-4,5-dihalogenpyridines were obtained by column chromatography [33,34]. The next nitration and rearrangement were carried out in one pot. Zinc powder in acetic acid was employed to reduce 2-amino-3-nitropyridine intermediates, otherwise, hydrogenation by Pd/C or even stannous chloride led to seriously dehalogenated products. Finally, the condensation of 2,3-diamino-4,5-dihalogenpyridine and diethyl oxalate were carried out under reflux for 6 h to give 7,8-dihalogen-5-azaquinoxaline-2,3-diones.

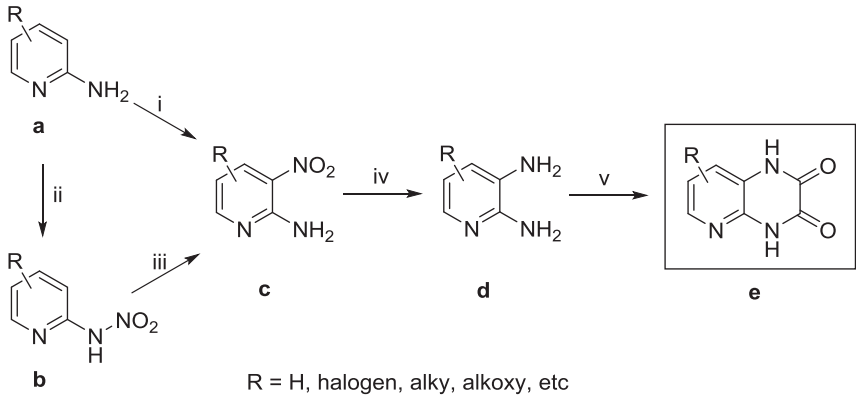
3. Results and discussion

3.1. Discovery of 5-azaquinoxaline-2,3-diones as novel D-amino acid oxidase inhibitors

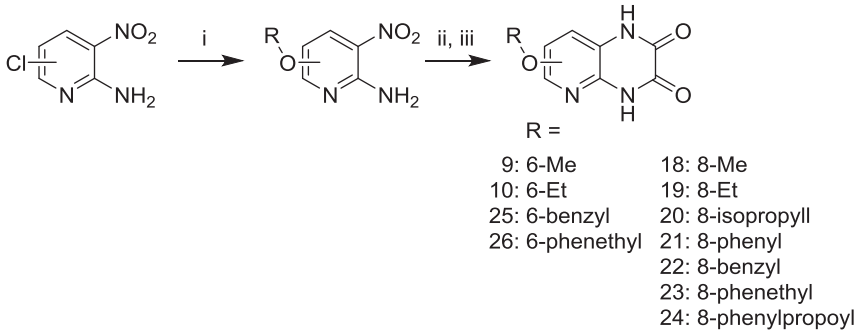
As noncarboxylic DAAO inhibitors 3-hydroxyquinolin-2-one [14] and newly discovered scaffolds in recent years, such as 1-hydroxybenzoimidazol-2-one [15], 3-hydroxypyridin-2-one [16], and 4-hydroxypyridazin-3-one [16] shared a common pharmacophore—α-hydroxylactam, by which a hydrogen bond (HB) net with DAAO residues including Arg283, Tyr 228 and Gly313 are constructed (Fig. 1).

Due to the possible amide-iminol tautomerism, we reported that quinoxaline-2,3-dione and its derivatives were potential α-hydroxylactam-based DAAO inhibitors [31]. Docking simulation demonstrated that quinoxaline-2,3-dione could bind to the active site of DAAO in a similar manner to that of 3-hydroxyquinolin-2-one in crystalline complex (Fig. 2A), which suggested that α-carbonlactam moiety could also function as α-hydroxylactam one, constructing corresponding HB net with DAAO. Regrettably, varying substitution on the benzene ring of quinoxaline-2,3-dione endowed these molecules with moderate DAAO inhibition (IC_{50} > 5 μM) [31]. To our surprise, in random screening, 5-azaquinoxaline-2,3-dione derivative, i.e., 8-methyl-1,4-dihydropyrido[2,3-*b*]pyrazine-2,3-dione exhibited marked DAAO inhibitory property (IC_{50} = 0.63 μM). The improved potency of this 5-azaquinoxaline-2,3-dione derivative seemed hardly to be explained only by molecular docking simulation because of its similarity to quinoxaline-2,3-dione in binding mode (Fig. 2B). We proposed that the weak potencies of quinoxaline-2,3-diones may be caused by the similar enolizability of di-lactam, and introduction of 5-nitrogen may be able to significantly break the similar enolization state of di-lactam and make it more favorable to form α-hydroxylactam pharmacophore. Furthermore, 4-amino (ortho position of pyridine) owing to the increased bond polarity may prefer to donate its hydrogen atom and form a stronger HB with carbonyl group of Gly313. Thus, 5-azaquinoxaline-2,3-dione derivatives are much likely to be a novel family of the potent DAAO inhibitors.

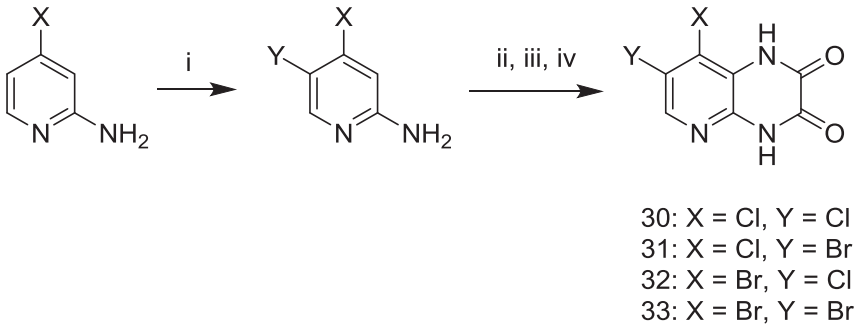
As expected, the inhibitory activities of 5-azaquinoxaline-2,3-diones on porcine kidney DAAO (pkDAAO) are widely superior to the corresponding quinoxaline-2,3-diones (Table 1). Some newly synthesized derivatives exhibited potent efficacy (IC_{50} = 100–500 nM) *in vitro* (compounds **7**, **13**, **16**, **17**, **27**–**29** and **31**–**33**). Limited by the restricted space around DAAO active site, small substituents such as methyl, trifluoromethyl and halogen atoms are favorable for potent inhibitory activity. Larger groups than methyl even if ethyl or methoxyl are detrimental to the



Scheme 1. General synthesis route of 5-azaquinoxalinedinones. Reagents and conditions: (i). 98% H_2SO_4, fuming HNO_3, −5 to 50 °C, 3 h; (ii). 98% H_2SO_4, fuming HNO_3, −5 to 0 °C, 15 min; (iii). 98% H_2SO_4, 0–50 °C, 2 h; (iv). H_2, 10% Pd–C, EtOAc/MeOH (V/V = 8/1), RT, overnight or $SnCl_2$, EtOH, reflux, 3–5 h; (v). $(COOEt)_2$, reflux, 4 h.



Scheme 2. Synthesis of compounds **9**, **10** and **18**–**26**. Reagents and conditions: (i). sodium alkoxide or alcohol/NaH, DMF, RT, 3 h; (ii). H_2, 10% Pd/C, EtOAc/MeOH (V/V = 8/1), RT, overnight or $SnCl_2$, EtOH, reflux, 3 h; (iii). $(COOEt)_2$, reflux, 4 h.



Scheme 3. Synthesis of compounds **30**–**33**. Reagents and conditions: (i). NCS or NBS, DMF or CH_3CN, −20 °C to RT, 16–24 h; (ii). 98% H_2SO_4, fuming HNO_3, −5 to 50 °C, 3 h; (iii). Zn powder, AcOH, RT, 3 h; (iv). $(COOEt)_2$, reflux, 6 h.

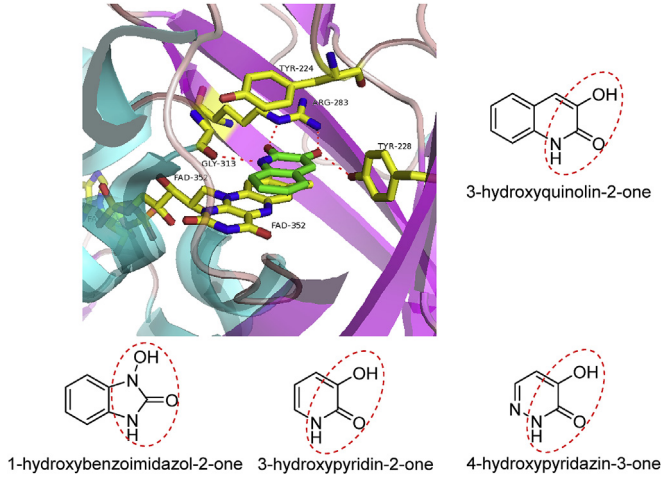


Fig. 1. The complex of DAAO with 3-hydroxyquinolin-2-one (3G3E) and the representative α-hydroxylactam-based inhibitors. FAD (flavin adenine dinucleotide) and residues are highlighted in yellow; the inhibitor and hydrogen bonds are highlighted in green and red, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

inhibitory activity (e.g. compounds **2**/**9**/**10** and **4**/**5**/**18**/**19**/**20**). For mono-substitution, the inhibitory activity of 8-substitution derivatives excelled that of 6 or 7-substitution derivatives (e.g. compounds **2**/**3**/**4** and **8**/**14**/**16**), and halogen atoms such as chloro and bromo at 8-position (compounds **16** and **17**) afforded the most potent efficacies, which reached the same level with the known DAAO inhibitors 6-chlorobenzo[*d*]isoxazol-3-ol (CBIO) [8] and 3-hydroxyquinolin-2-one [14]. When the 8-position was occupied by methyl or halogen, various halogen atoms at the 7-position also provided potent DAAO affinity (compounds **27**–**33**). Given the significant activation of 8-substitution, we believed that the 8-substituents with proper size may promote the formation of HB between the 4-amino and Gly313 by pushing the ligands nearer to Gly313. With an attempt to gain additional interactions on the "subpocket region" which was confirmed in the cases of 3-hydroxypyridin-2-one [16], 4-hydroxypyridazin-3-one [16] and Kojic acid inhibitors [17], we introduced side chains containing phenyl group into 6 or 8-position of 5-azaquinoxaline-2,3-dione, however these derivatives resulted in a complete loss of activity (compounds **21**–**26**).

For those 5-azaquinoxaline-2,3-diones with IC_{50} values below 300 nM against pkDAAO, we further tested them on DAAO originating from the spinal homogenates of rats (rsDAAO) and recombinant human DAAO (hDAAO). Noteworthy, 8-chloro-1,4-dihydropyrido[2,3-*b*]pyrazine-2,3-dione (compound **16)** exhibited potent inhibitory activities ($IC_{50} \leq 210$ nM) on three forms of DAAO, which are comparable to previously reported CBIO [8] and 3-hydroxyquinolin-2-one [14] (Table 2).

3.2. *In vivo blockade effects of pain and morphine analgesic tolerance*

Due to potent inhibition on pkDAAO, hDAAO and especially rsDAAO, compound **16** was therefore selected as a representative molecule for further investigation on its analgesic effects in laboratory mice or rats.

3.2.1. *Differential effects of compound 16 given intrathecally on formalin and NMDA-induced pain*

We previously showed that DAAO inhibitors such as CBIO blocked formalin-induced tonic pain [21–26]. To confirm compound **16**, as a novel DAAO inhibitor, also has such an effect, the analgesic effect of compound **16** on the formalin test was studied

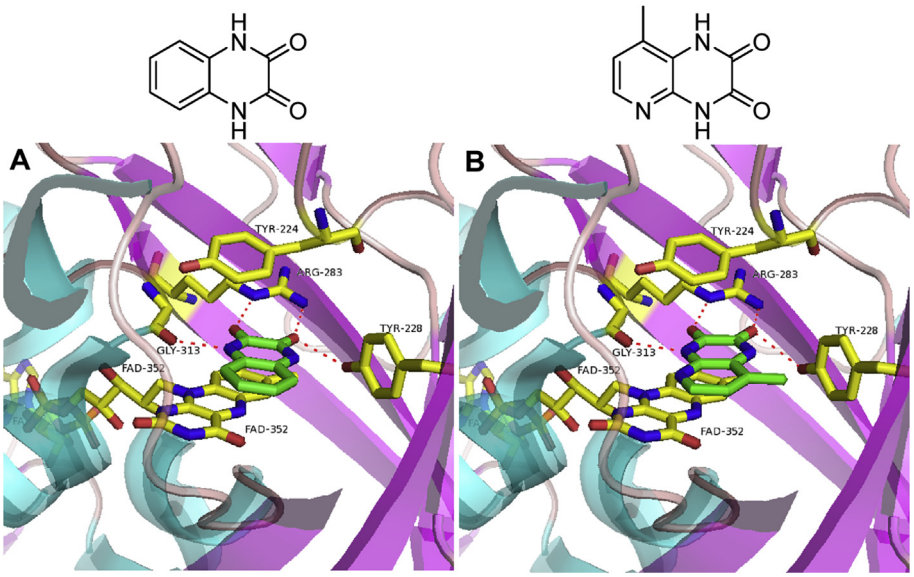


Fig. 2. Molecular docking simulation of quinoxaline-2,3-dione and 8-methyl-1,4-dihydropyrido[2,3-*b*]pyrazine-2,3-dione with DAAO. FAD (flavin adenine dinucleotide) and residues are highlighted in yellow; the inhibitor and hydrogen bonds are highlighted in green and red, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

via direct spinal cord administration in rats chronically implanted with intrathecal cannulas. Five groups of rats (n = 6 in each group) received intrathecal bolus injection of normal saline (10 μL) or compound **16** (0.1, 0.3, 1 or 3 μg) 30 min before formalin injection. Subcutaneous injection of formalin in control rats receiving intrathecal injection of normal saline produced a characteristic bi-phasic flinching responses consisting of an initial, rapidly decaying acute phase (within 10 min after formalin injection) followed by a slowly rising and long-lived (10–90 min) tonic phase as shown in Fig. 3A. Compared with the normal saline control, compound **16** up to 3 μg did not prevent formalin-induced flinch response in the acute phase, however it prevented formalin-induced pain in the tonic phase in a dose-dependent manner (Fig. 3A). The areas under the flinching response curve from 10 to 90 min ($AUC_{10-90\ min}$) were calculated for dose–response analysis by best fitting. Maximum inhibition (E_{max}) of formalin-induced tonic pain was 67.1% and half-effective dose (ED_{50}) was 0.25 μg (Fig. 3B).

It had been reported that a number of quinoxaline-2,3-diones showed antagonism at the NMDA glycine site [36,37]. In order to exclude the possibility that the analgesic property of compound **16** is due to its antagonism of the NMDA receptors, three groups of rats (n = 6 in each group) received intrathecal injection of saline (10 μL), the NMDA receptor antagonist MK-801 (0.1 μg) or compound **16** (10 μg) 30 min before intrathecal injection of 5 nmol NMDA. The pain behaviors were immediately measured for 10 min after NMDA injection. In the saline-pretreated control rats, intrathecal injection of NMDA produced immediate scratching, biting and licking responses, with measured pain duration approximately 200 s in 10 min. Pretreatment with intrathecal MK801 effectively inhibited NMDA-induced pain response by approximately 80%. However, compound **16** had no effects on NMDA-induced pain behaviors (Fig. 3C).

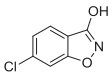
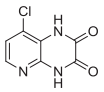
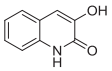
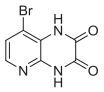
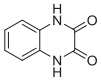
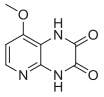
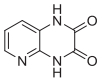
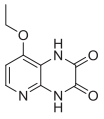
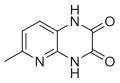
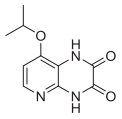
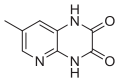
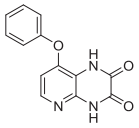
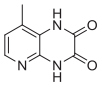
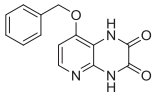
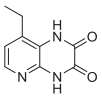
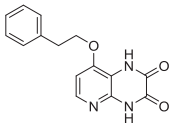
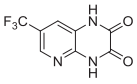
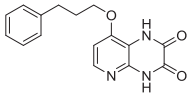
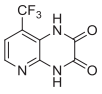
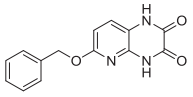
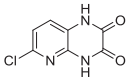
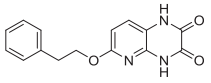
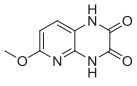
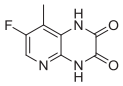
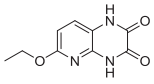
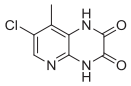
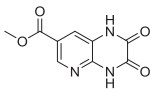
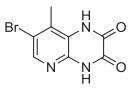
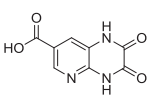
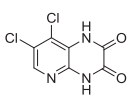
3.2.2. Analgesic effects of compound 16 given intrathecally on spinal nerve ligation-induced mechanical allodynia and heat hyperalgesia

To evaluate the effects of compound **16** on neuropathic pain, spinal nerve ligated neuropathic rats 7 days after surgery were divided into two groups (n = 6 in each group): saline (10 μL) and compound **16** (30 μg). The paw withdrawal thresholds to van frey monofilaments and withdrawal latency to redial heat were measured before and 0.5, 1, 2, 4 h after drug injection. Paw withdrawal thresholds were transformed into a % maximum possible effect (% MPE), which equals to 100 × [New threshold - baseline threshold]/[Contralateral threshold - baseline threshold] [38]. Compared with contralateral paws, spinal nerve ligation induced marked mechanical and thermal hypersensitivity. Intrathecal injection of compound **16** significantly increased both paw withdrawal thresholds to mechanical stimuli (Fig. 4A) and withdrawal latency to heat stimuli in ipsilateral paws (Fig. 4B) ($P < 0.05$, by two-way ANOVA followed by post hoc Dunnett's test). At 1 h after injection, the blockade effects for mechanical allodynia and heat hyperalgesia were 54.4± 5.2% and 80.8± 11.3% MPE, respectively. In contrast, compound **16** failed to influence paw withdrawal responses in contralateral paws.

3.2.3. Preventive and reversal effects of compound 16 given intrathecally on morphine analgesic tolerance in the mouse hot-plate test

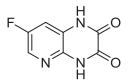
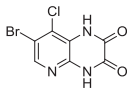
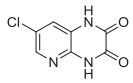
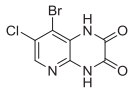
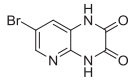
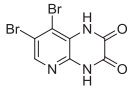
To evaluate the preventive and reversal effects of compound **16** on morphine analgesia tolerance, three groups of mice (n = 6 in each group) received intrathecal injections of saline (10 μL), morphine (10 μg), and compound **16** (10 μg) plus morphine (10 μg), twice daily with 12 h intervals for 7 days. On day 8, mice received a single bolus intrathecal administration of 10 μL saline or 10 μg compound **16** at 15 min before saline (10 μL) or morphine (10 μg) challenge. The latency of licking/biting/jumping was measured before and 0.5, 1, 2 h after saline or morphine injection on day 1, 3, 5, 7 and 8. The areas under the tail-flicking or licking/biting/jumping response curves over 2 h ($AUC_{0-2\ h}$) were calculated. As shown in Fig. 5A, multiple bi-daily intrathecal injections of morphine induced progressive and complete analgesic tolerance during the 7-day administration; coadministration of compound **16** significantly attenuated the development of morphine analgesic tolerance ($P < 0.05$ by two-way ANOVA followed by post-hoc student-Newman-Keuls test). Furthermore, single bolus injection of compound **16** significantly reversed the established morphine tolerance on day 8 ($P < 0.05$ by two-way ANOVA followed by post-hoc student-Newman-Keuls test) (Fig. 5B).

Table 1
Inhibitory effects of 5-azaquinoxaline-2,3-diones on porcine kidney D-amino acid oxidase (pkDAAO).

Compound	Structure	IC_{50} (μM)[a]	Compound	Structure	IC_{50} (μM)[a]
CBIO		0.21	**16**		0.18
3-hydroxyquinolin-2-one		0.10	**17**		0.22
quinoxaline-2,3-dione		33.6	**18**		85.7
1		5.7	**19**		>100
2		17.7	**20**		>100
3		36.7	**21**		>1000
4		0.63	**22**		>300
5		2.4	**23**		>300
6		22.9	**24**		>300
7		0.52	**25**		>300
8		20.3	**26**		>300
9		>100	**27**		0.15
10		>100	**28**		0.26
11		>1000	**29**		0.40
12		>1000	**30**		0.65

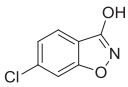
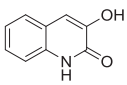
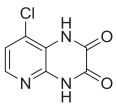
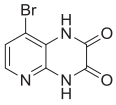
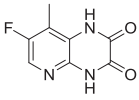
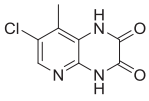
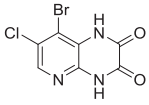
(*continued on next page*)

Table 1 (*continued*)

Compound	Structure	IC_{50} (μM)[a]	Compound	Structure	IC_{50} (μM)[a]
13		0.46	**31**		0.48
14		6.7	**32**		0.24
15		12.4	**33**		0.53

[a] The pyruvate production method was adopted [35]; all of the readings are means of triplicates, repeated two to three times.

Table 2
Inhibitory effects of partial 5-azaquinoxalinedinones on recombinant human D-amino acid oxidase (hDAAO), porcine kidney DAAO (pkDAAO) and rat spinal DAAO (rsDAAO).

Compound	Structure	IC_{50} (nM)[a]		
		pkDAAO	rsDAAO	hDAAO
CBIO		210 (188)	90	110
3-hydroxyquinolin-2-one		104	94 (215)	43 (4)
16		180	140	210
17		220	2240	230
27		150	150	340
28		260	4160	1130
32		240	7990	1380

[a] 5-Azaquinoxalinedinones with IC_{50} < 300 nM on pkDAAO were chosen; The pyruvate production method was adopted [35] to measure the DAAO enzymatic activity; all of the readings are means of triplicates, repeated two to three times; The data in brackets are reported by literatures [8,14].

3.2.4. Analgesic effects of compound 16 given subcutaneously and by oral gavage on formalin-induced pain

In order to determine whether compound **16** has a systemic exposure and oral bioavailability, we finally evaluated subcutaneous injection and oral gavage of compound **16** on formalin-induced pain in mice. Six groups of mice (n = 6 in each group) received subcutaneous injection of saline (10 mL/kg), compound **16** (0.3, 1, 3 and 10 mg/kg) and one group of 6 fasted mice received gavage of compound **16** (10 mg/kg) 30 min before formalin challenge. Subcutaneous injection of compound **16** did not prevent acute nociception (Fig. 6A), but inhibited tonic pain in a dose-dependent manner ($P < 0.05$ by one-way ANOVA followed by post-hoc student-Newman-Keuls test) (Fig. 6B), with an E_{max} of 49% inhibition and an ED_{50} of 1.8 mg/kg (Fig. 6C). Oral gavage of compound **16** (10 mg/kg) blocked formalin-induced tonic pain by approximate 30%, which was comparable to that of 3 mg/kg but significantly less than that of 10 mg/kg given subcutaneously, suggesting that compound **16** has an apparent oral bioavailability of approximately 30% (vs. subcutaneously).

4. Conclusions

In search of novel and potent DAAO inhibitors for pharmacological studies we synthesized and evaluated a series of 5-azaquinoxalinedinone derivatives. As a novel family of non-carboxylic DAAO inhibitor, 5-azaquinoxaline-2,3-diones exhibited significantly improved potency over quinoxaline-2,3-diones. Some newly synthesized derivatives were potent (IC_{50} of 100–500 nM) to inhibit pkDAAO *in vitro*. These results supported our speculation that introduction of 5-nitrogen into quinoxaline-2,3-dione could significantly break the similar enolization of di-lactam and make it more favorable to interact with guanidyl of Arg283, and 4-amino, owing to the increased bond polarity prefers to donate its hydrogen atom and form a stronger HB with carbonyl of Gly313.

We intensively studied the analgesic effects of compound **16**, which exhibited comparable DAAO inhibitory potency to the existing inhibitors such as CBIO and 3-hydroxypyridin-2-one on pkDAAO, rsDAAO and hDAAO. Given intrathecally, compound **16** can effectively and specifically block spinal nerve ligation-induced mechanical allodynia and heat hyperalgesia in neuropathic rats, prevent and reverse morphine analgesic tolerance, and relieve formalin-induced tonic pain without influencing the NMDA receptor activity. Moreover, compound **16** could also attenuate formalin-induced tonic pain by subcutaneous injection or oral gavage. This research not only confirmed the analgesic effects of DAAO inhibitors based on a new scaffold, but provided a new class of chemical entities with oral application potential for the treatment of chronic pain and morphine analgesic tolerance.

5. Experimental

5.1. Biology

5.1.1. Drugs and reagents

NMDA, MK-801 and 3-hydroxypyridin-2-one were purchased from Sigma (Sigma, St Louis, MO, USA). CBIO (5-chloro-benzo[*d*] isoxazol-3-ol) was manufactured from Maybridge (Cornwall, U.K.) and was a kind gift from Dr. Kenji Hashimoto at the Chiba

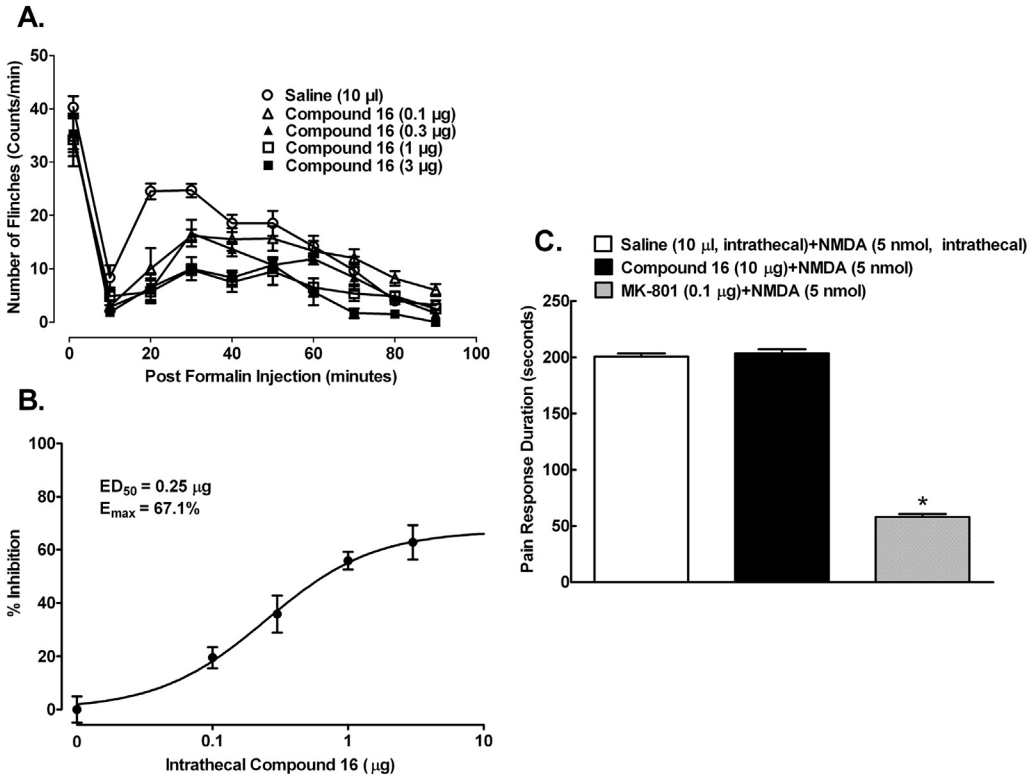


Fig. 3. Differential effects of intrathecal injection of compound **16** on formalin- and NMDA-induced pain. **A**. Effects on formalin-induced pain by intrathecal injection. Rats received intrathecal injection of normal saline (10 μL) or compound **16** (0.1, 0.3, 1, or 3 μg) 30 min before subcutaneous injection of 50 μL of 5% formalin. Nociceptive behavior was quantified by counting the number of the formalin-injected paw flinches in 1-min epochs; **B**. Dose response analysis on formalin-induced tonic pain; **C**. Effects on NMDA-induced pain by intrathecal injection. Rats received intrathecal injection of normal saline (10 μL), compound **16** (10 μg) or MK-801 (0.1 μg) 30 min before intrathecal NMDA (5 nmol). The pain behaviors were immediately measured for 10 min after NMDA injection. Data are presented as means ± S.E.M. (n = 6 in each group). * denotes statistically significant difference ($P < 0.05$ by one-way ANOVA followed by post-hoc Student-Newman-Keuls test), compared to the saline control group.

University Center for Forensic Mental Health, Japan. Porcine kidney DAAO (pkDAAO), recombinant human DAAO (hDAAO) and FAD (flavin adenine dinucleotide) were purchased from SERVA (Heidelberg, New York, USA), Sigma (Sigma, St Louis, MO, USA) and Novoprotein (Shanghai, China), and formalin and morphine hydrochloride injection were purchased from Sinopharm Group Chemical Reagent Co. (Shanghai, China) and Shenyang First Pharmaceutical Co. (Shenyang, Liaoning, China), respectively. All test drugs were freshly dissolved in sterile normal saline solution (Sinopharm Group Chemical Reagent Co., Ltd.) with pH adjusted to 7.3–7.5 by 1 N NaOH solution as needed. All enzymes were freshly dissolved in Tris–HCl (pH = 8.2).

5.1.2. Animals

Male Wistar rats (180–250 g) and Swiss mice (18–22 g) were obtained from the Shanghai Experimental Animal Institute for Biological Sciences (Shanghai, China). The animals were housed in a temperature- and humidity-controlled environment on a 12-h light/dark cycle (lights on 7:00 AM). Food and water were freely available. Animals were acclimated to the laboratory environment for 3–5 days before entering the study. Experimental study groups were assigned randomly, and the researcher was blind for behavior testing. The research protocols were approved by the Animal Care and Welfare Committee of Shanghai Jiao Tong University and followed the animal care guidelines of the National Institutes of Health. Efforts were made to reduce the number of animals used, and to minimize their sufferings.

5.1.3. DAAO enzymatic activity assay

The enzymatic activities of DAAO were determined according to the keto acid method [35]. DAAO inhibitors were assayed for their effects on rsDAAO, pkDAAO and hDAAO. In the control homogenates incubated for 5 min for pkDAAO and 60 min for both rsDAAO and hDAAO, the α-keto acid produced by D-alanine was determined by measurement of pyruvic acid. All enzymes and FAD were dissolved in 0.1 M Tris–HCl, pH = 8.2 (final concentration: 0.04 mg/mL for pkDAAO, 0.3 mg/mL for rsDAAO and 0.05 mg/mL for hDAAO, and 0.2 mg/mL for FAD). Fifty microliters of D-alanine (final concentration: 1 mM for both pkDAAO and hDAAO, and 6 mM for rsDAAO) was added to 25 μL of DAAO inhibitors solution (concentration as follow) and enzyme solution (50 μL of pkDAAO and rsDAAO, while 25 μL of FAD should be added to 25 μL of hDAAO). The mixture above was incubated (700 rpm) at 37 °C for 5 or 60 min. Trichloroacetic acid (25%; 50 μL) was then added to the assay mixture, mixed, and centrifuged (14,000 rpm, 5 min). The supernatant (50 μL) was mixed with 50 μL of 1 mM 2,4-dinitrophenylhydrazine (in 1 N HCl) and incubated (700 rpm) at 37 °C for 10 min. Finally, 100 μL of 1.5 M sodium hydroxide was added, mixed and incubated (700 rpm) at 37 °C for 10 min. The absorbance was read at 450 nm on an ELx800 Universal Microplate Reader (BioTek Instruments, Winooski, VT) against a blank sample consisting of the same homogenates without D-alanine. The activity of DAAO in the homogenates was quantified against the standard curve of pyruvic acid (from 100 to 800 μM; $R2 > 0.99$).

5.1.4. Rat intrathecal catheterization and injection

A 18-cm polyethylene catheter (PE-10: 0.28 mm i.d. and 0.61 mm o.d., Clay Adams, Parsippany, NJ, USA) with a volume of 13 μL was inserted into the rat lumbar level of the spinal cord as described elsewhere under inhale isoflurane anesthesia (4% for

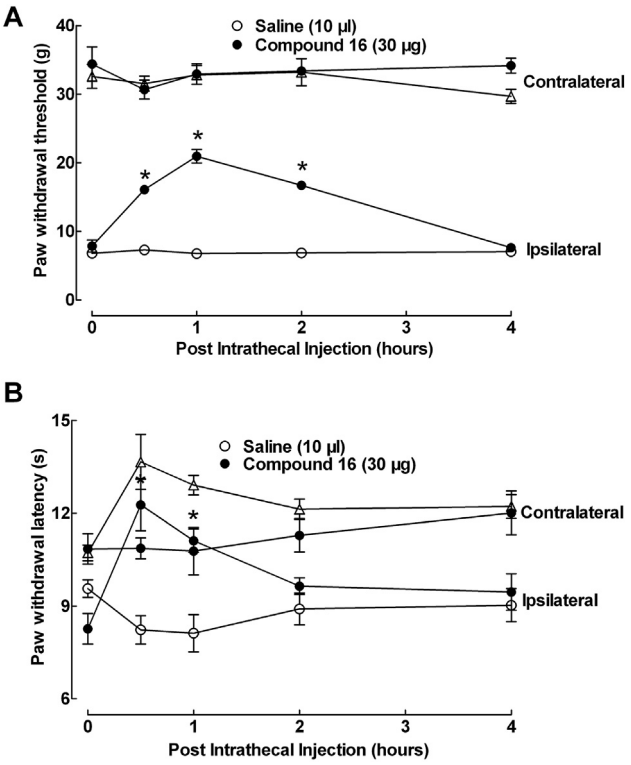


Fig. 4. Analgesic effects of compound **16** given intrathecally on mechanical allodynia and heat hyperalgesia. **A**. Analgesic effect on mechanical allodynia; **B**. Analgesic effect on heat hyperalgesia. Neuropathic rats 7 days after spinal nerve ligation received single intrathecal administration of saline (10 μL) or compound **16** (30 μg). The contralateral and ipsilateral hind limb withdrawal thresholds to mechanical stimuli and withdrawal latency to heat stimulus were measured at 0.5, 1, 2 or 4 h after intrathecal administration. Data are presented as means ± S.E.M. (n = 6 in each group). * denotes statistically significant difference ($P < 0.05$ by two-way ANOVA test) compared with the normal saline control.

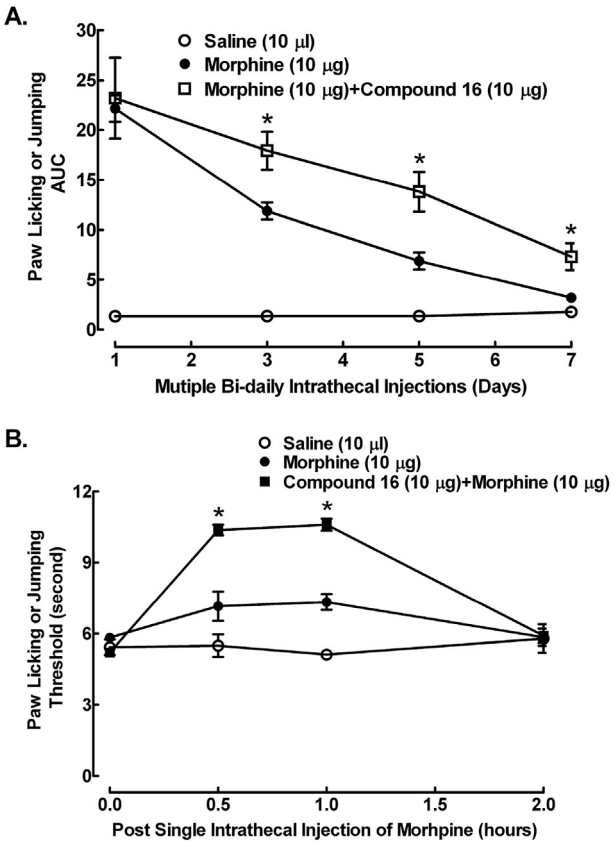


Fig. 5. Preventive and reversal effects of compound **16** on morphine analgesic tolerance in the hot-plate test. **A**. Preventive effect. Intrathecally cannulated rats received intrathecal injections of saline (10 μL), morphine (10 μg) or the combination of compound **16** (10 μg) and morphine (10 μg), twice daily with a 12 h-interval for 7 days. **B**. Reversal effect. On day 8, rats received a single intrathecal administration of saline (10 μL) or compound **16** (10 μg) 15 min before saline or morphine (5 μg)) challenge 30 min prior to the hot-plate test. Data are presented as means ± S.E.M. (n = 6 in each group). * denotes statistically significant difference ($P < 0.05$, by two-way ANOVA followed by post-hoc Student-Newman-Keuls test), compared to the morphine group.

induction and 1% for maintenance) run by an anesthesiameter (Ugo Basile Gas Anesthesia System, Comerio, Italy). Two days after recovery from anesthesia, the placing of the catheter in the spinal cord was verified by administering 4% lidocaine (10 μL followed by 15 μL of saline for flushing) with a 50-μL micro injector (Shanghai Anting Micro-Injector Factory, Shanghai, China). Only rats that had no motor impairment following intrathecal catheter were considered for the study; only rats that developed immediate bilateral paralysis of hind limbs following intrathecal administration of lidocaine were selected for the study.

5.1.5. The rat and mouse formalin test

Animals were acclimated individually to the observation cage for 30 min prior to testing. The formalin test was performed as previously described by injecting 50 μL (for rats) or 10 μL (for mice) of 5% formalin in 0.9% saline subcutaneously on the dorsal side of the right hindpaw and the animal was immediately placed in a transparent polycarbonate box. For rats, nociceptive behavior was manually quantified by counting the number of the formalin-injected paw flinches in 1-min epochs and measurements were taken at 10-min intervals beginning immediately after formalin injection and ending 90 min later. For mice, the duration of nociceptive behaviors (licking/biting) was manually quantified in the pooled durations at 0–5 min and 20–40 min, which were considered as the acute nociception and tonic pain, respectively.

5.1.6. The mouse hot-plate test

For the hot-plate test, pain reflexes in response to thermal stimulus were measured using YLS-6B Intelligence Hot Plate Analgesia Meter (Shandong Academy of Medical Sciences Device Co., Shandong, China). The surface of the hot-plate was heated to a constant temperature at 55 ± 0.1 °C, as measured by a built-in digital thermometer with an accuracy of 0.1 °C and verified by a surface thermometer. Mice were placed on the hot-plate, which was surrounded by a clear acrylic cage, and the Start/Stop button on a timer was activated. The latency to respond with either a hind-paw lick or flick, biting, or jump (whichever came first) was measured to the nearest 0.1 s by deactivating the timer when the response was observed. Trials were terminated if the animals did not respond within the 40 s cut-off period to avoid tissue damage.

5.1.7. The rat spinal nerve ligation-induced neuropathic pain model, and mechanical allodynia and heat hyperalgesia measurement

Spinal nerve ligation was used to induce a peripheral neuropathy and pain hypersensitivity. The unilateral ligation of 2 spinal nerves (L5 and L6) was performed under inhale isoflurane anesthesia (4% for induction and 1% for maintenance) run by an anesthesiameter (Ugo Basile Gas Anesthesia System, Comerio, Italy). The unilateral ligation of 2 spinal nerves (L5 and L6) was described in detail earlier. The left L5 and L6 spinal nerves were isolated and

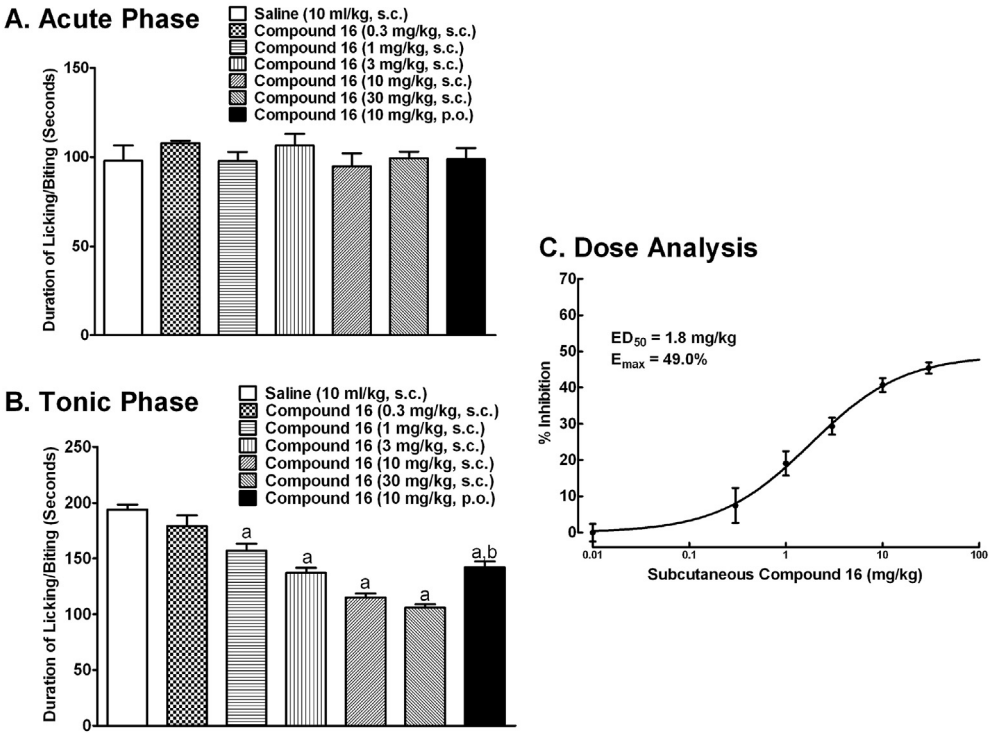


Fig. 6. Effects of systemic administration of compound **16** on formalin-induced pain. **A**. Effect on acute nociception. **B**. Effect of on tonic pain. **C**. Dose-response analysis on tonic pain. Mice received subcutaneous injection of normal saline (10 mL/kg) and compound **16** (0.3, 1, 3, 10 or 30 mg/kg), and compound **16** (10 mg/kg) for gavage 30 min before paw 5% formalin (10 μL) challenge. Cumulated biting duration from 0 to 5 min and 20–40 min after formalin injection represents acute phase nociception and tonic phase pain, respectively. Data are presented as means ± S.E.M. (n = 6 in each group). [a] and [b] denote statistically significant difference ($P < 0.05$ by one-way ANOVA followed by post-hoc Student-Newman-Keuls test), compared to the saline control group and compound **16** (10 mg/kg, subcutaneously) group, respectively.

tightly ligated with 6–0 silk thread. After ligation, the wound was sutured and the rats were allowed to recover. Of the nerve-ligated animals, only those with marked unilateral allodynia to mechanical stimulation (hind limb withdrawal thresholds in the operated side < 8 g) and with no major impairment were included in the study.

In order to evaluate mechanical allodynia, animals were acclimated for at least half an hour to the test environment, namely a plexiglass box on a metal grid (0.5 × 0.5 cm). The hind paw withdrawal threshold (PWT) was measured by a 2450 CE Electronic Von Frey hair (IITC Life Science Inc, Woodland Hill, CA, USA). An electronic hand-held transducer with a No.15 monofilament was applied perpendicularly to the medial, surface of the hind paws with the force increasing until the rat suddenly withdrew or licked the hind paw. The lowest force producing a withdrawal response was considered the threshold; this was based on three repeated measurements with a 10-min interval and the mean of these threshold values for each hind paw at each time point was used.

For assessment of heat hyperalgesia, rats were put in a plexiglass box on the elevated glass surface. Following an adaption period of 30 min at least, radiant heat was applied to the plantar medial surface of each hind paw. The hind paw withdrawal latency (PWL) was measured by the 390G Plantar Test Analgesia Meter (IITC Life Science Inc.). A cut-off time was 20 s to minimize tissue damage upon the stimulation. The paw withdrawal latency was defined as the time from the onset of radiant heat application to the withdrawal of hind paws. Both hind paws were tested independently for three times with a 10-min interval between trials. Each test was calculated as a mean of three repeated measurements.

5.2. Chemistry

5.2.1. General method

Unless otherwise noted all materials were obtained from commercial suppliers and used without further purification. Organic extracts were routinely dried over anhydrous sodium sulfate. If necessary silica gel column chromatography (200–300 mesh) were performed for purification of intermediate using PE/EtOAc or DCM/MeOH as mobile phase. NMR spectra were recorded on a Bruker 400 MHz spectrometer for ^{1}H NMR and 100 MHz for ^{13}C NMR. High-resolution mass spectroscopy (HRMS) values were recorded on a Waters Q-Tof Premier system. The purities of the tested compounds were determined to be above 95% by Agilent 1200 HPLC system (4.6 mm × 250 mm column, 5 μm, 5%, 10% or 20% MeOH to 95% MeOH in H_2O in 20 min at 1.0 mL/min and UV detection at 254 nm or 210 nm).

5.2.2. General procedure for synthesis of 5-azaquinoxaline-2,3-diones

Step 1. To 25–30 mL 98% H_2SO_4 was added portionwise 10.0 mmol substituted 2-aminopyridine at −5 °C. After the solid material was dissolved 0.6 mL fuming HNO_3 was added dropwise over 5 min maintaining internal temperature ≤0 °C. The mixture was allowed to warm to 50 °C over 2 h, and then stirred for another 1 h at this temperature before poured onto 150 g crushed ice. The aqueous solution was basified to pH = 9 with ammonia and extracted with ethyl acetate. The combine extracts were dried, concentrated and purified by column chromatography to give 2-amino-3-nitropyridine. Alternative method: To 25–30 mL 98% H_2SO_4 was added portionwise 10.0 mmol substituted 2-aminopyridine at −5 °C. After the solid material was dissolved,

0.6 mL fuming HNO_3 was added dropwise over 5 min maintaining internal temperature ≤0 °C. The mixture was stirred at this temperature for 10 min before poured onto 150 g crushed ice. Ammonia was used to adjust pH = 3–4, and the resulted white solid was filtered, washed with water and 10 mL 50–75% ethanol, and dried under vacuum to give 2-nitraminopyridine. To 25–30 mL 98% H_2SO_4 was added portionwise 2-nitraminopyridine above obtained at 0 °C. The mixture was allowed to warm to 50 °C over 1 h, and then stirred for another 1 h at this temperature before poured onto 150 g crushed ice. The aqueous solution was basified to pH = 9 with ammonia and extracted with ethyl acetate. The combine extracts were dried, concentrated and purified by column chromatography to give 2-amino-3-nitropyridine.

Step 2. To a solution of 2-amino-3-nitropyridine in mixed solvent of ethyl acetate and methanol (V/V = 8:1) was added 10–15% (m/m) 10% Pd/C. This mixture was vacuumed and backfilled with hydrogen and stirred overnight at room temperature before filtration. The filtrate was concentrated to give crude 2.3-diaminopyridine, which could be purified by column chromatography. Alternative method: To a solution of 10.0 mmol 2-amino-3-nitropyridine in 20 mL ethanol was added 30.0 mmol $SnCl_2$. This mixture was heated to reflux for 3–5 h before treatment with 2 M NaOH. The mixture was extracted with ethyl acetate, dried and concentrated to give 2,3-diaminopyridine.

Step 3. A mixture of 2,3-diaminopyridine in diethyl oxalate (10 mL/1 mmol phenyldiamine) was heated to reflux for 4 h before cooled to room temperature. The resulted solid was filtered, washed with ethyl acetate and 95% ethanol, decolorized with activated charcoal and recrystallized in water and DMF or DMSO to give 5-azaquinoxalinediones as white solid.

*5.2.2.1. 1,4-Dihydropyrido[2,3-b]pyrazine-2,3-dione (**1**).* The title compound was prepared from 2,3-aminopyridine and diethyl oxalate as white solid (527 mg, 75.0%). IR (KBr) ν 3215, 2741, 1697, 1625, 1465, 1381 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 7.13 (1H, dd, J = 8.0, 4.8 Hz, H-7), 7.46 (1H, dd, J = 8.0, 1.6 Hz, H-6), 8.07 (1H, dd, J = 4.8, 1.6 Hz, H-8), 11.97 (1H, s, NH-1), 12.32 (1H, s, NH-4); ^{13}C NMR (100 MHz, DMSO-d_6) δ 118.78, 121.60, 122.23, 139.01, 142.02, 154.68, 155.79; HRMS-EI $C_7H_5N_3O_2$ calcd $[M+Na]^+$ 186.0279, found 186.0278.

*5.2.2.2. 6-Methyl-1,4-dihydropyrido[2,3-b]pyrazine-2,3-dione (**2**).* The title compound was prepared from 2-amino-6-methylpyridine according to the general procedure as white solid (391 mg, 22.1%). IR (KBr) ν 3176, 3109, 1682, 1591, 1479, 1391 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 2.40 (3H, s, CH_3), 6.98 (1H, d, J = 8.0 Hz, H-7), 7.35 (1H, d, J = 8.0 Hz, H-8), 11.93 (1H, brs, NH-1), 12.22 (1H, brs, NH-4); ^{13}C NMR (100 MHz, DMSO-d_6) δ 23.08, 118.03, 119.15, 122.89, 138.24, 150.70, 154.65, 155.91; HRMS-EI $C_8H_7N_3O_2$ calcd $[M+Na]^+$ 200.0436, found 200.0435.

*5.2.2.3. 7-Methyl-1,4-dihydropyrido[2,3-b]pyrazine-2,3-dione (**3**).* The title compound was prepared from 2-amino-5-methylpyridine according to the general procedure as white solid (623 mg, 35.2%). IR (KBr) ν 3041, 2774, 1694, 1386 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 2.27 (3H, s, CH_3), 7.26 (1H, d, J = 1.6 Hz, H-6), 7.91 (1H, d, J = 1.6 Hz, H-8), 12.07 (2H, brs, NH-1 & 4); ^{13}C NMR (100 MHz, DMSO-d_6) δ 17.32, 121.41, 122.57, 128.04, 137.07, 142.00, 155.00, 155.75; HRMS-EI $C_8H_7N_3O_2$ calcd $[M+Na]^+$ 200.0436, found 200.0435.

*5.2.2.4. 8-Methyl-1,4-dihydropyrido[2,3-b]pyrazine-2,3-dione (**4**).* The title compound was prepared from 2-amino-4-methylpyridine according to the general procedure as white solid (420 mg, 23.7%). IR (KBr) ν 3048, 2745, 1682, 1623, 1386, 906 847, 721 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 2.37 (3H, s, CH_3), 6.99 (1H, d, J = 4.8 Hz, H-7), 7.95 (1H, d, J = 4.8 Hz, H-6), 11.48 (1H, brs, NH-1), 12.27 (1H, brs, NH-4); ^{13}C NMR (100 MHz, DMSO-d_6) δ 16.62, 120.35, 120.79, 132.84, 138.77, 141.79, 155.40, 155.62; HRMS-EI $C_8H_7N_3O_2$ calcd $[M+Na]^+$ 200.0436, found 200.0436.

*5.2.2.5. 8-Ethyl-1,4-dihydropyrido[2,3-b]pyrazine-2,3-dione (**5**).* The title compound was prepared from 2-amino-4-ethylpyridine according to the general procedure as white solid (308 mg, 16.1%). 2-Amino-4-ethylpyridine was synthesized according to the literature [39]. IR (KBr) ν 2755, 1704, 1690, 1622, 1387, 832 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 1.15 (3H, t, J = 7.6 Hz, CH_3), 2.79 (2H, q, J = 7.6 Hz, CH_2), 7.02 (1H, d, J = 5.2 Hz, H-7), 7.99 (1H, d, J = 5.2 Hz, H-6), 11.48 (1H, brs, NH-1), 12.27 (1H, brs, NH-4); ^{13}C NMR (100 MHz, DMSO-d_6) δ 13.57, 22.32, 118.96, 119.54, 138.47, 138.80, 142.08, 155.40, 155.51; HRMS-EI $C_9H_9N_3O_2$ calcd $[M+Na]^+$ 214.0592, found 214.0591.

*5.2.2.6. 7-Trifluoromethyl-1,4-dihydropyrido[2,3-b]pyrazine-2,3-dione (**6**).* The title compound was prepared from 2-amino-5-trifluoromethylpyridine according to the general procedure as white solid (635 mg, 27.5%). IR (KBr) ν 3055, 1717, 1698, 1627, 1342, 1165, 1094 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 7.63 (1H, d, J = 2.0 Hz, H-8), 8.44 (1H, s, H-6), 12.13 (1H, s, NH-1), 12.71 (1H, s, NH-4); ^{13}C NMR (100 MHz, DMSO-d_6) δ 118.74 (t), 120.04 (d), 122.52, 124.18 (d), 138.99 (t), 142.95, 154.95, 156.39; HRMS-EI $C_8H_4F_3N_3O_2$ calcd $[M+Na]^+$ 254.0153, found 254.0150.

*5.2.2.7. 8-Trifluoromethyl-1,4-dihydropyrido[2,3-b]pyrazine-2,3-dione (**7**).* The title compound was prepared from 2-amino-4-trifluoromethylpyridine according to the general procedure as white solid (178 mg, 7.7%). IR (KBr) ν 1700, 1458, 1374, 1188, 1137 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 7.40 (1H, d, J = 4.8 Hz, H-7), 8.20 (1H, d, J = 4.8 Hz, H-6), 11.63 (1H, brs, NH-1), 12.62 (1H, s, NH-4); ^{13}C NMR (100 MHz, DMSO-d_6) δ 114.72 (q), 118.58, 119.32 (m), 121.30, 124.03, 126.75, 141.43, 142.47, 155.28, 155.63; HRMS-EI $C_8H_4F_3N_3O_2$ calcd $[M+Na]^+$ 254.0153, found 254.0149.

*5.2.2.8. 6-Chloro-1,4-dihydropyrido[2,3-b]pyrazine-2,3-dione (**8**).* The title compound was prepared from 2-amino-6-chloropyridine according to the general procedure as white solid (176 mg, 8.9%). IR (KBr) ν 3117, 1688, 1625, 1587, 1469, 1390 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 7.19 (1H, d, J = 8.0 Hz, H-7), 7.46 (1H, d, J = 8.0 Hz, H-8), 12.05 (1H, s, NH-1), 12.51 (1H, s, NH-4); ^{13}C NMR (100 MHz, DMSO-d_6) δ 118.69, 121.66, 125.84, 139.49, 141.58, 154.95, 156.19; HRMS-EI $C_7H_4ClN_3O_2$ calcd $[M+H]^+$ 198.0070, found 198.0066.

*5.2.2.9. 6-Methoxy-1,4-dihydropyrido[2,3-b]pyrazine-2,3-dione (**9**).* To a solution of 2-amino-3-nitro-6-chloropyridine (300 mg, 1.73 mmol, an intermediate in preparation of **8**) in 5 mL anhydrous DMF was added sodium methoxide (187 mg, 3.46 mmol). This mixture was stirred at room temperature for 3 h then poured onto 20 g crushed ice and extracted with ethyl acetate. The extracts were dried, evaporated and purified by column chromatography on silica gel (PE/AcOEt = 5:1) to give 2-amino-3-nitro-6-methoxylpyridine (246 mg, 84.1%) as yellow solid. Next steps followed the general procedure, and the title compound was obtained as white solid (188 mg, 67.0% over 2 steps). IR (KBr) ν 3246, 3184, 1708, 1635, 1599, 1488, 1462, 1341, 1260, 1024, 839 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 3.81 (3H, s, CH_3), 6.56 (1H, d, J = 8.4 Hz, H-7), 7.42 (1H, d, J = 8.4 Hz, H-8), 11.82 (1H, s, NH-1), 12.25 (1H, s, NH-4); ^{13}C NMR (100 MHz, DMSO-d_6) δ 53.50, 104.56, 115.19, 126.61, 136.21, 154.35, 156.11, 158.55; HRMS-EI $C_8H_7N_3O_3$ calcd $[M+Na]^+$ 216.0385, found 216.0382.

*5.2.2.10. 6-Ethoxy-1,4-dihydropyrido[2,3-b]pyrazine-2,3-dione (**10**).* The title compound was prepared from 2-amino-3-nitro-6-chloropyridine and sodium ethoxide in a manner similar to **9** as white solid (160 mg, 44.9%). IR (KBr) ν 3174, 1712, 1688, 1594, 1467, 1261 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 1.29 (3H, t, $J = 7.2$ Hz, CH_3), 4.23 (2H, q, $J = 7.2$ Hz, CH_2), 6.53 (1H, d, $J = 8.4$ Hz, H-7), 7.41 (1H, d, $J = 8.4$ Hz, H-8), 11.81 (1H, s, NH-1), 12.21 (1H, s, NH-4); ^{13}C NMR (100 MHz, DMSO-d_6) δ 14.47, 61.58, 104.75, 115.05, 126.56, 136.16, 154.34, 156.11, 158.17; HRMS-EI $C_9H_9N_3O_3$ calcd $[M+Na]^+$ 230.0542, found 230.0540.

*5.2.2.11. Methyl 2,3-dioxo-1,2,3,4-tetrahydropyrido[2,3-b]pyrazine-7-carboxylate (**11**).* The title compound was prepared from methyl 6-aminonicotinate according to the general procedure as white solid (818 mg, 37.0%). IR (KBr) ν 3233, 3062, 2846, 2757, 1724, 1691, 1615, 1434, 1391, 1319, 1229 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 3.86 (3H, s, CH_3), 7.86 (1H, s, H-6), 8.55 (1H, s, H-8), 12.08 (1H, s, NH-1), 12.66 (1H, s, NH-4); ^{13}C NMR (100 MHz, DMSO-d_6) δ 52.33, 120.29, 121.67, 122.08, 142.54, 143.11, 154.51, 155.97, 164.69; HRMS-EI $C_9H_7N_3O_4$ calcd $[M+Na]^+$ 244.0334, found 244.0334.

*5.2.2.12. 2,3-Dioxo-1,2,3,4-tetrahydropyrido[2,3-b]pyrazine-7-carboxylic acid (**12**).* A mixture of compound **11** (300 mg, 1.36 mmol), water (15 mL) and sodium hydroxide (0.1 g) was heated to 60 °C and stirred at this temperature for 1–2 h. The mixture was cooled to with ice-water and adjusted to pH = 5–6 with 2 M HCl. The resulted precipitate was filtered, washed with water, and dried under vacuum to give the title compound as white solid (243 mg, 86.5%). IR (KBr) ν 3174, 3043, 2977, 1716, 1673, 1613, 1414, 1399, 1219 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 7.88 (1H, s, H-6), 8.56 (1H, s, H-8), 12.09 (1H, s, NH-1), 12.63 (1H, s, NH-4); ^{13}C NMR (100 MHz, DMSO-d_6) δ 121.48, 121.56, 122.45, 142.22, 143.38, 154.57, 155.97, 165.71; HRMS-EI $C_8H_5N_3O_4$ calcd $[M-H]^-$ 206.0202, found 206.0199.

*5.2.2.13. 7-Fluoro-1,4-dihydropyrido[2,3-b]pyrazine-2,3-dione (**13**).* The title compound was prepared from 2-amino-5-fluoropyridine according to the general procedure as white solid (530 mg, 29.3%). IR (KBr) ν 3150, 3042, 1708, 1683, 1621, 1458, 1380 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 7.30 (1H, d, $J = 8.4$ Hz, H-6), 8.07 (1H, s, H-8), 12.24 (2H, brs, NH-1 & 4); ^{13}C NMR (100 MHz, DMSO-d_6) δ 109.58 (d), 122.64 (d), 128.74 (d), 136.02 (d), 153.95, 154.70, 155.30, 156.40; HRMS-EI $C_7H_4FN_3O_2$ calcd $[M+Na]^+$ 204.0185, found 204.0184.

*5.2.2.14. 7-Chloro-1,4-dihydropyrido[2,3-b]pyrazine-2,3-dione (**14**).* The title compound was prepared from 2-amino-5-chloropyridine according to the general procedure as white solid (433 mg, 21.9%). IR (KBr) ν 3190, 3137, 3051, 2753, 1716, 1608, 1445, 1392 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 7.44 (1H, d, $J = 2.0$ Hz, H-6), 8.09 (1H, d, $J = 2.0$ Hz, H-8); ^{13}C NMR (100 MHz, DMSO-d_6) δ 121.22, 122.83, 124.32, 138.19, 139.69, 154.58, 155.53; HRMS-EI $C_7H_4ClN_3O_2$ calcd $[M+H]^+$ 198.0070, found 198.0069.

*5.2.2.15. 7-Bromo-1,4-dihydropyrido[2,3-b]pyrazine-2,3-dione (**15**).* The title compound was prepared from 2-amino-5-bromopyridine according to the general procedure as white solid (620 mg, 25.6%). IR (KBr) ν 3199, 3138, 3045, 2751, 1715, 1603, 1446, 1389 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 7.55 (1H, d, $J = 1.6$ Hz, H-6), 8.15 (1H, d, $J = 1.6$ Hz, H-8); ^{13}C NMR (100 MHz, DMSO-d_6) δ 112.36, 123.20, 123.80, 138.46, 141.83, 154.55, 155.58; HRMS-EI $C_7H_4BrN_3O_2$ calcd $[M+H]^+$ 241.9565, found 241.9567.

*5.2.2.16. 8-Chloro-1,4-dihydropyrido[2,3-b]pyrazine-2,3-dione (**16**).* The title compound was prepared from 2-amino-4-chloropyridine according to the general procedure as white solid (284 mg, 14.4%). IR (KBr) ν 3044, 2743, 1697, 1594, 1373, 1276 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 7.29 (1H, d, $J = 5.6$ Hz, H-7), 8.01 (1H, d, $J = 5.6$ Hz, H-6), 11.74 (1H, s, NH-1), 12.50 (1H, s, NH-4); ^{13}C NMR (100 MHz, DMSO-d_6) δ 119.18, 119.79, 126.98, 140.28, 142.13, 155.13, 155.57; HRMS-EI $C_7H_4ClN_3O_2$ calcd $[M+H]^+$ 198.0070, found 198.0064.

*5.2.2.17. 8-Bromo-1,4-dihydropyrido[2,3-b]pyrazine-2,3-dione (**17**).* The title compound was prepared from 2-amino-4-bromopyridine according to the general procedure as white solid (334 mg, 13.8%). IR (KBr) ν 3126, 3087, 3040, 2728, 1714, 1697, 1589, 1384, 1275 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 7.43 (1H, d, $J = 5.2$ Hz, H-7), 7.92 (1H, d, $J = 5.2$ Hz, H-6), 11.37 (1H, s, NH-1), 12.46 (1H, s, NH-4); ^{13}C NMR (100 MHz, DMSO-d_6) δ 116.87, 121.20, 122.46, 139.86, 142.16, 155.17, 155.50; HRMS-EI $C_7H_4BrN_3O_2$ calcd $[M+H]^+$ 241.9565, found 241.9561.

*5.2.2.18. 8-Methoxy-1,4-dihydropyrido[2,3-b]pyrazine-2,3-dione (**18**).* To a solution of 2-amino-3-nitro-4-chloropyridine (300 mg, 1.73 mmol, an intermediate in preparation of compound **16**) in 5 mL anhydrous DMF was added sodium methoxide (187 mg, 3.46 mmol). This mixture was stirred at room temperature for 3 h then poured onto 20 g crushed ice and extracted with ethyl acetate. The extracts were dried, evaporated and purified by column chromatography on silica gel (DCM/MeOH = 40:1) to give 2-amino-3-nitro-4-methoxylpyridine (255 mg, 87.3%) as yellow solid. The next steps followed the general procedure and gave the title compound as white solid (166 mg, 57.1% over 2 steps). IR (KBr) ν 3028, 2870, 2750, 1714, 1688, 1621, 1506, 1378, 1140, 981 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 3.93 (3H, s, CH_3), 6.91 (1H, d, $J = 6.0$ Hz, H-7), 8.00 (1H, d, $J = 6.0$ Hz, H-6), 11.50 (1H, s, NH-1), 12.23 (1H, s, NH-4); ^{13}C NMR (100 MHz, DMSO-d_6) δ 56.33, 102.87, 110.64, 139.22, 143.62, 152.30, 154.66, 155.97; HRMS-EI $C_8H_7N_3O_3$ calcd $[M+Na]^+$ 216.0385, found 216.0384.

*5.2.2.19. 8-Ethoxy-1,4-dihydropyrido[2,3-b]pyrazine-2,3-dione (**19**).* The title compound was prepared from 2-amino-3-nitro-4-chloropyridine and sodium ethoxide in a manner similar to **18** as white solid (144 mg, 40.3%). IR (KBr) ν 1697, 1623, 1386, 1139 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 1.39 (3H, t, $J = 7.2$ Hz, CH_3), 4.21 (2H, q, $J = 7.2$ Hz, CH_2), 6.89 (1H, d, $J = 5.6$ Hz, H-7), 7.97 (1H, d, $J = 5.6$ Hz, H-6), 11.43 (1H, s, NH-1), 12.22 (1H, s, NH-4); ^{13}C NMR (100 MHz, DMSO-d_6) δ 14.17, 64.70, 103.34, 110.62, 139.27, 143.61, 151.55, 154.68, 155.98; HRMS-EI $C_9H_9N_3O_3$ calcd $[M+Na]^+$ 230.0542, found 230.0542.

*5.2.2.20. 8-Isopropoxy-1,4-dihydropyrido[2,3-b]pyrazine-2,3-dione (**20**).* The title compound was prepared from 2-amino-3-nitro-4-chloropyridine, isopropanol and sodium hydride in a manner similar to **18** as white solid (137 mg, 35.8%). IR (KBr) ν 2757, 1692, 1621, 1500, 1370, 1280 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 1.33 (6H, d, $J = 6.0$ Hz, CH_3), 4.82 (1H, m, CH), 6.91 (1H, d, $J = 5.6$ Hz, H-7), 7.95 (1H, d, $J = 5.6$ Hz, H-6), 11.38 (1H, brs, NH-1), 12.21 (1H, brs, NH-4); ^{13}C NMR (100 MHz, DMSO-d_6) δ 21.46, 71.46, 104.05, 111.20, 139.53, 143.51, 150.65, 154.68, 156.00; HRMS-EI $C_{10}H_{11}N_3O_3$ calcd $[M+Na]^+$ 244.0698, found 244.0700.

*5.2.2.21. 8-Phenoxy-1,4-dihydropyrido[2,3-b]pyrazine-2,3-dione (**21**).* The title compound was prepared from 2-amino-3-nitro-4-chloropyridine and sodium phenolate in a manner similar to **18** as white solid (76 mg, 17.2%). IR (KBr) ν 1716, 1688, 1621, 1493, 1381, 1202 cm^{-1}; ^1HNMR (400 MHz, DMSO-d_6) δ 6.49 (1H, d, $J = 5.6$ Hz, H-7), 7.20 (2H, m, C_6H_5-2′ & 6′), 7.29 (1H, m, C_6H_5-4′), 7.49 (2H, m, C_6H_5-3′ & 5′), 7.94 (1H, d, $J = 5.6$ Hz, H-6), 11.91 (1H, s, NH-1), 12.38

(1H, s, NH-4); ^{13}C NMR (100 MHz, DMSO-d_6) δ 106.48, 112.50, 120.19, 125.30, 130.25, 140.57, 143.21, 150.35, 154.04, 154.76, 156.00; HRMS-EI $C_{13}H_9N_3O_3$ calcd $[M+Na]^+$ 278.0542, found 278.0544.

*5.2.2.22. 8-(Benzyloxy)-1,4-dihydropyrido[2,3-b]pyrazine-2,3-dione (**22**).* The title compound was prepared from 2-amino-3-nitro-4-chloropyridine, benzyl alcohol and sodium hydride in a manner similar to **18** as white solid (105 mg, 22.5%). IR (KBr) ν 1694, 1621, 1501, 1426, 1379, 1275, 1129 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6) δ 5.34 (2H, s, CH_2), 6.98 (1H, d, J = 6.0 Hz, H-7), 7.29–7.44 (3H, m, C_6H_5-2′, 4′ & 6′), 7.57 (2H, d, J = 8.4 Hz, C_6H_5-3′ & 5′), 7.95 (1H, d, J = 6.0 Hz, H-6), 11.62 (1H, s, NH-1), 12.26 (1H, s, NH-4); ^{13}C NMR (100 MHz, DMSO-d_6) δ 70.46, 104.44, 111.46, 128.20, 128.43, 128.74, 136.23, 140.02, 143.83, 151.63, 155.23, 156.40; HRMS-EI $C_{14}H_{11}N_3O_3$ calcd $[M+Na]^+$ 292.0698, found 292.0697.

*5.2.2.23. 8-Phenylethoxy-1,4-dihydropyrido[2,3-b]pyrazine-2,3-dione (**23**).* The title compound was prepared from 2-amino-3-nitro-4-chloropyridine, 2-phenylethanol and sodium hydride in a manner similar to **18** as white solid (178 mg, 36.4%). IR (KBr) ν 2862, 2754, 1703, 1620, 1502, 1386, 1281, 1139 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.14 (2H, t, J = 6.8 Hz, CH_2CH_2O), 4.35 (2H, t, J = 6.8 Hz, CH_2CH_2O), 6.92 (1H, d, J = 5.6 Hz, H-7), 7.20–7.25 (1H, m, C_6H_5-4′), 7.31 (2H, t, J = 7.2 Hz, C_6H_5-2′ & 6′), 7.40 (2H, d, J = 7.8 Hz, C_6H_5-3′ & 5′), 7.95 (1H, d, J = 5.6 Hz, H-6), 11.48 (1H, s, NH-1), 12.23 (1H, s, NH-4); ^{13}C NMR (100 MHz, DMSO-d_6) δ 34.36, 69.73, 103.50, 110.64, 126.36, 128.29, 129.08, 138.10, 139.32, 143.60, 151.47, 154.75, 155.97; HRMS-EI $C_{15}H_{13}N_3O_3$ calcd $[M+Na]^+$ 306.0855, found 306.0855.

*5.2.2.24. 8-Phenylpropoxy-1,4-dihydropyrido[2,3-b]pyrazine-2,3-dione (**24**).* The title compound was prepared from 2-amino-3-nitro-4-chloropyridine, 3-phenylpropanol and sodium hydride in a manner similar to **18** as white solid (153 mg, 29.8%). IR (KBr) ν 3027, 2945, 2872, 2756, 1708, 1694, 1623, 1507, 1391, 1276, 1134 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.03–2.13 (2H, m, $CH_2CH_2CH_2O$), 2.85 (2H, t, J = 7.6 Hz, $CH_2CH_2CH_2O$), 4.14 (2H, t, J = 6.0 Hz, $CH_2CH_2CH_2O$), 6.87 (1H, d, J = 5.6 Hz, H-7), 7.16–7.22 (1H, m, C_6H_5-4′), 7.25–7.33 (4H, m, C_6H_5-2′, 3′, 5′ & 6′), 7.96 (1H, d, J = 5.6 Hz, H-6), 11.59 (1H, s, NH-1), 12.26 (1H, s, NH-4); ^{13}C NMR (100 MHz, DMSO-d_6) δ 29.98, 31.31, 68.31, 103.25, 110.66, 125.76, 128.26, 128.38, 139.37, 141.60, 143.60, 151.72, 154.88, 156.01; HRMS-EI $C_{16}H_{15}N_3O_3$ calcd $[M+Na]^+$ 320.1011, found 320.1012.

*5.2.2.25. 6-(Benzyloxy)-1,4-dihydropyrido[2,3-b]pyrazine-2,3-dione (**25**).* The title compound was prepared from 2-amino-3-nitro-6-chloropyridine, benzyl alcohol and sodium hydride in a manner similar to **9** as white solid (121 mg, 26.0%). IR (KBr) ν 3028, 2952, 1697, 1623, 1594, 1476, 1456, 1340, 1261 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6) δ 5.29 (2H, s, CH_2), 6.61 (1H, d, J = 8.4 Hz, H-7), 7.28–7.40 (3H, m, C_6H_5-2′, 4′ & 6′), 7.44 (1H, d, J = 8.4 Hz, H-8), 7.48 (2H, d, J = 7.2 Hz, C_6H_5-3′ & 5′), 11.85 (1H, brs, NH-1), 12.28 (1H, brs, NH-4); ^{13}C NMR (100 MHz, DMSO-d_6) δ 67.25, 104.91, 115.42, 126.72, 127.85, 128.31, 128.34, 136.08, 136.92, 154.37, 156.15, 157.82; HRMS-EI $C_{14}H_{11}N_3O_3$ calcd $[M+Na]^+$ 292.0698, found 292.0697.

*5.2.2.26. 6-Phenethoxy-1,4-dihydropyrido[2,3-b]pyrazine-2,3-dione (**26**).* The title compound was prepared from 2-amino-3-nitro-6-chloropyridine, 2-phenylethanol and sodium hydride in a manner similar to **9** as white solid (89 mg, 18.1%). IR (KBr) ν 3028, 2945, 1703, 1681, 1600, 1482, 1464, 1340, 1269 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.01 (2H, t, J = 7.2 Hz, CH_2CH_2O), 4.38 (2H, t, J = 7.2 Hz, CH_2CH_2O), 6.54 (1H, d, J = 8.4 Hz, H-7), 7.18–7.35 (5H, m, C_6H_5), 7.42 (1H, d, J = 8.4 Hz, H-8), 11.83 (1H, s, NH-1), 12.24 (1H, s, NH-4); ^{13}C NMR (100 MHz, DMSO-d_6) δ 34.67, 66.61, 104.75, 115.22, 126.27, 126.63, 128.29, 128.97, 136.19, 138.21, 154.36, 156.13, 158.02; HRMS-EI $C_{15}H_{13}N_3O_3$ calcd $[M+Na]^+$ 306.0855, found 306.0856.

*5.2.2.27. 7-Fluoro-8-methyl-1,4-dihydropyrido[2,3-b]pyrazine-2,3-dione (**27**).* The title compound was prepared from 2-amino-4-methyl-5-fluoropyridine according to the general procedure as white solid (441 mg, 22.6%). IR (KBr) ν 3047, 2971, 2866, 2783, 1718, 1625, 1523, 1454, 1381, 1275 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.3 (3H, d, J = 1.6 Hz, CH_3), 8.04 (1H, s, H-6), 11.64 (1H, s, NH-1), 12.33 (1H, s, NH-4); ^{13}C NMR (100 MHz, DMSO-d_6) δ 8.73 (d), 120.27 (d), 121.30 (d), 128.21, 128.49, 135.39 (d), 154.38 (d), 154.85, 155.31; HRMS-EI $C_8H_6FN_3O_2$ calcd $[M+H]^+$ 196.0522, found 196.0516.

*5.2.2.28. 7-Chloro-8-methyl-1,4-dihydropyrido[2,3-b]pyrazine-2,3-dione (**28**).* The title compound was prepared from 2-amino-4-methyl-5-chloropyridine according to the general procedure as white solid (325 mg, 15.4%). IR (KBr) ν 3040, 2861, 2771, 1704, 1687, 1613, 1449, 1381 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.42 (3H, s, CH_3), 8.11 (1H, s, H-6), 11.62 (1H, brs, NH-1), 12.43 (1H, brs, NH-4); ^{13}C NMR (100 MHz, DMSO-d_6) δ 13.71, 121.47, 125.86, 130.31, 137.75, 140.05, 155.07, 155.28; HRMS-EI $C_8H_6ClN_3O_2$ calcd $[M+H]^+$ 212.0227, found 212.0218.

*5.2.2.29. 7-Bromo-8-methyl-1,4-dihydropyrido[2,3-b]pyrazine-2,3-dione (**29**).* The title compound was prepared from 2-amino-4-methyl-5-bromopyridine according to the general procedure as white solid (279 mg, 10.9%). IR (KBr) ν 3032, 2854, 2762, 1697, 1609, 1435, 1379 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.45 (3H, s, CH_3), 8.19 (1H, s, H-6), 11.58 (1H, brs, NH-1), 12.40 (1H, brs, NH-4); ^{13}C NMR (100 MHz, DMSO-d_6) δ 16.68, 116.67, 121.61, 131.80, 138.28, 142.51, 155.18, 155.31; HRMS-EI $C_8H_6BrN_3O_2$ calcd $[M+H]^+$ 255.9722, found 255.9723.

*5.2.2.30. 7,8-Dichloro-1,4-dihydropyrido[2,3-b]pyrazine-2,3-dione (**30**).* To a solution of 2-amino-4-chloropyridine (1.28 g, 10.0 mmol) in DMF (40 mL) at −20 °C was added NCS (2.67 g, 20.0 mmol). This mixture was allowed to warm to room temperature and stirred for 24 h, and then poured into 300 mL ice-water and extracted with ethyl acetate. The extracts were washed with 1 M NaOH and brine, dried and evaporated. The residue was purified by column chromatography on silica gel to give 4,5-dichloropyridin-2-amine (1.12 g, 69.0%).

To 30 mL 98% H_2SO_4 was added portionwise 4,5-dichloropyridin-2-amine (1.0 g, 6.13 mmol) at −5 °C. After the solid material was completely dissolved, 3.7 mL fuming HNO_3 was added dropwise over 5 min maintaining internal temperature ≤0 °C. The mixture was allowed to warm to 50 °C over 2 h, and then stirred for another 1 h at this temperature before poured onto 150 g crushed ice. The aqueous solution was basified to pH = 7.3 with ammonia and extracted with ethyl acetate. The combined extracts were dried, concentrated and purified by column chromatography to give 4,5-dichloro-3-nitropyridin-2-amine (0.80 g, 62.6%).

To a solution of 4,5-dichloro-3-nitropyridin-2-amine (300 mg, 1.44 mmol) in 5 mL AcOH was added portionwise zinc dust (472 mg, 7.2 mmol) over 20 min. After addition, the mixture was stirred for another 3 h then added dropwise to 100 mL saturated Na_2CO_3 and extracted with ethyl acetate. The combined extracts were washed with brine, dried, evaporated and purified by column chromatography to give 4,5-dichloropyridine-2,3-diamine (148 mg, 57.5%).

A mixture of 4,5-dichloropyridine-2,3-diamine (148 mg, 0.83 mmol) in 8 mL diethyl oxalate was heated to reflux for 6 h before cooled to room temperature. The resulted solid was filtered and washed with ethyl acetate. The solid product was decolored with activated charcol and recrystallized in water and DMF to give

the title compound as white solid (51 mg, 26.5%). IR (KBr) ν 3113, 3040, 1727, 1704, 1592, 1372 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 8.24 (1H, s, H-6), 11.86 (1H, s, NH-1), 12.60 (1H, s, NH-4); ^{13}C NMR (100 MHz, DMSO-d_6) δ 121.36, 123.38, 125.00, 138.97, 140.33, 155.04, 155.09; HRMS-EI $C_7H_3Cl_2N_3O_2$ calcd $[M+Na]^+$ 253.9500, found 253.9502.

*5.2.2.31. 7-Bromo-8-chloro-1,4-dihydropyrido[2,3-b]pyrazine-2,3-dione (**31**).* To a solution of 2-amino-4-chloropyridine (1.28 g, 10.0 mmol) in anhydrous CH_3CN (40 mL) was added portionwise N-bromosuccinimide (1.96 g, 11.0 mmol). The reaction mixture was stirred at room temperature for 24 h then poured into 100 mL ice-water and extracted with ethyl acetate. The combined extracts were washed with 1 M NaOH and brine, dried and evaporated. The residue was purified by column chromatography on silica gel to give 5-bromo-4-dichloropyridin-2-amine (1.53 g, 60.8%). Next steps followed the procedure of **30** and gave the title compound as white solid (63 mg, 12.5% over 3 steps). IR (KBr) ν 3108, 3033, 1703, 1587, 1398, 1367 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 8.30 (1H, s, H-6), 11.82 (1H, s, NH-1), 12.59 (1H, s, NH-4); ^{13}C NMR (100 MHz, DMSO-d_6) δ 114.00, 121.88, 127.18, 139.80, 143.23, 155.49, 155.61; HRMS-EI $C_7H_3BrClN_3O_2$ calcd $[M+Na]^+$ 297.8995, found 297.8993.

*5.2.2.32. 8-Bromo-7-chloro-1,4-dihydropyrido[2,3-b]pyrazine-2,3-dione (**32**).* The title compound was prepared from 2-amino-4-bromopyridine in a manner similar to **30** as white solid (19 mg, 6.9%). IR (KBr) ν 3236, 1767, 1687, 1582, 1419, 1194, 693 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 8.20 (1H, s, H-6), 11.49 (1H, s, NH-1), 12.58 (1H, s, NH-4); ^{13}C NMR (100 MHz, DMSO-d_6) δ 117.63, 123.34, 126.14, 138.88, 140.48, 155.49, 155.56; HRMS-EI $C_7H_3BrClN_3O_2$ calcd $[M+Na]^+$ 297.8995, found 297.8991.

*5.2.2.33. 7,8-Dibromo-1,4-dihydropyrido[2,3-b]pyrazine-2,3-dione (**33**).* The title compound was prepared from 2-amino-4-bromopyridine in a manner similar to **31** as white solid (17 mg, 5.2%). IR (KBr) ν 1703, 1577, 1387, 1355, 1260 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 8.27 (1H, s, H-6), 11.45 (1H, s, NH-1), 12.57 (1H, s, NH-4); ^{13}C NMR (100 MHz, DMSO-d_6) δ 116.86, 119.75, 123.49, 139.19, 143.00, 155.59; HRMS-EI $C_7H_3Br_2N_3O_2$ calcd $[M+Na]^+$ 341.8490, found 341.8489.

Acknowledgments

This study was supported by the National Natural Science Foundation of China [No: 81072623], the Shanghai Innovation Project (No: 11ZR1416400, 12JC1404800) and the Shanghai Industrial and Translational Project (No: 12431900603). We thank Dr. Kenji Hashimoto at the Chiba University Center for Forensic Mental Health in Japan for his kind gift of CBIO.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2016.04.017.

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