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Discovery and development of novel pyrimidine and pyrazolo/thieno-fused pyrimidine derivatives as potent and orally active inducible nitric oxide synthase dimerization inhibitor with efficacy for arthritis

Liu Zeng Chen [a, b, 1], Hai Yang Shu [a, b, 1], Jing Wu [a, b], Yun Long Yu [a, b], Duo Ma [a, b], Xin Huang [a, b], Ming Ming Liu [a, b], Xin Hua Liu [a, b, *], Jing Bo Shi [a, b, **]

[a] School of Pharmacy, Anhui Medical University, Hefei, 230032, PR China
[b] Inflammation and Immune Mediated Diseases Laboratory of Anhui Province, Hefei, PR China

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ABSTRACT

In order to discover and develop drug-like anti-inflammatory agents against arthritis, based on "Hit" we found earlier and to overcome drawbacks of toxicity, twelve series of total 89 novel pyrimidine, pyrazolo[4,3-*d*]pyrimidine and thieno[3,2-*d*]pyrimidine derivatives were designed, synthesized and screened for their anti-inflammatory activity against NO and toxicity for normal liver cells (LO2). Relationships of balance toxicity and activity have been summarized through multi-steps, and title compounds **22o, 22l** were found to show lower toxicity (against LO2: IC_{50} = 2934, 2301 μM, respectively) and potent effect against NO release (IR = 98.3, 97.67%, at 10 μM, respectively). Furthermore, compound **22o** showed potent iNOS inhibitory activity with value of IC_{50} is 0.96 μM and could interfere stability and formation of the active dimeric iNOS. It's anti-inflammatory activity *in vivo* was assessed by AIA rat model. Furthermore, the results of metabolic stability, CYP, PK study *in vivo*, acute toxicity study and subacute toxicity assessment indicated this compound had good drug-like properties for treatment.

1. Introduction

In mammals, nitric oxide (NO) is an important cellular signaling molecule and participates in many physiological functions. NO was produced by nitric oxide synthases (NOSs) [1]. They have three isoforms, including inducible nitric oxide synthase (iNOS), endothelial NOS (eNOS) and neuronal NOS (nNOS). Among them, iNOS is not always activated in cells. When cells are induced or stimulated by bacterial lipopolysaccharide (LPS) and/or proinflammatory cytokines, iNOS will express [2,3]. After activation, overexpression amounts of NO is generated through iNOS and excessive NO can defend against invading pathogens. Thus, iNOS is crucial for the innate immune system and the inflammatory response. However, inappropriately high NO concentration which is overexpressed or dysregulated by iNOS, is associated with varieties of human diseases, such as rheumatoid arthritis, inflammatory bowel disease, and septic shock et al. [4,5] Dimerization of NOS proteins is therefore essential for their activity [6]. However, the three isoforms appear to differ in the cellular turnover rates of their respective dimers and the relative dimer strengths were eNOS ≫ nNOS > iNOS [7,8]. These reports suggest the superior isoform selectivity could be achieved by targeting the dimerization process.

In the past decades, several NOS dimerization inhibitors have been discovered such as miconazole, clotrimazole, KLYP961, PPA250, BBS-1 and BBS-4 [9–13]. However, nearly all of the inhibitors have imidazole moiety, which would also disturb with P450 functions and result some side effect. Fewer non-imidazole-based inhibitors, KLYP961 and KLYP956, which can inhibit dimerization of iNOS, were reported [7,14]. Consequently, discovery of non-imidazole moiety iNOS inhibitors with high activity, low-toxicity is a pressing need.

Previously, we described the identification of a non-imidazole-based iNOS dimerization inhibitor compound **D27** through third

* Corresponding author. School of Pharmacy, Anhui Medical University, Hefei, 230032, PR China.
** Corresponding author. School of Pharmacy, Anhui Medical University, Hefei, 230032, PR China.
E-mail addresses: xhliuhx@163.com (X.H. Liu), sjbo616@126.com (J.B. Shi).
[1] These authors contributed equally to this work. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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rounds structural optimization [15]. This compound **D27** was shown both in in *vitro* and *vivo* activities in adjuvant-induced arthritis rat model. To further develop compound **D27**, the drug-like properties of it were studied. Unfortunately, the rats were given 200 mg/kg by intragastric administration and the rats were well tolerated without acute toxicity. However, all rats have signs of poisoning at dosage 500 mg/kg. The rats stopped eating, weight loss and their fur was soft. Most rats die within 1–3 days. Pathological analysis showed that liver necrosis occurred in rats. This compound displayed high toxicity (200 mg/kg < LD_{50} < 500 mg/kg) and several drawbacks associated with ADME. The results strongly suggesting further development of analogs based on this chemical structure with improved potency, lower toxicity and drug like properties. Herein, new series of compounds were designed, synthesized based on previous SARs and drug-like improvement step by step (Fig. 1).

2. Results and discussion

2.1. Design and optimization

Based on the balance between toxicity and anti-inflammatory activity, aiming to improve the physicochemical properties and to enhance drug-like properties, hit to lead were carried out step-by-step on basis of anti-inflammatory activity and their toxicity against normal liver cells (LO2 cells) (Fig. 1).

In the process of structural optimization, replacing the moiety **A** (3,4,5-trimethoxyphenyl moiety) with polar furan-2-yl moiety seemed to be favorable for solubility. Therefore, we first focused on the introduction of polar groups at moiety **A**. Among them, the nitrogen heterocycle is one of the privileged structures in drug discovery and has been used as a good phenyl bioisostere to improve solubility and potency. In general, combination a basic group to a molecule can promote its water solubility [16–22]. Therefore, first, on basis of improving solubility, the 3,4,5-trimethoxyphenyl group (**moiety A**) was replaced by pyridin-3-yl, 3,5,6-trimethylpyrazin-2-yl and pyridine 1-oxide, resulting in compound (**6b)** with moderate inhibitory activity and lower toxicity was discovered (LO2: IC_{50} = 574 μM) (**step 1**). In order to study the influences of vinyl as a link against the activity, the linker (compound **6b)** was changed into ethyl, the compound with weak potency and similar toxicity was found (compound **8b**, **step 2**). This result indicated the linker of vinyl should be keep in the scaffold.

The preliminary results of metabolic stability indicated that hit compound **D27** could inhibit CYP enzyme activity (with IC_{50} < 1.0 μM against CYP1A2, CYP2C9, CYP2C19, and with IC_{50} < 3.0 μM against CYP 2D6 and CYP3A4/5). We speculated that interaction with CYP should be contribute by pyrazolo[4,3-*d*]pyrimidine core. So, we then carried out further modification on core moiety. The core was replaced with pyrimidine, substituted pyrimidine, 1,3,5-triazin-2-ol and thieno[3,2-*d*]pyrimidine [23–27], respectively. Among them, one compound, which was thieno[3,2-*d*]pyrimidine core, showed lower toxicity against LO2 cell with IC_{50} value > 1000 μM (**step 3**).

So, based on above findings, thieno[3,2-*d*]pyrimidine scaffold was fixed, moieties **A** and **B** were substituted with difference groups in light of results of previous structure-activity relationships. At last, after several rounds of structural optimization and structure-activity relationship summary, the lead compound with good anti-inflammatory activity and lower toxicity against LO2 cells was discovered.

2.2. Chemistry

4-Amino-2-methyl-5-propylpyrazole-3-carboxamide (compound **1**) was chose as starting material as well as our previous work [15,28,29]. Series of compounds **5a~g**, **6a~g**, **7a~g** and **8a~g** were prepared based on the protocol outlined in Scheme 1.

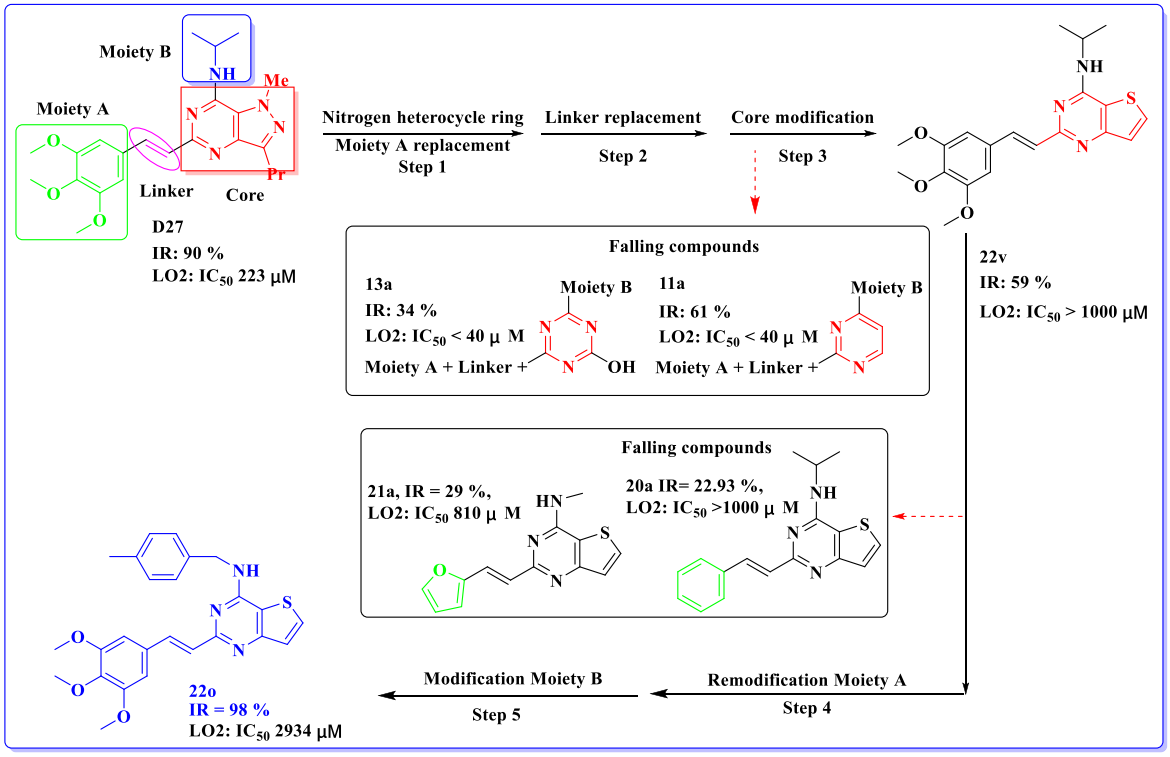
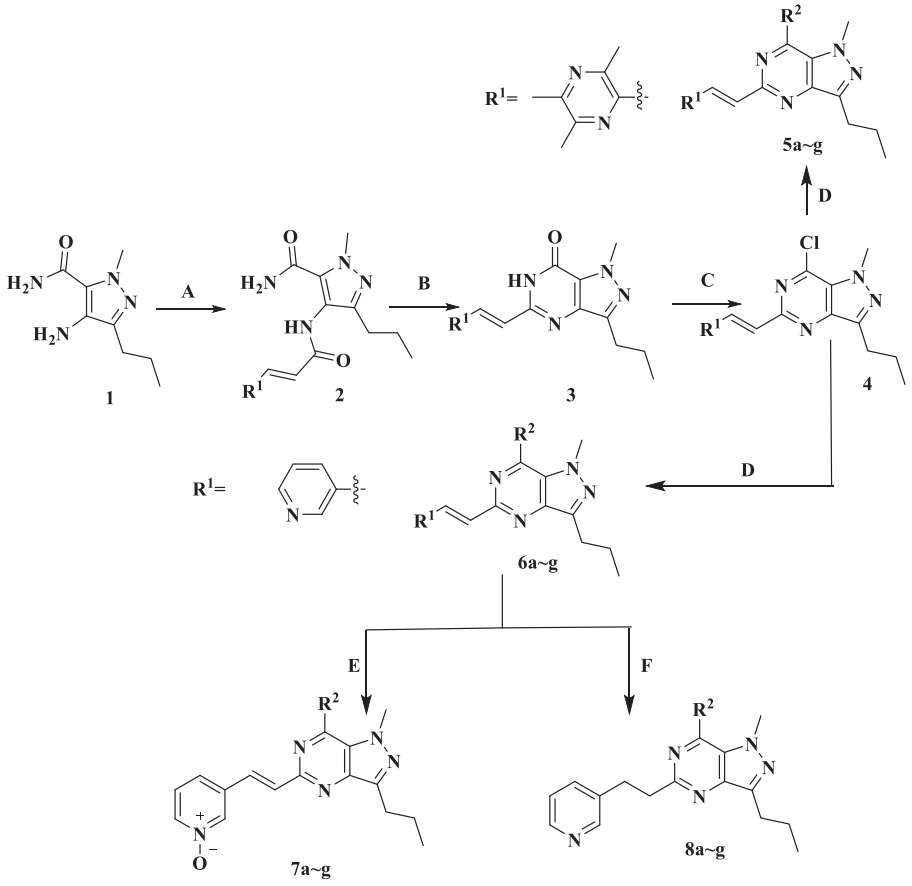


Fig. 1. Hit to lead through structural optimization step by step.

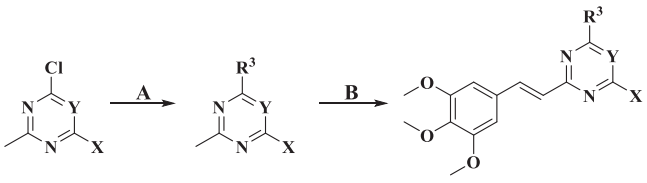


Scheme 1. Synthesis of compounds **5a~g**, **6a~g**, **7a~g** and **8a~g**[a].

The starting material **1** was coupled to the corresponding substituted carboxylic acid with EDCI, HOBT and TEA in DCM to yield amide products **2** with good yield, respectively. Compounds **3** were afforded through intramolecular cyclization reaction. Subsequent treatment of pyrimidine-derivatives **3** with refluxing $POCl_3$ provided the corresponding chloride **4**, thereby completing the preparation of the pyrazolo[4,3-*d*]pyrimidine core. The amine-side chain of **5a~g** and **6a~g** were introduced via S_NAr reaction with compound **4** in the presence of various amines. The pyridine-*N*-oxides **7a~g** were synthesized by oxidation of the corresponding pyridines **6a~g** with 10 equiv. amount of potassium peroxymonosulfate (oxone) in a acetone-H_2O mixture [30]. Compounds **8a~g** were obtained from **6a~g** by hydrogenation with 5% Pd/C under 2.2 atm hydrogen for 8 h at room temperature in 95% yield [31].

[a] Reaction conditions and Reagents: (**A**) substituted carboxylic acid, EDCI, HOBt, TEA, rt.; (**B**) NaOMe, EtOH, reflux, 12 h; (**C**) $POCl_3$, reflux, 3–8 h; (**D**) amine derivatives, TEA, isopropanol, reflux, 3–8 h; (**E**) $NaHCO_3$, $KHSO_5$, H_2O, acetone, rt.; (**F**) THF, H_2, 5% Pd/C, rt.

The preparation of **11a~c**, **12a~c** and **13a~c** most follows the synthetic route as shown in Scheme 2. Commercial compounds **9a~c** was treated with amine derivatives in 2-propanol facilitated a S_NAr reaction to obtained compounds **10a~c**. The arylvinyl pyrimidines **11a~c**, **12a~c** and **13a~c** have been carried out by condensation reaction between methylpyrimidines derivatives **10a~c** and trimethoxybenzaldehyde in boiling aqueous dilute hydrochloric acid for 5 h in moderate yield [32].

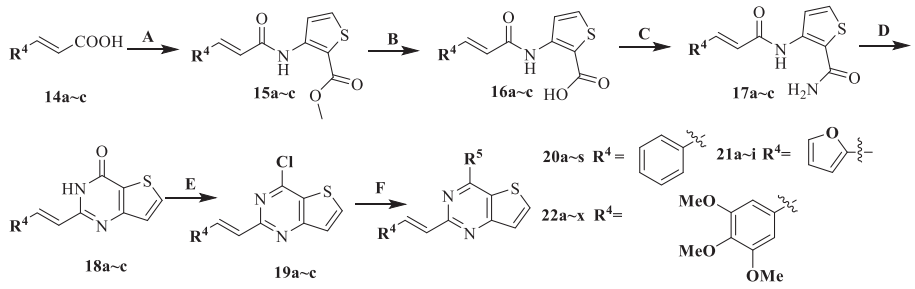


Scheme 2. Synthesis of compounds **11a~c**, **12a~c**, and **13a~c**[a].

[a] Reaction conditions and Reagents: (**A**) amine derivatives, TEA, isopropanol, reflux, 3–8 h; (**B**) trimethoxybenzaldehyde, dilute hydrochloric acid, reflux, 6 h.

The synthetic route of compounds **20a~t**, **21a~i** and **22a~x** most likely follows described in Scheme 3.

Aryl acrylic acid **14a~c** was reacted with oxalyl chloride($(COCl)_2$) in DCM and catalyzed by *N, N*-dimethylformamide (DMF) to obtain corresponding arylacryloyl chloride. A brown syrup was obtained as crude and subsequently it coupled with methyl 3-amino-2-thiophenecarboxylate in TEA and DCM to yield acrylamide derivatives **15a~c** in good yield, respectively. Compounds **15a~c** were hydrolyzed with sodium hydroxide and then followed by acidification gave acid **16a~c** which were converted into acyl chloride with $(COCl)_2$ in DCM and then directly reacted with ammonia in acetone to form amide compounds **17a~c**. Compounds **19a~c** were afforded through intramolecular cyclization reaction and chlorination as well as compound **4** shown in Scheme 1. Lastly,



Scheme 3. Synthesis of compounds **20a~t**, **21a~i** and **22a~x**[a].

compounds **19a~c** was reacted with amine derivatives in 2-propanol facilitated a S_NAr reaction to obtained compounds **20a~t**, **21a~i** and **22a~x**, respectively.

[a] Reagents and conditions: **(A)** (1) oxalyl chloride, DMF, CH_2Cl_2, rt, 1.5 h; (2)methyl 3-amino-2-thiophenecarboxylate, TEA, CH_2Cl_2, rt, 6 h; **(B)** NaOH (aq), CH_3OH, reflux, 3 h; **(C)** (1) oxalyl chloride, DMF, CH_2Cl_2, rt, 2.5 h;

(**2**) $NH_3 \cdot H_2O$, Acetone, rt, 12 h; **(D)** sodium methoxide, CH_3OH, reflux, 3 h; **(E)** $POCl_3$, 90 °C, 12 h; **(F)** amine derivatives, IPA, reflux, 12 h.

Compound **22c** was further confirmed by X-ray. Crystal data: colorless crystals, yield = 51.5%; mp 184–185 °C; $C_{20}H_{21}N_3O_3S$, Orthorhombic, space group *P*bcn; $a = 30.4741(8)$, $b = 8.3751(3)$, $c = 14.6462(3)$ (Å); $\alpha = 90$, $\beta = 90$, $\gamma = 90$ (deg), $V = 3738.06(18)$ nm^3, $T = 293$ K, $Z = 8$, $Dc = 1.359$ g/cm^3, $F(000) = 1608.0$, reflections collected/unique = 11384/3670, data/restraints/parameters = 3670/0/244, goodness of fit on F [2] = 1.004, fine, $R_1 = 0.0490$, w$R(F$ [2]$) = 0.1540$. The structure was shown in Fig. 2.

2.3. Inhibition of NO production in RAW 264.7 cells

Anti-inflammatory activity screening was carried out against LPS-induced NO production (Tables 1–5) [33]. Compounds **5a~g** and **6a~g** were substituted with 3,5,6-trimethylpyrazin-2-yl, pyridin-3-yl at moiety **A** and substituted with isopropylamine, cyclopropylamino et al., at moiety **B**, respectively. It could be observed that these polar nitrogen heterocycles derivatives showed moderate inhibitory activity against NO release induced by LPS at 10 μM (inhibitory rates (IR) were range from 48.39 to 77.42%). Compounds **7a~g** were substituted with pyridine 1-oxide at moiety **A**. Most of them showed weak inhibitory activity. In contrast, compounds **7e** and **7g**, analog with phenethylamino, and 4-fluorobenzylamino at moiety **B**, respectively, resulted in moderate inhibitory activity (IR = 72.92%, 64.58%, respectively). For the purpose of examination the toxicity of the title compounds, toxicity against normal liver cells (LO2 cells) was assessed. As for LO2 cells, most of compounds showed lower toxicity than compound **D27**. Values of some compounds were even over 1000 μM. Fewer compounds **5d**, **5e**, **7f** and **7g** show stronger toxicity than compound **D27**. Base on the finding, it is obviously to find that polar nitrogen heterocycles substituted at moiety **A** are disadvantageous to anti-inflammatory potency, but beneficial to their toxicity for normal liver cell.

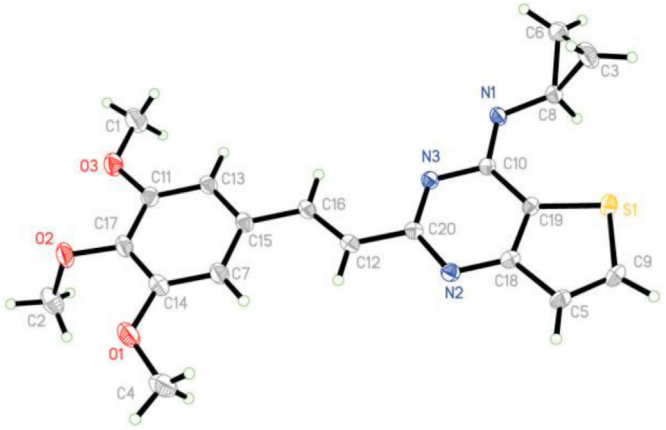


Fig. 2. ORTEP drawing of compound **22c**.

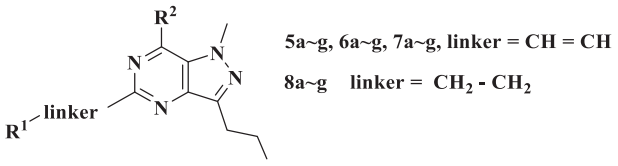
In addition, another series compounds **8a~g** with the same scaffold (compounds **6a~g**), but with the different linker ethyl, which showed significant decrease anti-inflammatory activity compared to **6a~g**. These results indicated the linker must be keep vinyl in the scaffold, which was similar with our previous found. At the same time, all compounds but **8g** showed lower toxicity than that of compound **D27**.

In view of the preliminary SARs results, 3,4,5-trimethoxystyryl may be the most critical moiety for inhibitory activity. In the subsequent optimization, series of derivatives based on the pyrimidine fragment were synthesized to extended compound library (Table 2) via various approaches, including ring bioisostere strategy (**22v ~ x**) and scaffold-hopping strategy (**11a~c**, **12a~c**, **13a~c**). Compounds **11a~c**, **12a~c**, **13a~c** and **22v ~ x** which were replaced the pyrazolo [4,3-*d*]pyrimidine core with pyrimidine (substituted pyrimidine), 1,3,5-triazin-2-ol, thieno[3,2-*d*]pyrimidine and substituted with isopropylamine, 2-phenylethan-1-amine or 2-(4-fluorophenyl) ethan-1-amine at moiety **B**. As described in Table 2, most of compounds showed weak to moderate potencies (IR ranged from 26% to 85%) and higher toxicities (IC_{50} against LO2 cells < 40 μM). From Table 2, compound **22v**, with thieno[3,2-*d*]pyrimidine core, showed lower toxic effect on normal cell line LO2 with IC_{50} value of over 1000 μM and moderate potencies. In light of this, for keep the balance between toxicity and anti-inflammatory activity, thieno [3,2-*d*]pyrimidine core should be a privileged scaffold.

Based on above clue, the thieno[3,2-*d*]pyrimidine scaffold may be important for toxicity. In order to further confirm structural and toxicity relationship in this scaffold, compounds **20a~s**, aryl vinyl substituted at C-2, were synthesized and screened for their toxicities and anti-inflammatory activities. Most of compound **20a~s** substituted with styrene at C-2 showed very weak potencies (IR ranging from 3.57 to 35.20%) and indicated styrene at C-2 was not promising, as well as our previously reported. Amazingly, almost all of compounds showed very low toxicity with IC_{50} values over 1000 μM. The results further confirmed that thieno[3,2-*d*]pyrimidine scaffold may show low contribution to toxicity against LO2 cells.

Inspired by these, further modifications were stared. Compounds **21a~i**, substituted with 2-(furan-2-yl)vinyl group at C-2 position and substituted with amino derivatives at C-4 position were prepared. Most of them showed weak potencies and low

Table 1
Inhibitory effects of compounds **5a~g**, **6a~g**, **7a~g** and **8a~g** against NO production in RAW 264.7 cells and against LO2 cells [a].



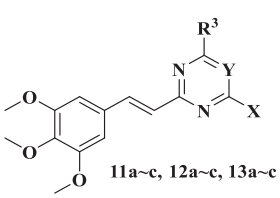
5a~g, 6a~g, 7a~g, linker = CH = CH

8a~g linker = CH_2 - CH_2

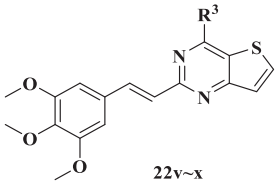
Compd	R^1	R^2	NO [b] inhibition (%) ± SD	LO2 IC_{50} (μM)
5a			48.39 ± 3.28	705.69 ± 30.37
5b			54.84 ± 4.25	342.29 ± 41.65
5c			74.19 ± 2.36	370.00 ± 24.13
5d			51.61 ± 4.56	<40
5e			52.69 ± 3.16	<40
5f			68.82 ± 3.25	786.31 ± 90.07
5g			63.44 ± 3.98	>1000
6a			49.46 ± 4.52	261.46 ± 33.55
6b			74.19 ± 4.18	574.37 ± 83.93
6c			69.89 ± 2.69	535.33 ± 57.06
6d			60.22 ± 4.36	472.50 ± 65.39
6e			77.42 ± 3.96	>1000
6f			66.67 ± 4.21	>1000
6g			61.29 ± 3.25	939.77 ± 27.71
7a			29.17 ± 6.32	580.17 ± 54.99
7b			45.83 ± 4.55	324.16 ± 24.25
7c			20.83 ± 5.02	817.20 ± 91.00
7d			3.13 ± 6.32	>1000
7e			72.92 ± 3.22	392.19 ± 64.45
7f			37.5 ± 5.24	162.17 ± 24.29
7g			64.58 ± 3.23	<40
8a			7.29 ± 6.12	561.63 ± 87.20
8b			30.21 ± 6.23	514.12 ± 73.57
8c			27.08 ± 5.48	858.67 ± 73.85
8d			15.63 ± 6.12	>1000
8e			36.46 ± 4.55	783.41 ± 84.16
8f			37.5 ± 3.25	611.63 ± 43.63
8g			18.75 ± 6.32	<40
Dex[c]	–	–	51.81 ± 3.25	4620.58 ± 67.43
Cisplatin[c]	–	–	[d]_	99.43 ± 12.37

[a] The cells were treated with compounds for 1 h and stimulated with LPS (0.5 μg/mL) for 24 h. The NO level in the culture medium were measured by nitrite and nitrate assay. All of compounds showed no toxicities on RAW 264.7 cells at 20 μM ($p > 0.05$). All the data displayed are at least three independent experiments.
[b] The test concentration of compounds was 10 μM.
[c] Dex: Dexamethasone as Positive control.
[d] No anti-inflammatory activity.

Table 2
Inhibitory effects of compounds **11a~c**, **12a~c**, **13a~c** and **22v ~ x** against NO production in RAW 264.7 cells and against LO2 cells [a].



11a~c, 12a~c, 13a~c



22v~x

Compd	X	Y	R^3	NO [b] inhibition (%) ± SD	LO2 IC_{50} (μM)
11a	H	CH		61 ± 3.23	<40
11b	H	CH		85 ± 3.65	<40
11c	H	CH		47 ± 4.52	543.94 ± 6.69
12a	Cl	CH		29.03 ± 6.95	<40
12b	Cl	CH		31.18 ± 7.25	<40
12c	Cl	CH		55.91 ± 6.23	<40
13a	OH	N		34.0 ± 5.42	<40
13b	OH	N		56.98 ± 3.25	<40
13c	OH	N		36.55 ± 6.21	483.32 ± 45.25
22v	[d]_	–		59.13 ± 4.12	>1000
22w	-	–		46.23 ± 5.36	<40
22x	–	–		26.88 ± 6.21	<40
Dex[c]	–	–	–	51.81 ± 3.25	4620.58 ± 67.43
Cisplatin	–	–	–	–	99.43 ± 12.37

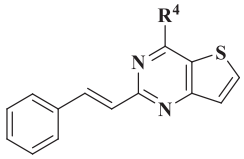
[a] Described as Table 1.

toxicities on LO2 cells (IC_{50} values > 1000 μM). Interestingly, compounds **21f** and **21h**, substituted *N*-benzyl and *N*-4-fluorobenzyl at C-4, had divergence in inhibitory activity (64.14% and 59.24%) and lower toxicities (IC_{50} values > 1000 μM). These results indicated substituted with benzyl groups at C-2 position may have favorable for anti-inflammatory activity. After all, most of compounds **21a~i** showed weak potencies and indicated 2-(furan-2-yl)vinyl group at C-2 position may be not a privileged moiety to enhance anti-inflammatory activity. However, data of toxicity against LO2 cells were further indicated that thieno[3,2-*d*]pyrimidine scaffold may be a privileged scaffold (Table 4).

Based on the re-summarized SARs and confirmed the donation of 3,4,5-trimethoxyphenyl fragment to the activity. At the end of campaign, we synthesized trimethoxystyryl moiety at C-2 of thieno[3,2-*d*]pyrimidine core and substituted with diverse amino derivatives, compounds **22a~g** were designed and synthesized. Most of them showed weak or moderate activity and low toxicity against LO2 cells. Especially, compound **22c**, substituted with cyclopropanamine showed excellent activity (IR = 90.70%) and lower toxicity (IC_{50} values > 1000 μM). Compounds **22h ~ m**, which is substituted or unsubstituted *N*-phenyl at C-4, reflected significant difference potencies. Among them, compounds **22h** and **22i**, with *N*-p-tolyl, *N*-4-isopropylphenyl moiety at C-4, respectively, showed weak activity. However, compounds **22j ~ l** with *N*-4-methoxyphenyl, *N*-4-trifluoromethyl and *N*-phenol group at C-4 indicated moderate or excellent activities (52.50%, 55.36%, 97.67%, respectively). Substitution with an electron donating or withdrawing group (-Me, -OMe, $-CF_3$, −OH) in 4-position of *N*-phenyl had significant influence against activity and toxicity.

Compounds **22n~t**, with substituted or unsubstituted aryl

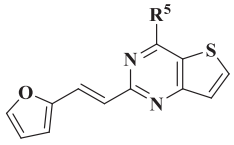
Table 3
Inhibitory effects of compounds **20a~s** against NO production and against LO2 cells [a].



Compd	R^4	NO [b] inhibition (%) ± SD	LO2 IC_{50} (μM)
20a		22.92 ± 4.56	>1000
20b		21.93 ± 3.26	>1000
20c		3.57 ± 10.23	>1000
20d		22.44 ± 4.52	>1000
20e		25.00 ± 6.51	>1000
20f		34.14 ± 3.26	>1000
20g		18.53 ± 3.25	198.40 ± 97.08
20h		20.97 ± 7.45	>1000
20i		24.87 ± 2.12	>1000
20j		19.51 ± 10.23	>1000
20k		28.78 ± 3.22	>1000
20l		21.95 ± 6.23	>1000
20m		30.10 ± 3.52	>1000
20n		23.41 ± 4.18	>1000
20o		19.51 ± 7.45	>1000
20p		35.20 ± 3.25	>1000
20q		30.67 ± 5.55	>1000
20r		29.75 ± 4.52	>1000
20s		29.75 ± 6.32	>1000
Dex[c]	[d]–	51.81 ± 3.25	4620.58 ± 67.43
Cisplatin	–	–	99.43 ± 12.37

[a] Described as Table 1.

Table 4
Inhibitory effects of compounds **21a~i** against NO production and against LO2 cells [a].



Compd	R^5	NO [b] inhibition (%) ± SD	LO2 IC_{50} (μM)
21a		29.08 ± 6.12	810.81 ± 80.24
21b		33.33 ± 6.34	>1000
21c		28.06 ± 9.26	>1000
21d		43.87 ± 4.23	>1000
21e		31.66 ± 5.12	>1000
21f		64.14 ± 3.21	>1000
21g		13.00 ± 6.59	>1000
21h		59.24 ± 4.97	>1000
21i		11.66 ± 9.11	>1000
Dex	–	51.81 ± 3.25	4620.58 ± 67.43
Cisplatin	–	–	99.43 ± 12.37

[a] Described as Table 1.

methylamine at C-4 position, showed moderate or higher potencies than compounds **22h ~ m**. Among them, compounds **22n~q**, with a group (-H, -Me, -OMe, Cl) in 4-position of phenylmethylamine, had better potencies (71.92%, 86.89%, 73.68%, 65.54%, respectively) and low toxicities (IC_{50} value > 1000 μM). However, compound **22r** (IR = 21.92%) with a group (Cl) in 2-position of phenylmethylamine, showed worse activity than that of compound **22q** (IR = 65.54%). Compounds **22s ~ t**, substituted with furan-2-ylmethanamine and pyridin-4-ylmethanamine, showed moderate toxicity.

To our delight, compound **22o** with a balance of toxicity and anti-inflammatory activity was finally discovered, which showed excellent activity (IR = 98.38%, which superior to that of positive Dexamethasone) and lower toxicity (IC_{50} value > 2900 μM, compare with **D27** IC_{50} value = 233 μM) (Table 5, Fig. 2).

2.4. Inhibitory activity of some compounds on iNOS

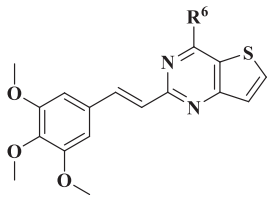
For the purpose of exploration anti-inflammatory mechanism, inhibitory effect of the compounds on iNOS was determined. Some compounds which shown higher NO inhibitory activity were selected to assess their inhibitory activity against iNOS (Table 6). As described in Table 6, some acquired significant inhibitory activities on NO and iNOS. As we anticipated, compound **22o** showed the most potent inhibitory activity (iNOS: IC_{50} = 0.96 μM, NO: IC_{50} = 1.86 μM) compared with dexamethasone (iNOS: IC_{50} > 10 μM, NO: IC_{50} = 8.71 μM). To check relationships between the inhibitory effects of compounds on NO release, iNOS activity and cell viability, MTT assay was adopted. Compound **22o** showed no cytotoxicity on RAW 264.7 cells (without or with LPS, IC_{50} values > 50 μM) and LO2 cells (IC_{50}values > 2900 μM) with the most potent activity. On the results of anti-inflammatory activity and cellular viability *in vitro*, compound **22o** was chose to evaluated in the further studies.

2.5. Inhibition of iNOS dimer formation

We determined that compound **22o** had good inhibitory effect against NO on the cells and enzyme. NO can promote the occurrence and development of inflammation related diseases, which is closely involved with the regulation and expression of iNOS [34,35]. Therefore, we first analyzed the inhibitory effect of **22o** on LPS mediated iNOS expression by Western blot in Raw264.7 cells. The western blot results showed that compound **22o** did not affect the expression of total iNOS protein (Fig. 3). In previously, our studies have confirmed that small molecules could inhibited the formation of iNOS protein dimers. Due to the inhibition of iNOS dimer, the secretion of NO was inhibited. To confirm this, Native Gel Sample Loading Buffer low-temperature SDS-PAGE was used to study the formation of iNOS protein dimer in the presence of compound **22o**. From Fig. 4, within a certain period of time, the dimer iNOS was separated based on its unique mobility. This result indicated that compound **22o** exhibited anti-inflammatory activity through inhibiting the formation of iNOS dimers.

[a] Detection of total iNOS protein expression by Western blot.

Table 5
Inhibitory effects of compounds **22a~u** against NO production [a].



Compd	R^6	NO [b] inhibition (%) ± SD	LO2 IC_{50} (μM)
22a		39.47 ± 4.32	>1000
22b		27.19 ± 9.32	>1000
22c		90.70 ± 4.24	>1000
22d		33.21 ± 4.35	680.99 ± 30.91
22e		39.31 ± 8.24	>1000
22f		46.66 ± 4.03	>1000
22g		12.24 ± 6.23	>1000
22h		19.16 ± 8.22	<40
22i		13.33 ± 9.56	369.52 ± 13.3
22j		52.50 ± 6.23	278.92 ± 35.6
22k		55.36 ± 5.23	435.69 ± 5.44
22l		97.67 ± 5.23	>1000
22m		32.45 ± 6.51	766.98 ± 28.27
22n		71.92 ± 3.12	>1000
22o		98.30 ± 3.62	>1000
22p		73.68 ± 4.33	>1000
22q		65.54 ± 4.25	>1000
22r		21.92 ± 5.23	>1000
22s		46.49 ± 5.26	189.39 ± 23.85
22t		76.00 ± 3.12	373.74 ± 82.49
22u		33.76 ± 6.33	>1000
Dex	–	51.81 ± 3.25	4620.58 ± 67.43
Cisplatin	–	-	99.43 ± 12.37

[a] Described as Table 1.

Different concentrations of compound **22o** (0.5 μM, 1 μM, 2 μM) were used to treat cells; β-actin was used as loading control; $^{###}p < 0.001$ compared with control. Data were obtained by at least three independent experiments and each was performed in duplicate.

2.6. Compound **22o** improved AIA-adjuvant induced arthritis

As an experimental model established for adjuvant arthritis, AIA is often used to test anti-inflammatory drugs [36–38]. In the AIA model, approximately 80%–90% of rats will develop to adjuvant arthritis within 14–21 days after injected by complete Freund's adjuvant. The rats were randomly divided into 5 groups, each with 10 animals: normal, AIA, sinomenine (60 mg/kg), compound **22o** H (80 mg/kg) and compound **22o** L (40 mg/kg) treatment groups. On days 14–28 after AIA immunization, compound **22o** was administered intragastrically. The body weight and swollen fingers of the feet were measured every 3 days. First of all, the therapeutic effect of compound **22o** in AIA rats was judged by measuring the paw swelling index and the weight change of the rats (Fig. 5 A, **B**). The rats in the model group showed joint swelling, bone stiffness, severe edema and erythema during the 4-week experiment comparing with normal group. Pathological nodules appeared in the tail, ears and nose of rats, especially a large number of pathological nodules in the tail (Fig. 5, C). However, the swelling and edema of the ankle joint of the rats treated with compound **22o** were significantly reduced after two weeks, and some rats recovered almost normal foot physiological state at 3–4 weeks (Fig. 5, C).

with AIA. (**A**) Effects of compound **22o** on body weight loss in rats with AIA. (**B**) Effects of compound **22o** on hind paw swelling in rats with AIA. Sinomenine (60 mg/kg) was the positive control. (**C**) Representative photos of hind paws on 30th day after inducing arthritis. The arrow indicated the location of the pathological nodule. Data are the mean ± sd, n = 10 per group. Sinomenine was the positive control. $^{###}p < 0.001$ compared with control; $***p < 0.001$, $**p < 0.01$, $*p < 0.05$ *vs* AIA group.

Use safranin O (SO) staining, hematoxylin and eosin (H&E) staining and immunohistochemical staining to assess the degree of cartilage degradation and inflammation during the pathogenesis of AIA (Fig. 6). The cartilage tissue of untreated AIA rats was compared with normal and compound **22o**-treated rats to evaluate the destruction of inflammatory cells and articular cartilage. The H&E staining of normal group rats did not show any histological changes at any site (Fig. 6, **A**, Normal), while untreated AIA rats showed synovial hyperplasia, bone or cartilage destruction and inflammatory cell infiltration after the 4th week (Fig. 6, **A**, AIA). The positive control sinomenine treatment group showed inflammatory cell infiltration. AIA rats treated with compound **22o** L showed moderate synovial hyperplasia and inflammatory cell infiltration. The group treated with compound **22o** H improved cartilage destruction and synovial hyperplasia, although there was still slight inflammatory cell infiltration (Fig. 6, **A**, **22o** H). In SO staining, compared with the AIA group, after 2 weeks of treatment, significant positive staining of SO was shown in synovial tissue sections from compound **22o**-treated rats. The round mature chondrocytes were surrounded by glycosaminoglycan (GAG; dark red) deposits, and obvious regeneration of GAG was found at the boundary between bone and cartilage at 4th week (Fig. 6, **B**, **22o** H). We could saw that the cartilage is damaged and the SO stain is lighter in the AIA group. Mature chondrocytes have a smaller staining area (Fig. 6, **B**, AIA).

These results indicate that compound **22o** can reduce the damage of the ankle joint in rats and increase the regeneration of cartilage tissue. In addition, use immunohistochemistry analysis to check the expression of iNOS in different experimental groups (Fig. 6, **C**). The model group has a large amount of iNOS expression. After administration with compound **22o**, the expression level of iNOS decreased significantly (Fig. 6, **C**, **22o** H). In summary, these experimental results strongly suggest that compound **22o** has protective effect on AIA rat model, and that compound **22o** could be a powerful drug for the treatment of arthritis.

In order to detect serum inflammatory factors of rats, we found that the serum levels of IL-6, IL-1β, and TNF-α in the AIA group were significantly increased than normal group ($p < 0.001$). Compared with the AIA group, the serum IL-6, IL-1β, and TNF-α concentrations of AIA model rats treated with compound **22o** (40, 80 mg/kg) decreased in a dose dependent manner. Sinomenine has similar inhibitory effects as the positive control (Fig. 7). These data confirm again the therapeutic effect of compound **22o** on arthritis.

Table 6
IC_{50} values of selected compounds against NO.

Compd	NO IC_{50} (μM)[a]	iNOS IC_{50} (μM)[c]	Cytotoxicity IC_{50} (μM)		LO2 IC_{50} (μM)
			without LPS	with LPS	
22c	6.03[b]	4.32	>50	>50	2356.21 ± 124.32
22j	10.71	5.12	>50	>50	278.92 ± 35.6
22k	9.36	4.20	>50	>50	435.69 ± 5.44
22l	4.07	1.89	>50	>50	2301.51 ± 224.33
22n	8.26	3.62	>50	>50	1796.21 ± 76.48
22o	1.86	0.96	>50	>50	2934.33 ± 73.12
22p	7.54	3.26	>50	>50	2051.21 ± 88.62
22q	9.05	4.23	>50	>50	4316.21 ± 325.47
22t	6.35	3.12	>50	>50	373.74 ± 82.49
Dex[d]	8.71	>10	>50	>50	4620.58 ± 67.43

[a] Inhibition (%) = [LPS (OD_{540}) − compounds (OD_{570})]/[LPS (OD_{540}) - control (OD_{540})] × 100.
[b] First calculate the inhibition rates for different concentrations. The IC_{50s} of various compounds were calculated by regression analysis through SPSS.
[c] First calculate the inhibition rates for different concentrations. The IC_{50s} of various compounds were calculated by regression analysis through SPSS.
[d] The positive control.

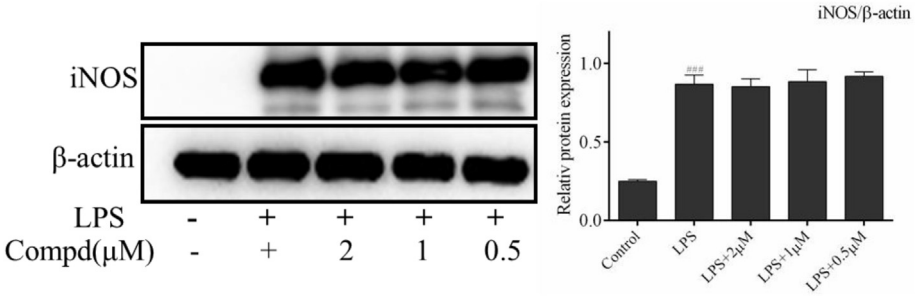


Fig. 3. Compound **22o** inhibits the expression of total iNOS protein [a].

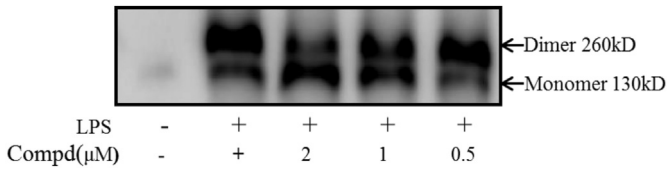


Fig. 4. Compound **22o** inhibits the stabilization of iNOS dimers in cells [a]. [a] The RAW 264.7 cells were pretreated with different concentrations of compound **22o**.

2.7. Metabolic stability

In order to evaluate the drug-like properties, the metabolic stability of compound **22o** were determined in human and rat hepatocytes. As shown in the Table 7, this compound showed high stability to the metabolism of rat and human hepatocytes. It is more stable in human hepatocytes than rats, with half-lives ($t_{1/2}$) of 326.7 and 55.2 min, respectively. In conclusion, the in *vitro* PK analysis indicated that this compound had oral bioavailability.

It is well known that metabolic enzymes (such as cytochrome P450, CYP) in the liver can metabolize most drugs in organisms [39]. Drugs can directly interact with CYP enzyme and inhibit the activity of CYP enzyme (CYP inhibition). It can also activate nuclear receptors to interact with CYP enzymes, thereby inducing its gene expression (CYP induction) [40]. So, when different drugs are used together, metabolism mediated drug interaction (DDI) may occur, resulting in reduced therapeutic effect or adverse reactions.

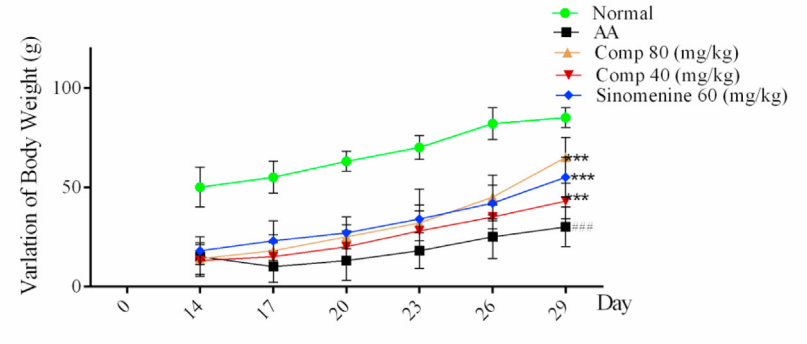
In order to evaluate whether compound **22o** has the potential of drug, we further studied the inhibitory effect of it on major CYP metabolizing enzymes. The selective inhibitors were used as positive control. The results showed that this compound had no significant inhibition on CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4/5 ($IC_{50} \leq 1.00$ μM, the value is regarded as a potent inhibition; 1.00 μM $\leq IC_{50} \leq$ 10.0 μM, it is regarded as a moderate inhibition; $IC_{50} >$ 10.0 μM, it is regarded as a No or weak inhibition). Among them, CYP2C19, CYP2D6 and CYP3A subtypes participate in the metabolism of approximately 90% of commercially available drugs. CYP3A and CYP2D6 are the most important [41]. Compound **22o** inhibited CYP3A and CYP2D6 with $IC_{50} >$ 50 μM. This suggests that side effects in liver and drug interactions induced by compound **22o** is unlikely to happen (Table 8).

2.8. In vivo PK study

Based on cellular activities and biochemical studies, the pharmacokinetic (PK) characteristics of compound **22o** through i.v. and p.o. pathways were further evaluated. The results showed that compound **22o** exhibited moderate PK property. As shown in Table 9, this compound showed longer half-lives of about 6.21 h (i.v.) and 4.91 h (p.o.). Also exhibited a higher plasma clearance of about 42.9 mL/min/kg, and it also had a high systemic exposure with areas under the curves (AUC) of 401 h ng/mL (iv) and 1152 h ng/mL (p.o.) with an oral bioavailability (F) of 29.9%. Additionally, compound **22o** showed a maximum plasma concentration (C_{max}) of 131 μg/L via the oral administration (10 mg/kg). These results indicated that compound **22o** had good drug properties.

2.9. Acute toxicity study of compound **22o** in healthy rats

In previous study, acute toxicity of hit compound **D27** was tested, which showed toxicity with value of lethal dose (LD_{50}) was ranged from 200 to 500 mg/kg. The pathological section study found that the liver damage was severe in rats in this study. From



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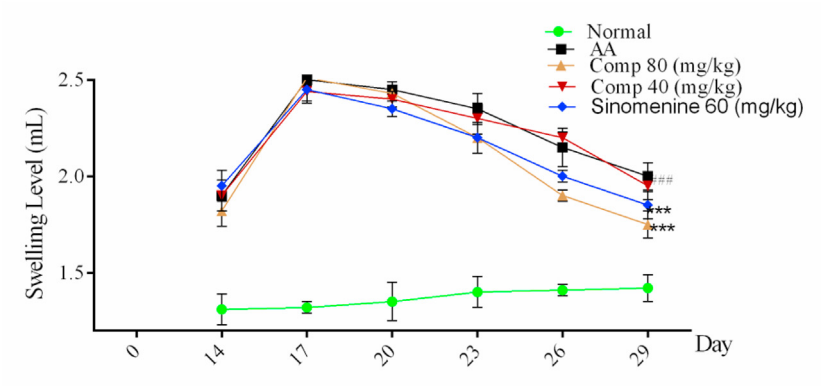

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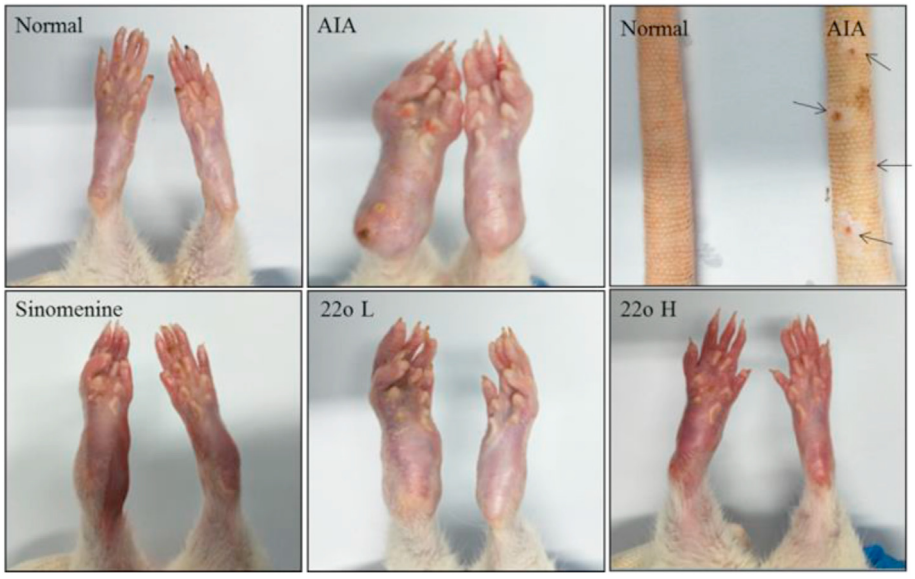
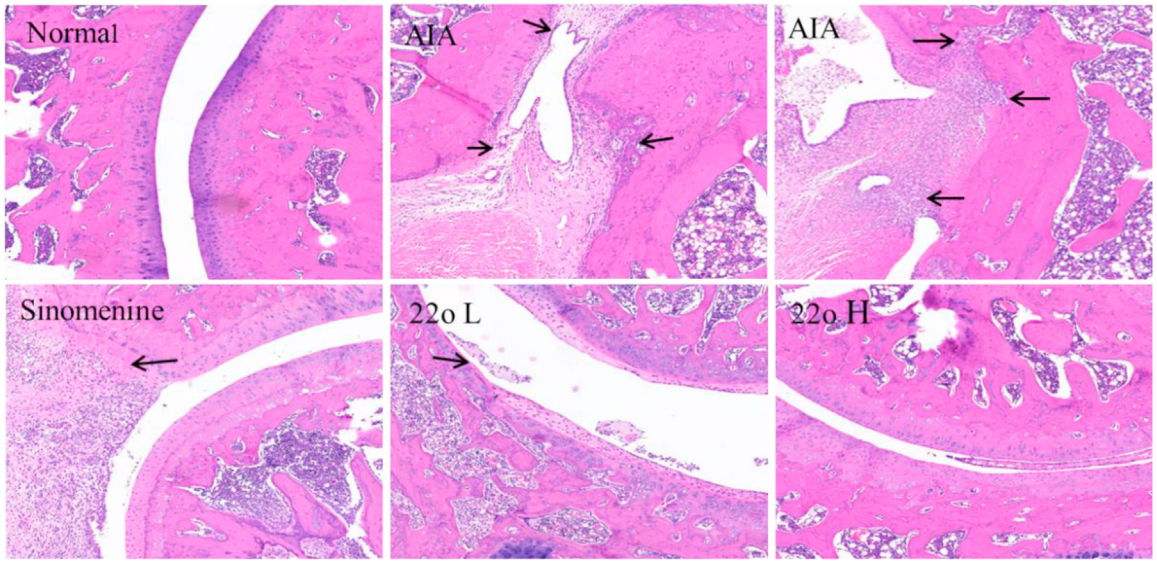


C

Fig. 5. Effects of compound **22o** on hind paw swelling and body weight loss in rats.

the pathological section, we found that the liver of the rats had already appeared massive necrosis (Fig. 8).

Above studies found that compound **22o** has relatively low cytotoxicity (LO2: IC_{50} = 2934.33 μM). In order to verify whether compound **22o** has the disadvantage of toxicity, the acute toxicity of single dose was studied in healthy rats. After a single intragastric administration at dosage 2000 mg/kg, no rats died and there were no abnormal behaviors (drowsiness, weight loss, reduced exercise, anorexia, fur wrinkles, etc.). All rats grew normally in the administration group, as well as in the Normal group. Overall, the weight of male and female rats gradually increased within 1 week with no statistical difference in weight gain (Fig. 9). Therefore, compound **22o** is well tolerated at dosage 2000 mg/kg and shown without acute toxicity.



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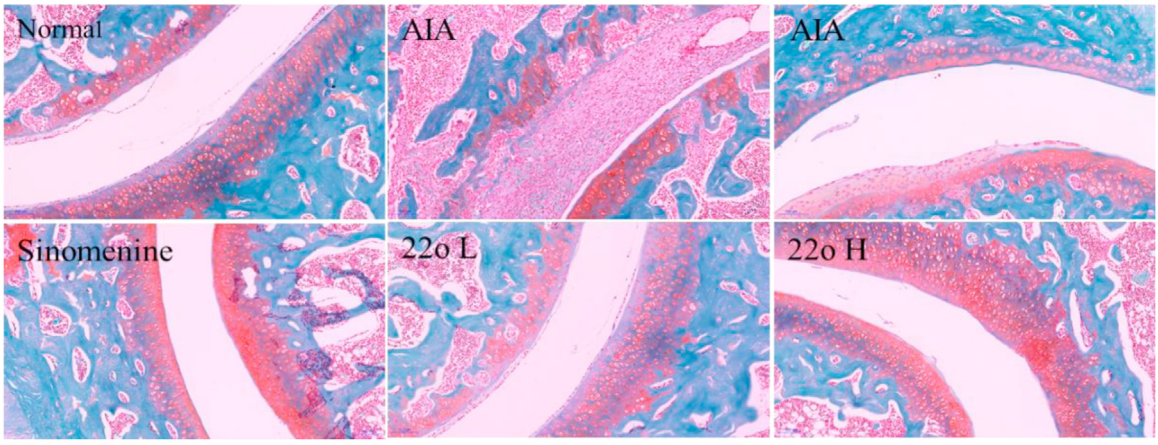

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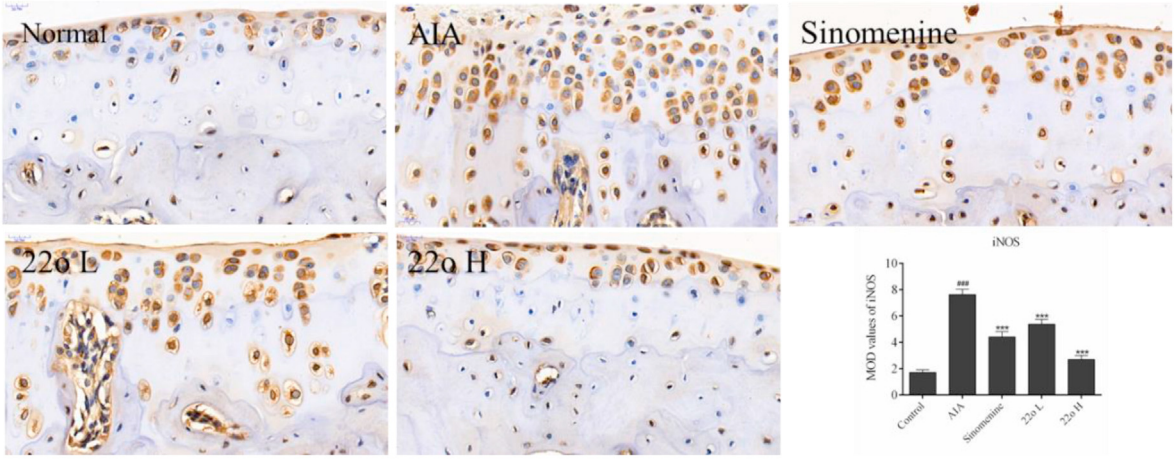

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Fig. 6. Histological analysis of therapeutic effect of compound **22o** on AIA rats. The rats were randomly divided into 5 groups: Normal, AIA, sinomenine, compound **22o** L and compound **22o** H. (**A**) H&E staining for histological changes of joints of **22o**-treated AIA rats. (**B**) Safranin O (SO) staining images of AIA rats after administered intragastrically of compound **22o** at 4th week. (**C**) Immunohistochemistry analysis on iNOS. Mean optical density (MOD) values of iNOS. The data were expressed as mean ± sd. n = 10 for all groups. $^{###}p < 0.001$ compared with control; $^{***}p < 0.001$ *vs* AIA group.

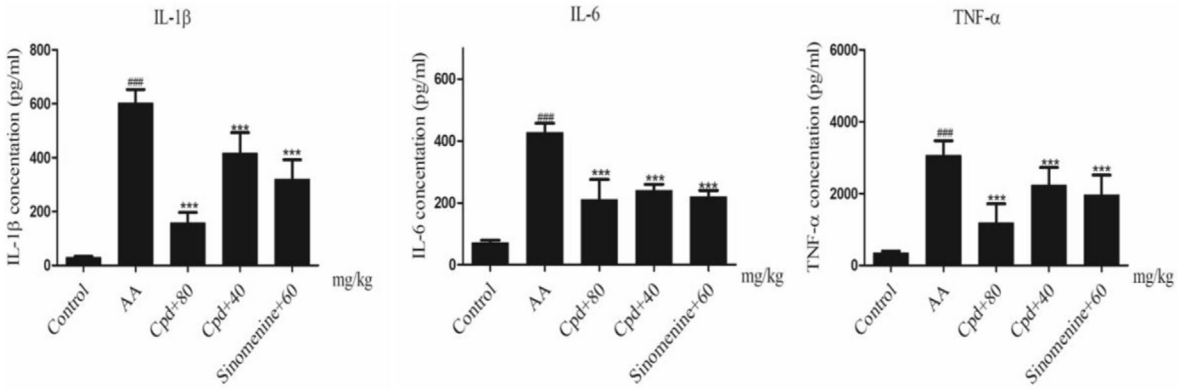

Fig. 7. The protein levels of various cytokines in AIA of rat induced by CFA were detected by ELISA. Data are the mean ± sd, n = 10 per group. Sinomenine was the positive control. $^{###}p < 0.001$ compared with Normal group; $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ *vs* AIA group.

Table 7
Study on *in vitro* metabolic stability of hepatocytes.

Compd	Human			Rat		
	$T_{1/2}$(min)	CL (μL/min/million cells)	Remaining @120 min (%)	$T_{1/2}$(min)	CL (μL/min/million cells)	Remaining @120 min (%)
22o	326.7	2.1	77.5	55.2	12.6	22.2
Phenacetin	43.8	15.8	–	32.6	21.3	–
Diclofenac	9.1	75.8	–	13.6	50.9	–
Dextromethorphan	14.5	47.8	–	4.6	150.1	–
Omeprazole	28.2	24.6	–	13.5	51.4	–
Midazolam	16.3	42.6	–	3.9	176.2	–
7-EC	7.5	92.1	–	16.6	41.7	–

Table 8
Inhibitory activity of compound **22o** on CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4/5.

Substrate	CYP subtypes	Selective inhibitors	Inhibitory activity (IC_{50}, μM)	Compd	Inhibitory activity (IC_{50}, μM)
Phenacetin	1A2	β-Naphthoflavone	0.0835	**22o**	42.2
Diclofenac	2C9	Sulfaphenazole	0.367	**22o**	8.91
Mephenytoin	2C19	Tranylcypromine	9.25	**22o**	7.76
Dextromethorphan	2D6	Quinidine	0.0408	**22o**	>50
Midazolam	3A4/5	Ketoconazole	0.0125	**22o**	>50

Table 9
In vivo PK study of compound **22o**.

Dose/routes	T_{max} (h)	$AUC_{0-\infty}$ (hr*ng/mL)	C_{max} (μg/L)	$t_{1/2}$ (h)	Vss (L/kg)	CL (mL/min/kg)	F (%)
1 mg/kg (i.v.)	/	401	388	6.21	11.3	42.9	/
10 mg/kg (p.o.)	3.33	1152	131	4.91	/	60.0	29.9

*2.10. Evaluation of the subacute toxicity of compound **22o** to healthy rats*

To further evaluate the safety of compound **22o** *in vivo*, we studied its subacute toxicity in healthy rats. Compound **22o** were intragastric administration to rats at dosage of 100 mg/kg and 500 mg/kg every other day for 14 days [42,43]. Every two days, the rats' weight changes, food intake, and activity levels were recorded. The experimental results showed that no rats died, and no abnormal behaviors (anorexia, clonic convulsions, drowsiness and fur wrinkles) and body weight changes were viewed during the administration stage. The weight of female and male rats in the experimental group gradually increased. There was no statistical difference between the experimental and the Normal groups. All rats were anesthetized on the 14th day. After dissecting the rat, the main organs (heart, spleen, liver, lung, etc.) were tested through hematoxylin-eosin (HE) staining. We found that there was no obvious pathological damage at dosage of 100 mg/kg and 500 mg/kg. The results of subacute toxicity assessment further indicated that compound **22o** had good safety *in vivo*. (Figs. 10–11).

3. Conclusion

In summary, aiming to design and synthesize novel molecules with potent drug-like anti-inflammatory activity, based on compound **D27** and NO inhibitory activity screening, guided by relationships of balance toxicity and anti-inflammatory activity, 12 series of total 89 novel compounds have been designed, synthesized step by step. After multiple rounds structural optimization through "hit" to "lead", (*E*)-*N*-(4-methylbenzyl)-2-(3,4,5-trimethoxystyryl)thieno[3,2-*d*]pyrimidin-4-amine (title compound **22o**) was discovered as lead compound, which displayed

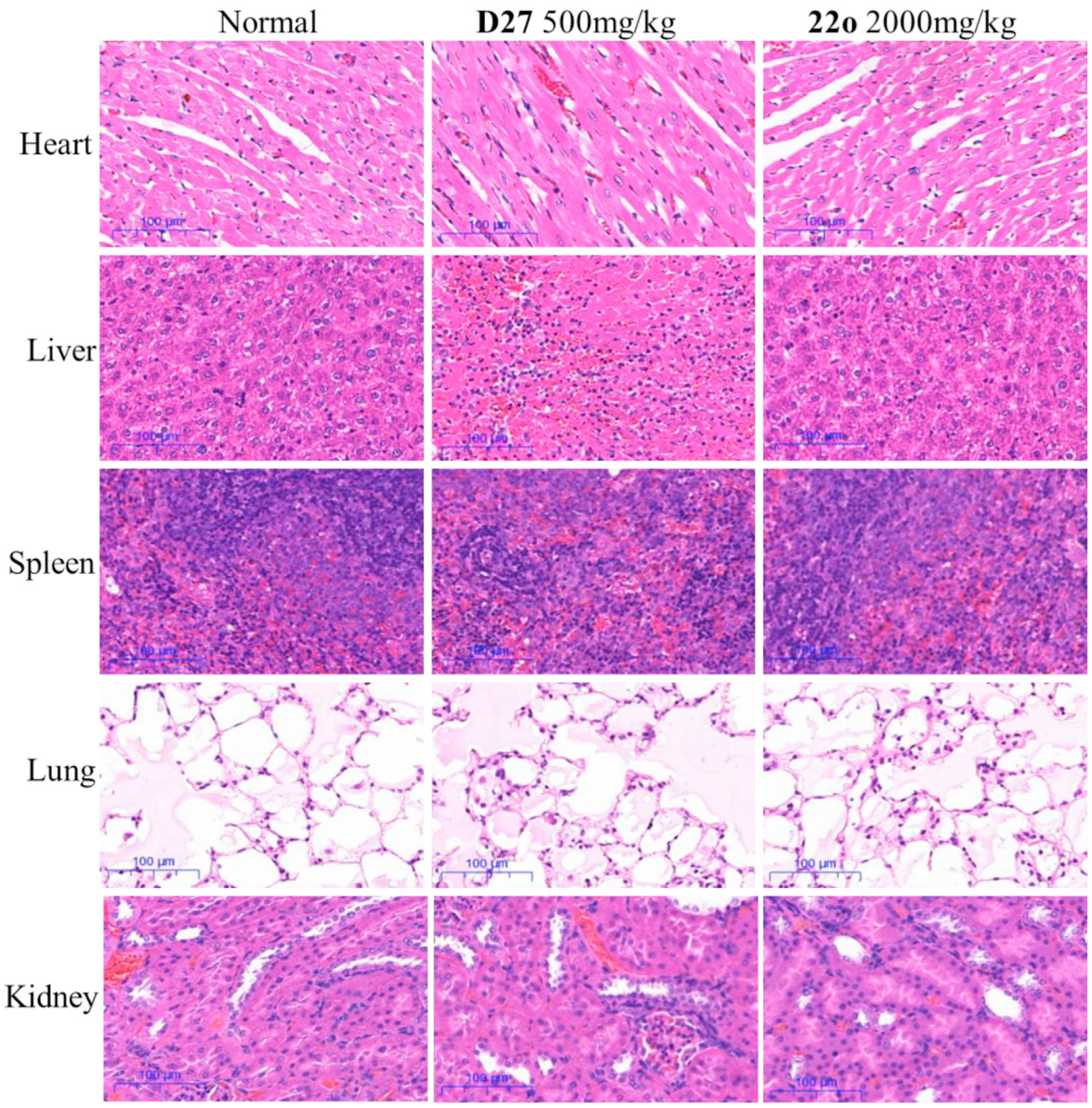

Fig. 8. Histopathology (H&E) of important organs (heart, liver, spleen, lungs, and kidneys) of treated groups rats after 7 day.

potent NO production and iNOS inhibitory activity with lower cytotoxicity against LO2 cells (IC_{50} = 2934.33 μM), resulting in interference iNOS dimerization. Its anti-inflammatory activity was assessed by adjuvant-induced arthritis rat model *in vivo*. Furthermore, the PK characteristics of compound through i.v. and p.o. pathways *in vitro* were evaluated, the results indicated that this title compound had good drug properties with higher oral bioavailability. Inhibitory effect on major CYP suggested title compound is less likely to induce drug-drug interactions and side effects in liver, acute toxicity study and subacute toxicity assessment indicated this lead compound had good drug-like properties for treatment of arthritis. We believe above findings would further support this compound as efficient iNOS regulatory inhibitor in the future.

4. Experimental section

4.1. General methods for chemistry

Reaction progresses were usually determined by thin layer chromatography (TLC) using silica plates. Chromatographic purifications were performed using silica gel (300–400 mesh) bought from Qingdao Haiyang Chemical Co., Ltd. Determination of melting point was conducted on a XT4MP apparatus (From Taike Corp., Beijing, China). ^{1}H and ^{13}C NMR spectra were recorded on Bruker AM-300 (^{1}H, 300 MHz; ^{13}C, 75 MHz) or Agilent DD2 600 MHz (^{1}H, 600 MHz; ^{13}C, 151 MHz) spectrometer using DMSO-d_6 or $CDCl_3$ and TMS as the internal standard. HR-MS were determined on a Micro Mass GCT CA 055 instrument.

4.2. General procedure for synthesis of compounds ***5a~g***

(*E*)-3-(3,5,6-trimethylpyrazin-2-yl)acrylic acid (0.96 g,

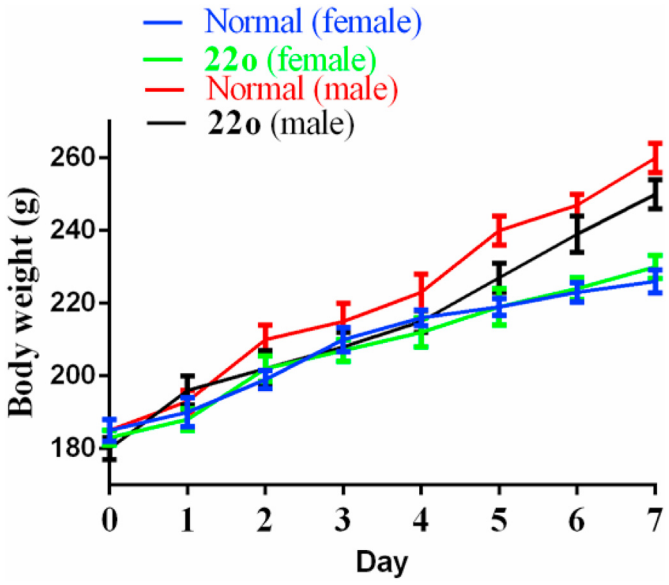

Fig. 9. Body weight of all rats in four groups (g)-time (day).

5.00 mmol), EDCI (1.43 g, 7.50 mmol) and HOBT (1.10 g, 7.5 mmol) were dissolved in dry CH_2Cl_2 (12 mL) and stirred at about 25 °C for 30 min. Compound **1** (1.001 g, 5.51 mmol) and TEA (2.073 mL, 15.00 mmol) were added slowly and then stirred at 25–30 °C overnight. The mixture was evaporated partly and the precipitate was filtered and washed with water. At last the crude was dried to give compound **2**.

Compound **2** (1.715 g, 5 mmol) and sodium methoxide (30%, 10.5 mL) were added to methanol (20 mL) solution and refluxed for 8–12 h. After that time, the reaction mixture was concentrated under vacuum and acidified with 2 N HCl to pH = 7. The crude precipitate was successively filtered off, washed with water, dried process. At the end of operation, we obtained compound **3**.

Compound **3** (1.69 g, 5.00 mmol) was slowly added to phosphorus oxychloride ($POCl_3$) (11 mL) in ice bath. Stirring was continued for 8–12 h at 100 °C. After that time, $POCl_3$ was evaporated partly under vacuum to get a dark brown oil. The mixture was carefully poured into ice/H_2O and then extracted with DCM (25 mL × 3). We separated the organic layer and it was washed with saturated $NaHCO_3$, saturated brine. After drying over anhydrous Na_2SO_4, the solvent was evaporated to give the key intermediate **4**.

Intermediate **4** (101 mg, 0.282 mmol), cyclopropanamine (0.022 mL, 0.31 mmol) and TEA (0.117 mL, 0.843 mmol) were added to isopropanol (20 mL) and heated to reflux for 6–8 h. The isopropanol was removed under vacuum. Obtained residue was purified by silica gel column, eluting with EtOAc/PE(50/50 v/v) to give compound **5a**.

Compounds **5b ~ g** were obtained using the similar procedures as compound **5a**.

*(E)-N-cyclopropyl-1-methyl-3-propyl-5-(2-(3,5,6-trimethylpyrazin-*2-yl*)vinyl)-1H-pyrazolo[4,3-d]pyrimidin-7-amine* (**5a**). Yellow solid, yield: 80.3%, mp: 174–175 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.70 (s, 1H), 8.29 (d, J = 14.9 Hz, 1H), 7.79 (d, J = 14.9 Hz, 1H), 4.25 (s, 3H), 3.30–3.25 (m, 1H), 2.90 (t, J = 7.5 Hz, 2H), 2.61 (s, 3H), 2.54–2.47 (m, 6H), 1.75–1.66 (m, 2H), 1.03–0.89 (m, 7H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 156.59, 150.81, 150.71, 149.65, 147.63, 144.46, 144.32, 142.48, 134.04, 129.39, 121.45, 39.47, 27.69, 24.49, 22.08, 21.94, 21.90, 20.82, 14.36, 7.05 (2C). HR-MS (ESI): calcd for $C_{21}H_{28}N_7$ $[M + H]^+$, 378.2041; found 378.2043.

*(E)-N-isopropyl-1-methyl-3-propyl-5-(2-(3,5,6-trimethylpyrazin-*2-yl*)vinyl)-1H- pyrazolo[4,3-d]pyrimidin-7-amine* **(5b).** Yellow solid, yield: 81.6%, mp: 160–161 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.38 (s, 1H), 8.23 (d, J = 14.9 Hz, 1H), 7.79 (d, J = 14.9 Hz, 1H), 4.86–4.77 (m, 1H), 4.31 (s, 3H), 2.91 (t, J = 7.5 Hz, 2H), 2.63 (s, 3H), 2.52 (d, 6H), 1.75–1.66 (m, 2H), 1.43 (d, J = 6.6 Hz, 6H), 0.96 (t, J = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 156.55, 150.76, 149.61, 149.13, 147.56, 144.45, 144.32, 142.47, 134.23, 129.02, 121.33, 42.80, 39.49, 27.68, 22.21 (2C), 22.09, 21.91, 21.88, 20.82, 14.35. HR-MS (ESI): calcd for $C_{21}H_{30}N_7$ $[M + H]^+$, 380.2557; found 380.2559.

*(E)-4-(1-methyl-3-propyl-5-(2-(3,5,6-trimethylpyrazin-*2-yl*)vinyl)-1H-pyrazolo[4,3-d]pyrimidin-*7-yl*)morpholine* **(5c).** Yellow solid, yield: 77.5%, mp: 195–196 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.18 (d, J = 15.0 Hz, 1H), 7.81 (d, J = 15.0 Hz, 1H),4.12 (s, 3H), 3.99–3.96 (m, 4H), 3.83–3.81 (m, 4H), 2.96 (t, J = 7.5 Hz, 2H), 2.65 (s, 3H), 2.52 (d, 6H), 1.78–1.69(m, 2H), 0.98 (t, J = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6 + Pyridine-d_5) δ 156.08, 153.63, 150.95, 149.60, 147.76, 146.06, 144.67, 144.16, 133.35, 129.82, 123.96, 66.12 (2C), 49.98 (2C), 39.21, 27.76, 21.97, 21.83, 21.80, 20.82, 14.29. HR-MS (ESI): calcd for $C_{22}H_{30}N_7O$ $[M + H]^+$, 408.2506; found 408.2510.

*(E)-4-(1-methyl-3-propyl-5-(2-(3,5,6-trimethylpyrazin-*2-yl*)vinyl)-1H-pyrazolo[4,3-d]pyrimidin-*7-yl*)thiomorpholine 1,1-dioxide* **(5d).** Yellow solid, yield: 76.5%, mp: 173–174 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.07 (d, J = 15.1 Hz, 1H), 7.74 (d, J = 15.1Hz, 1H), 4.18–4.15 (m, 4H), 4.14 (s, 3H), 3.47–3.73 (m, 4H), 2.93 (t, J = 7.5 Hz, 2H), 2.64 (s, 3H), 2.52 (d, 6H), 1.82–1.73 (m, 2H), 0.97 (t, J = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 155.78, 152.55, 151.12, 149.69, 147.84, 146.02, 145.09, 144.00, 132.97, 129.95, 123.66, 50.70 (2C), 48.10 (2C), 39.38, 27.70, 21.97, 21.92 (2C), 20.89, 14.38. HR-MS (ESI): calcd for $C_{22}H_{30}N_7O_2S$ $[M + H]^+$, 456.2176; found

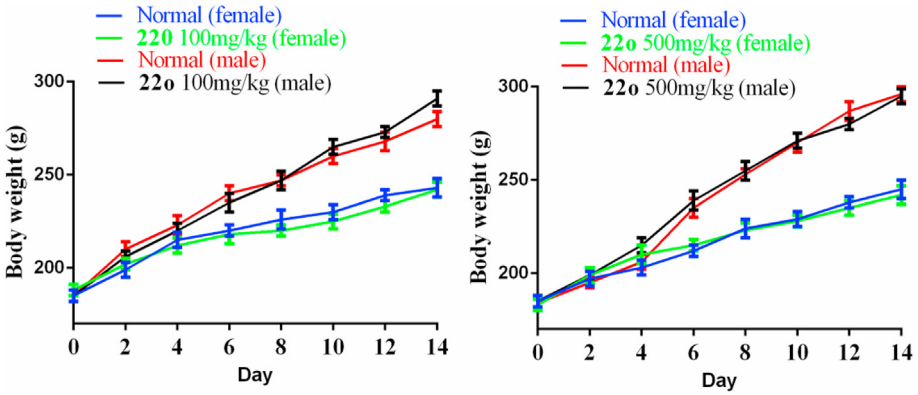

Fig. 10. Body weight of all rats in eight groups (g)-time (day).

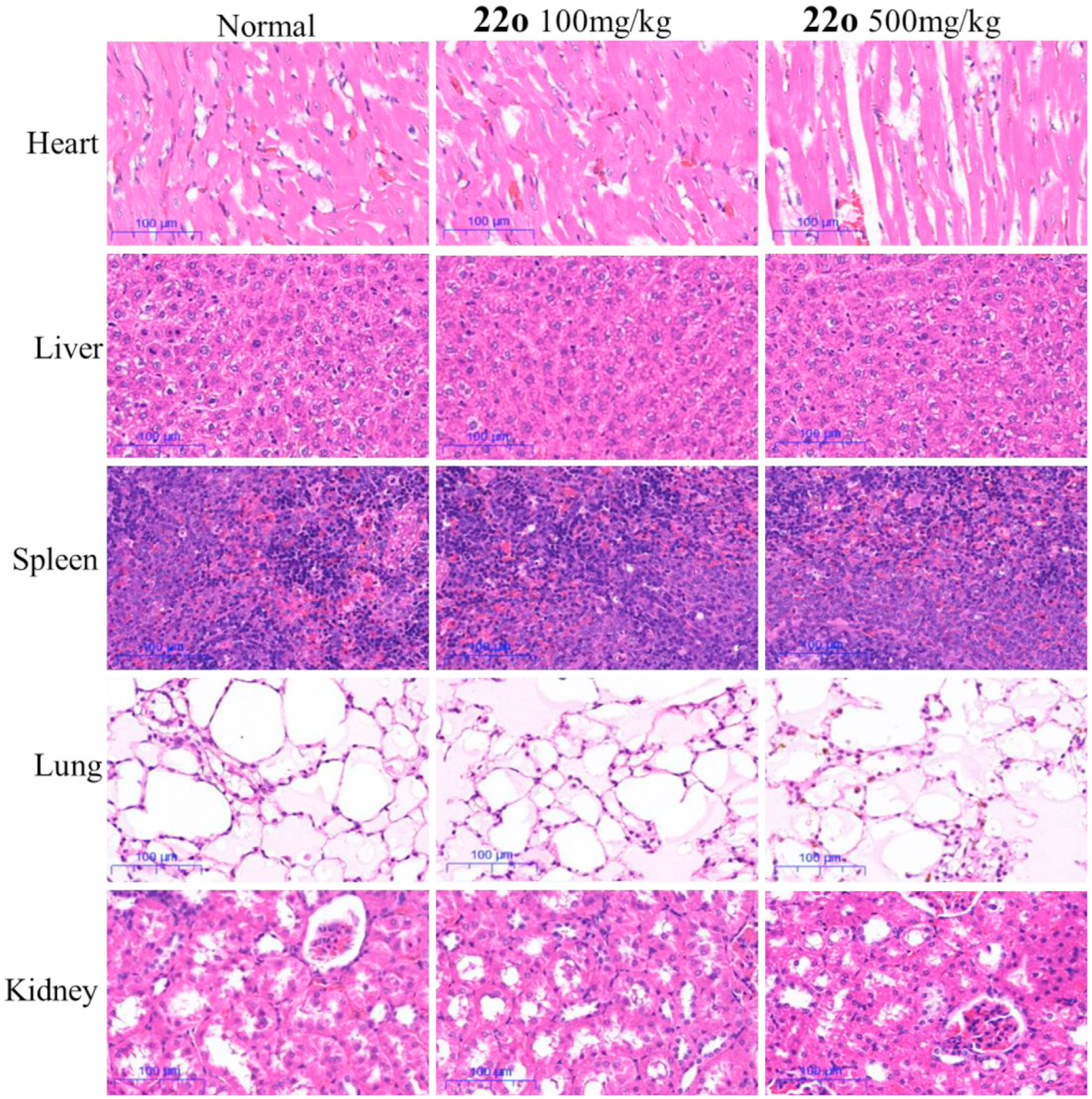

Fig. 11. Histopathology (H&E) of important organs (heart, liver, spleen, lungs and kidneys) of treated groups rats after 14 days.

456.2178.

(E)-1-methyl-N-phenethyl-3-propyl-5-(2-(3,5,6-trimethylpyrazin-2-yl)vinyl)-1H-pyrazolo[4,3-d]pyrimidin-7-amine **(5e)**. Yellow solid, yield: 78.6%, mp: 180–181 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.80 (s, 1H), 8.14 (d, J = 14.9 Hz, 1H), 7.71 (d, J = 14.9 Hz, 1H), 7.32 (dd, J = 13.0, 5.6 Hz, 4H), 7.23 (t, J = 7.0 Hz, 1H), 4.26 (s, 3H), 3.98–3.92 (m, 2H), 3.08–3.00 (m, 2H), 2.85 (t, J = 7.5 Hz, 2H), 2.61 (s, 3H), 2.51 (s, 6H), 1.75–1.66 (m, 2H), 0.95 (t, J = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6 + Pyridine-d_5) δ 156.44, 150.85, 149.58, 149.54, 147.64, 144.30, 144.17, 141.48, 140.07, 133.68, 129.59, 129.09 (2C), 128.85 (2C), 126.62, 121.42, 42.72, 39.58, 35.23, 27.74, 22.16, 21.83 (2C), 20.82, 14.27. HR-MS (ESI): calcd for $C_{26}H_{32}N_7$ $[M + H]^+$, 442.2714; found 442.2713.

(E)-N-(3-chlorophenethyl)-1-methyl-3-propyl-5-(2-(3,5,6-trimethylpyrazin-2-yl)vinyl)-1H-pyrazolo[4,3-d]pyrimidin-7-amine **(5f)**. Yellow solid, yield: 75.9%, mp: 142–143 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.92 (d, J = 15.2 Hz, 1H), 7.55 (d, J = 15.2 Hz, 1H), 7.49 (s, 1H), 7.42–7.25 (m, 4H), 4.16 (s, 3H), 3.87–3.81 (m, 2H), 3.08–3.00 (m, 2H), 2.80 (t, J = 7.5 Hz, 2H), 2.56 (s, 3H), 2.49 (s, 3H), 2.46 (s, 3H), 1.79–1.71 (m, 2H), 0.95 (t, J = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6 + Pyridine-d_5) δ 156.67, 150.71, 149.53, 149.50, 147.60, 144.49, 144.43, 142.69, 142.31, 134.22, 133.64, 130.55, 129.25, 128.97, 127.84, 126.61, 121.43, 42.18, 39.50, 34.79, 27.76, 22.15, 21.79 (2C), 20.88, 14.26. HR-MS (ESI): calcd for $C_{26}H_{31}ClN_7$ $[M + H]^+$, 476.2324; found 476.2321.

(E)-N-(4-fluorobenzyl)-1-methyl-3-propyl-5-(2-(3,5,6-trimethylpyrazin-2-yl)vinyl)-1H-pyrazolo[4,3-d]pyrimidin-7-amine **(5g)**. Yellow solid, yield: 79.2%, mp: 151–152 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.93 (t, J = 5.9 Hz, 1H), 7.76 (d, J = 15.2 Hz, 1H), 7.55 (dd, J = 8.4, 5.6 Hz, 2H), 7.47 (d, J = 15.2 Hz, 1H), 7.15 (t, J = 8.9 Hz, 2H), 4.79 (d, J = 5.7 Hz, 2H), 4.25 (s, 3H), 2.80 (t, J = 7.5 Hz, 2H), 2.53 (s, 3H), 2.47 (s, 3H), 2.45 (s, 3H), 1.80–1.71 (m, 2H), 0.94 (t, J = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 162.50 (d, J = 242.40 Hz), 156.46, 150.81, 149.60, 149.13, 147.56, 144.47, 144.25, 142.47, 136.65 (d, J = 3.03 Hz), 134.04, 129.60 (d, J = 8.08 Hz, 2C), 129.15, 121.23, 115.36 (d, J = 21.21 Hz, 2C), 43.61, 39.63, 27.69, 22.12, 21.91, 21.88,

20.86, 14.36. HR-MS (ESI): calcd for $C_{25}H_{29}FN_7$ $[M + H]^+$, 446.2463; found 446.2458.

4.3. General procedure for synthesis of compounds **6a~g**

Compounds **6b ~ g** were obtained according to the similar procedures as compound **5a**.

(E)-N-cyclopropyl-1-methyl-3-propyl-5-(2-(pyridin-3-yl*)vinyl)-1H-pyrazolo[4,3-d*]

pyrimidin-7-amine (**6a**). Yellow solid, yield: 86.5%, mp: 117−118 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.17 (s, 1H), 8.82 (d, J = 5.2 Hz, 1H), 8.60 (d, J = 7.9 Hz, 2H), 8.20 (d, J = 15.8 Hz, 1H), 7.93 (dd, J = 8.1, 5.3 Hz, 1H), 7.81 (d, J = 15.9 Hz, 1H), 4.26 (s, 3H), 3.42−3.36 (m, 1H), 2.94 (t, J = 7.5 Hz, 2H), 1.77−1.68 (m, 2H), 1.07−0.87 (m, 7H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 156.61, 150.74, 149.49, 149.37, 144.34, 142.34, 133.88, 132.52, 131.55, 131.41, 124.35, 121.45, 39.48, 27.69, 24.54, 22.16, 14.36, 7.01 (2C). HR-MS (ESI): calcd for $C_{19}H_{23}N_6$ $[M + H]^+$, 335.1979; found 335.1977.

(E)-N-isopropyl-1-methyl-3-propyl-5-(2-(pyridin-3-yl*)vinyl)-1H-pyrazolo[4,3-d]pyrimidin-7-amine* (**6b**). White solid, yield: 86.7%, mp: 146−147 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.83 (d, J = 2.2 Hz, 1H), 8.49 (dd, J = 4.7, 1.6 Hz, 1H), 8.16 (dt, J = 8.1, 2.0 Hz, 1H), 7.73 (d, J = 16.0 Hz, 1H), 7.40 (dd, J = 8.0, 4.7 Hz, 1H), 7.26 (d, J = 16.0 Hz, 1H), 6.69 (d, J = 7.7 Hz, 1H), 4.71−4.66 (m, 1H), 4.19 (s, 3H), 2.80 (t, J = 7.5 Hz, 2H), 1.80−1.70 (m, 2H), 1.34 (d, J = 6.6 Hz, 6H), 0.94 (t, J = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 156.60, 149.47, 149.33, 149.15, 144.33, 142.38, 133.89, 132.52, 131.73, 131.05, 124.33, 121.32, 42.50, 39.51, 27.69, 22.36, 22.17 (2C), 14.35. HR-MS (ESI): calcd for $C_{19}H_{25}N_6$ $[M + H]^+$, 337.2135; found 337.2133.

(E)-4-(1-methyl-3-propyl-5-(2-(pyridin-3-yl*)vinyl)-1H-pyrazolo [4,3-d]pyrimidin*-7-yl*)morpholine* (**6c**). White solid, yield: 81.3%, mp: 130−131 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.14 (s, 1H), 8.73 (d, J = 3.9 Hz, 1H), 8.67 (d, J = 8.2 Hz, 1H), 7.94 (d, J = 16.0 Hz, 1H), 7.84 (dd, J = 8.1, 5.3 Hz, 1H), 7.59 (d, J = 16.0 Hz, 1H), 4.10 (s, 3H), 3.85−3.82 (m, 4H), 3.67−3.66 (m, 4H), 2.89 (t, J = 7.5 Hz, 2H), 1.83−1.74 (m, 2H), 0.97 (t, J = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 156.06, 153.68, 149.63, 149.40, 145.94, 144.56, 133.93, 132.32, 131.94, 130.72, 124.31, 123.93, 66.15 (2C), 50.00 (2C), 39.27, 27.75, 22.02, 14.38. HR-MS (ESI): calcd for $C_{20}H_{25}N_6O$ $[M + H]^+$, 365.2084; found 365.2088.

(E)-4-(1-methyl-3-propyl-5-(2-(pyridin-3-yl*)vinyl)-1H-pyrazolo [4,3-d]pyrimidin*-7-yl*)thiomorpholine 1,1-dioxide* (**6d**). Light yellow solid, yield: 75.3%, mp: 192−193 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.88 (d, J = 2.2 Hz, 1H), 8.52 (dd, J = 4.8, 1.6 Hz, 1H), 8.21 (dt, J = 8.0, 2.0 Hz, 1H), 7.82 (d, J = 16.0 Hz, 1H), 7.46−7.43 (m, 1H), 7.40 (d, J = 16.0 Hz, 1H), 4.12 (s, 3H), 4.05−4.02 (m, 4H), 3.44−3.41 (m, 4H), 2.88 (t, J = 7.5 Hz, 2H), 1.84−1.75 (m, 2H), 0.97 (t, J = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 155.90,152.63, 149.76, 149.54, 145.89, 144.99, 134.04, 132.27, 132.24, 130.48, 124.37, 123.69, 50.76 (2C), 48.12 (2C), 39.41, 27.72, 22.04, 14.39. HR-MS (ESI): calcd for $C_{20}H_{25}N_6O_2S$ $[M + H]^+$, 413.1754; found 413.1779.

(E)-1-methyl-N-phenethyl-3-propyl-5-(2-(pyridin-3-yl*)vinyl)-1H-pyrazolo[4,3-d]pyrimidin-7-amine* (**6e**). White solid, yield: 81.9%, mp: 136−137 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.82 (d, J = 2.4 Hz, 1H), 8.53 (d, J = 4.3 Hz, 1H), 7.97 (d, J = 8.0 Hz, 1H), 7.88 (d, J = 15.9 Hz, 1H), 7.44−7.35 (m, 2H), 7.37−7.26 (m, 5H), 5.15 (t, J = 5.6 Hz, 1H), 4.06−4.01 (m, 2H), 4.03 (s, 3H), 3.11 (t, J = 6.7 Hz, 2H), 2.95 (t, J = 7.7 Hz, 2H), 1.89−1.82 (m, 2H), 1.04 (t, J = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 156.61, 149.48, 149.45, 149.21, 144.37, 142.36, 140.13, 133.81, 132.50, 131.74, 131.08, 129.16 (2C), 128.86 (2C), 126.59, 124.34, 121.31, 42.57, 39.45, 35.17, 27.70, 22.09, 14.34. HR-MS (ESI): calcd for $C24H_{27}N_6$ $[M + H]^+$, 399.2292; found 399.2291.

(E)-N-(3-chlorophenethyl)-1-methyl-3-propyl-5-(2-(pyridin-3-yl*) vinyl)-1H-pyrazolo[4,3-d]pyrimidin-7-amine* (**6f**). White solid, yield: 80.8%, mp: 141−142 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.82 (s, 1H), 8.53 (d, J = 3.9 Hz, 1H), 7.98 (d, J = 7.9 Hz, 1H), 7.87 (d, J = 15.9 Hz, 1H), 7.36−7.28 (m, 5H), 7.19 (d, J = 7.0 Hz, 1H), 5.20 (t, J = 5.8 Hz, 1H), 4.11 (s, 3H), 4.02 (m, 2H), 3.10 (t, J = 6.9 Hz, 2H), 2.95 (t, J = 7.7 Hz, 2H), 1.88−1.86 (m, 2H), 1.04 (t, J = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 156.59, 149.53, 149.39, 149.34, 144.37, 142.77, 142.36, 133.83, 133.45, 132.48, 131.64, 131.17, 130.67, 129.09, 128.04, 126.61, 124.35, 121.28, 42.15, 39.47, 34.75, 27.71, 22.16, 14.38. HR-MS (ESI): calcd for $C_{24}H_{26}ClN_6$ $[M + H]^+$, 433.1902; found 433.1905.

(E)-N-(4-fluorobenzyl)-1-methyl-3-propyl-5-(2-(pyridin-3-yl*)vinyl)-1H-pyrazolo-*

[4,3-d]pyrimidin-7-amine (**6g**). White solid, yield: 88.3%, mp: 172 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.78 (d, J = 2.2 Hz, 1H), 8.52 (dd, J = 4.8, 1.6 Hz, 1H), 7.93 (dt, J = 7.9, 2.0 Hz, 1H), 7.80 (d, J = 15.9 Hz, 1H), 7.49−7.40 (m, 2H), 7.35−7.27 (m, 2H), 7.13−7.05 (m, 2H), 5.48 (t, J = 5.5 Hz, 1H), 4.94 (d, J = 5.4 Hz, 2H), 4.24 (s, 3H), 3.01−2.92 (m, 2H), 1.91−1.83(m, 2H), 1.04 (t, J = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 161.55 (d, J = 243.41 Hz), 156.52, 149.52, 149.34, 149.16, 144.36, 142.43, 136.69 (d, J = 3.03 Hz), 133.84, 132.48,131.48, 131.40, 129.87 (d, J = 8.08 Hz, 2C), 124.35, 121.24, 115.35 (d, J = 21.21 Hz, 2C), 43.54,39.63, 27.70, 22.18, 14.36. HR-MS (ESI): calcd for $C_{23}H_{24}FN_6$ $[M + H]^+$, 403.2041; found 403.2042.

4.4. General procedure for synthesis of compounds **7a~g**

Compound **6a** (101 mg, 0.297 mmol) was dissolved in the mixture of acetone (6 mL) and water (3 mL). $NaHCO_3$ (749 mg, 8.918 mmol) and $KHSO_5$ (912 mg, 2.97 mmol) were added three times within 1 h. Follow the operation, stirring was kept for 4 h at room temperature. After that time, the acetone was removed by evaporation. The crude was purified by silica gel column, eluting with EtOAc/PE (75/25 v/v) to give compound **7a**. Compounds **7b ~ g** were prepared describe as the similar procedures as compound **7a**.

(E)-3-(2-(7-(cyclopropylamino)-1-methyl-3-propyl-1H-pyrazolo [4,3-d]pyrimidin-5-yl*)vinyl)pyridine 1-oxide* (**7a**). Yellow solid, yield: 61.7%, mp: 220−221 °C. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.59 (s, 1H), 8.14 (d, J = 6.5 Hz, 1H), 7.75 (d, J = 8.2 Hz, 1H), 7.71 (d, J = 15.7 Hz, 1H), 7.44−7.39 (m, 1H), 7.33 (d, J = 15.9 Hz, 1H), 7.28 (d, J = 3.0 Hz, 1H), 4.15 (s, 3H), 3.13−3.06 (m, 1H), 2.80 (t, J = 7.5 Hz, 2H), 1.81−1.70 (m, 2H), 0.94 (t, J = 7.3 Hz, 3H), 0.92−0.88 (m, 2H), 0.73−0.66 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 156.18, 150.74, 144.46, 142.30, 138.21, 137.93, 136.35, 133.73, 129.49, 126.86, 123.39, 121.48, 39.52, 27.69, 24.58, 22.14, 14.36, 6.98 (2C). HR-MS (ESI): calcd for $C_{19}H_{23}N_6O$

$[M + H]^+$, 351.1928; found 351.1931.

(E)-3-(2-(7-(isopropylamino)-1-methyl-3-propyl-1H-pyrazolo [4,3-d]pyrimidin-5-yl*)vinyl)pyridine 1-oxide* (**7b**). Yellow solid, yield: 66.7%, mp: 236−237 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.59 (t, J = 1.7 Hz, 1H), 8.14 (dd, J = 6.3, 1.0 Hz, 1H), 7.75 (d, J = 8.4 Hz, 1H), 7.63 (d, J = 15.9 Hz, 1H), 7.41 (dd, J = 8.0, 6.4 Hz, 1H), 7.30 (d, J = 16.0 Hz, 1H), 6.72 (d, J = 7.8 Hz, 1H), 4.72−4.64 (m, 1H), 4.20 (s, 3H), 2.79 (t, J = 7.5 Hz, 2H), 1.79−1.70 (m, 2H), 1.33 (d, J = 6.5 Hz, 6H), 0.93 (t, J = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 156.18, 149.14, 144.44, 142.30, 138.18, 137.90, 136.37, 133.85, 129.19, 126.85, 123.47, 121.34, 42.47, 39.50, 27.68, 22.39 (2C), 22.15, 14.35. HR-MS (ESI): calcd for $C_{19}H_{25}N_6O$ $[M + H]^+$, 353.2084; found 353.2083.

(E)-3-(2-(1-methyl-7-morpholino-3-propyl-1H-pyrazolo[4,3-d] pyrimidin-5-yl*)vinyl)pyridine 1-oxide* (**7c**). Yellow solid, yield: 66.5%, mp: 209−210 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.43 (s, 1H), 8.17 (d, J = 6.3 Hz, 1H), 7.68 (d, J = 15.9 Hz, 1H), 7.57 (d, J = 8.0 Hz, 1H), 7.37 (d, J = 15.9 Hz, 1H), 7.32 (dd, J = 8.1, 6.4 Hz, 1H), 4.14 (s, 3H), 4.00−3.93 (m, 4H), 3.63 (t, J = 4.7 Hz, 4H), 3.05−2.97 (m, 2H), 1.94−1.85 (m, 2H), 1.06 (t, J = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 155.66, 153.62, 146.02, 144.50, 138.37, 138.04, 136.17, 132.86, 130.14, 126.89, 123.95, 123.42, 66.14 (2C), 49.96 (2C), 39.40,

27.75, 22.01, 14.39. HR-MS (ESI): calcd for $C_{20}H_{25}N_6O_2$ $[M + H]^+$, 381.2034; found 381.2038.

(E)-3-(2-(7-(1,1-dioxidothiomorpholino)-1-methyl-3-propyl-1H-pyrazolo[4,3-d].

pyrimidin-5-yl*)vinyl)pyridine 1-oxide* (**7d**). White solid, yield: 60.9%, mp: 240–241 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.64 (t, J = 1.8 Hz, 1H), 8.15 (dd, J = 6.3, 1.0 Hz, 1H), 7.79 (d, J = 8.8 Hz, 1H), 7.71 (d, J = 16.0 Hz, 1H), 7.47–7.40 (m, 2H), 4.11 (s, 3H), 4.05–4.03 (m, 4H), 3.43–3.38 (m, 4H), 2.87 (t, J = 7.5 Hz, 2H), 1.83–1.74 (m, 2H), 0.96 (t, J = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 155.30, 152.61, 146.10, 144.65, 138.40, 137.91, 136.14, 132.53, 130.19, 127.16, 125.23, 123.71, 50.69 (2C), 47.99 (2C), 39.01, 27.54, 22.01, 14.26. HR-MS (ESI): calcd for $C_{20}H_{25}N_6O_3S$ $[M + H]^+$, 429.1703; found 429.1703.

(E)-3-(2-(1-methyl-7-(phenethylamino)-3-propyl-1H-pyrazolo [4,3-d]pyrimidin-5- yl)vinyl)pyridine 1-oxide (**7e**). Light yellow solid, yield: 66.8%, mp: 182–183 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.56 (s, 1H), 8.16 (d, J = 6.3 Hz, 1H), 7.72 (d, J = 8.1 Hz, 1H), 7.66 (d, J = 15.9 Hz, 1H), 7.46–7.37 (m, 2H), 7.39–7.29 (m, 5H), 7.27–7.16 (m, 1H), 4.16 (s, 3H), 3.88–3.78 (m, 2H), 3.06–2.98 (m, 2H), 2.79 (t, J = 7.6 Hz, 2H), 1.82–1.68 (m, 2H), 0.94 (t, J = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 156.20, 149.46, 144.47, 142.25, 140.13, 138.23, 137.75, 136.33, 133.87, 129.25 (2C), 129.21, 128.87 (2C), 126.93, 126.61, 123.50, 121.32, 42.49, 39.51, 35.17, 27.70, 22.14, 14.38. HR-MS (ESI): calcd for $C_{24}H_{27}N_6O$ $[M + H]^+$, 415.2241; found 415.2239.

(E)-3-(2-(7-((3-chlorophenethyl)amino)-1-methyl-3-propyl-1H-pyrazolo[4,3-d].

pyrimidin-5-yl*)vinyl)pyridine 1-oxide* (**7f**). White solid, yield: 64.2%, mp: 207–209 °C. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.57 (s, 1H), 8.14 (d, J = 6.5 Hz, 1H), 7.73 (d, J = 8.0 Hz, 1H), 7.65 (d, J = 15.9 Hz, 1H), 7.42 (m, 2H), 7.39–7.23 (m, 5H), 4.15 (s, 3H), 3.88–3.85 (m, 2H), 3.03 (t, J = 7.4 Hz, 2H), 2.79 (t, J = 7.6 Hz, 2H), 1.75 (m, 2H), 0.94 (t, J = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 156.16, 149.41, 144.48, 142.74, 142.30, 138.21, 137.81, 136.34, 133.80, 133.43, 130.56, 129.23, 129.16, 128.06, 126.85, 126.55, 123.39, 121.29, 42.00, 39.47, 34.81, 27.70, 22.14, 14.37. HR-MS (ESI): calcd for $C_{24}H_{26}ClN_6O$ $[M + H]^+$, 449.1851; found 449.1856.

(E)-3-(2-(7-((4-fluorobenzyl)amino)-1-methyl-3-propyl-1H-pyrazolo[4,3-d]pyrimidin-5-yl*)vinyl)pyridine 1-oxide* (**7g**). Light yellow solid, yield: 71.1%, mp: 221–222 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.57 (t, J = 1.7 Hz, 1H), 8.14 (dd, J = 6.3, 1.0 Hz, 1H), 7.94 (t, J = 5.9 Hz, 1H), 7.71 (d, J = 8.2 Hz, 1H), 7.63–7.51 (m, 3H), 7.41 (dd, J = 8.0, 6.4 Hz, 1H), 7.27 (d, J = 16.0 Hz, 1H), 7.20–7.11 (m, 2H), 4.82 (d, J = 5.7 Hz, 2H), 4.24 (s, 3H), 2.79 (t, J = 7.5 Hz, 2H), 1.79–1.69 (m, 2H), 0.93 (t, J = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 161.57 (d, J = 243.41 Hz), 156.07, 149.18, 144.49, 142.37, 138.23, 137.81, 136.63 (d, J = 3.03 Hz),136.30, 133.61, 130.01 (d, J = 8.08 Hz, 2C), 129.41, 126.90, 123.48, 121.27, 115.32 (d, J = 21.21 Hz, 2C), 43.42, 39.64, 27.68, 22.15, 14.35. HR-MS (ESI): calcd for $C_{23}H_{24}FN_6O$ $[M + H]^+$, 419.1990; found 419.1989.

4.5. General procedure for synthesis of compounds ***8a~g***

Compound **6a** (102.5 mg, 0.30 mmol) was added to THF (6 mL) and hydrogenated (2.2 atm) at room temperature for 10 h using 5% Pd/C (3.14 mg, 0.0297 mmol) as catalyst. After the reaction was completed, the Pd/C catalyst was filtered off and THF was removed by evaporation. The crude was purified by silica gel column, eluting with EtOAc/PE (75/25 v/v) to give compound **8a**. Compounds **8b ~ g** were prepared using the similar procedures as compound **8a**.

N-cyclopropyl-1-methyl-3-propyl-5-(2-(pyridin-3-yl*)ethyl)-1H-pyrazolo[4,3-d*].

pyrimidin-7-amine (**8a**). Colorless and transparent oil, yield: 64.1%. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.44 (d, J = 2.2 Hz, 1H), 8.34 (dd, J = 4.7, 1.6 Hz, 1H), 7.65 (d, J = 7.8, 1H), 7.25 (dd, J = 7.8, 4.7 Hz, 1H), 7.12 (d, J = 2.9 Hz, 1H), 4.09 (s, 3H), 3.14 (t, J = 7.3 Hz, 2H), 3.06 (t, J = 7.7 Hz, 2H), 2.96–2.94 (m, 1H), 2.73 (t, J = 7.5 Hz, 2H), 1.73–1.66 (m, 2H), 0.89 (t, J = 7.3 Hz, 3H), 0.83–0.75 (m, 2H), 0.65–0.61 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 161.14, 151.00, 150.18, 147.37, 143.46, 142.14, 137.74, 136.33, 123.70, 121.25, 39.78, 39.34, 31.13, 27.63, 24.45, 22.09, 14.27, 6.99 (2C). HR-MS (ESI): calcd for $C_{19}H_{25}N_6$

$[M + H]^+$, 337.2135; found 337.2130.

N-isopropyl-1-methyl-3-propyl-5-(2-(pyridin-3-yl*)ethyl)-1H-pyrazolo[4,3-d]pyrimidin-7-amine* (**8b**). White solid, yield: 69.4%, mp: 59–60 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.41 (d, J = 1.4 Hz, 1H), 8.34 (dd, J = 4.8, 1.7 Hz, 1H), 7.64 (dt, J = 7.8, 2.0 Hz, 1H), 7.27–7.23 (m, 1H), 6.58 (d, J = 7.8 Hz, 1H), 4.53–4.41 (m, 1H), 4.14 (s, 3H), 3.09 (t, J = 6.8 Hz, 2H), 3.05–2.97 (m, 2H), 2.73 (t, J = 7.5 Hz, 2H), 1.74–1.65 (m, 2H), 1.26 (d, J = 6.6 Hz, 6H), 0.89 (t, J = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 161.17, 150.09, 149.43, 147.40, 143.48, 142.21, 137.67, 136.29, 123.73, 121.09, 42.35, 39.78, 39.40, 31.26, 27.64, 22.34 (2C), 22.10, 14.30. HR-MS (ESI): calcd for $C_{19}H_{27}N_6$ $[M + H]^+$, 339.2292; found 339.2293.

4-(1-methyl-3-propyl-5-(2-(pyridin-3-yl*)ethyl)-1H-pyrazolo[4,3-d]pyrimidin*-7-yl).

morpholine (**8c**). Yellow oil, yield: 68.0%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.50 (d, J = 2.3 Hz, 1H), 8.44 (dd, J = 4.9, 1.6 Hz, 1H), 7.68–7.65 (m, 1H), 7.25 (dd, J = 7.4, 5.2 Hz, 1H), 4.08 (s, 3H), 3.92–3.86 (m, 4H), 3.55–3.48 (m, 4H), 3.32–3.18 (m, 4H), 3.02–2.92 (m, 2H), 1.89–1.80 (m, 2H), 1.02 (t, J = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 160.99, 153.75, 149.74, 146.91, 146.83, 144.90, 137.39, 136.33, 124.20, 123.34, 66.38 (2C), 49.88 (2C), 39.54, 38.43, 31.64, 27.79, 22.16, 14.05. HR-MS (ESI): calcd for $C_{20}H_{27}N_6O$ $[M + H]^+$, 367.2242; found 367.2236.

4-(1-methyl-3-propyl-5-(2-(pyridin-3-yl*)ethyl)-1H-pyrazolo[4,3-d]pyrimidin*-7-yl).

thiomorpholine 1,1-dioxide (**8d**). Yellow solid, yield: 61.5%, mp: 114 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.46 (dd, J = 4.9, 1.6 Hz, 1H), 8.42 (d, J = 2.2 Hz, 1H), 7.68 (dt, J = 7.8, 2.0 Hz, 1H), 7.34–7.27 (m, 1H), 4.11–4.02 (m, 4H), 4.08 (s, 3H), 3.32–3.29 (m, 2H), 3.24–3.22 (m, 2H), 3.17 (t, J = 5.3 Hz, 4H), 3.01–2.93 (m, 2H), 1.90–1.81 (m, 2H), 1.04 (t, J = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 160.80, 151.88, 149.32, 147.31, 146.88, 145.91, 137.19, 136.66, 123.66, 123.56, 50.54 (2C), 47.76 (2C), 39.42, 38.43, 31.71, 27.76, 22.09, 14.06. HR-MS (ESI): calcd for $C_{20}H_{27}N_6O_2S$ $[M + H]^+$, 415.1911; found 415.1915.

1-methyl-N-phenethyl-3-propyl-5-(2-(pyridin-3-yl*)ethyl)-1H-pyrazolo[4,3-d*].

pyrimidin-7-amine (**8e**). White solid, yield: 70.6%, mp: 97–98 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.43 (s, 1H), 8.34 (d, J = 3.1 Hz, 1H), 7.64 (d, J = 7.8 Hz, 1H), 7.36–7.24 (m, 5H), 7.25–7.20 (m, 2H), 4.11 (s, 3H), 3.76–3.66 (m, 2H), 3.13 (t, J = 6.7 Hz, 2H), 3.04 (t, J = 6.7 Hz, 2H), 2.98–2.90 (m, 2H), 2.74 (t, J = 7.5 Hz, 2H), 1.77–1.61 (m, 2H), 0.90 (t, J = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 161.23, 150.09, 149.72, 147.45, 143.50, 142.17, 140.13, 137.70, 136.29, 129.15 (2C), 128.87 (2C), 126.59, 123.76, 121.07, 42.50, 39.53, 39.37, 35.19, 31.33, 27.67, 22.10, 14.34. HR-MS (ESI): calcd for $C_{24}H_{29}N_6$

$[M + H]^+$, 401.2448; found 401.2450.

N-(3-chlorophenethyl)-1-methyl-3-propyl-5-(2-(pyridin-3-yl*) ethyl)-1H-pyrazolo[4,3-d]pyrimidin-7-amine* (**8f**). White solid, yield: 66.3%, mp: 103–104 °C. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.40 (d, J = 2.3 Hz, 1H), 8.32 (dd, J = 4.8, 1.6 Hz, 1H), 7.61 (dt, J = 7.8, 2.1 Hz, 1H), 7.35–7.26 (m, 2H), 7.25–7.19(m, 4H), 4.08 (s, 3H), 3.73–3.67 (m, 2H), 3.09 (t, J = 7.7 Hz, 2H), 3.05–2.98 (m, 2H), 2.92 (t, J = 7.3 Hz, 2H), 2.71 (t, J = 7.5 Hz, 2H), 1.68 (m, 2H), 0.88 (t, J = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 161.21, 150.09, 149.68, 147.44, 143.53, 142.78, 142.20, 136.28, 133.40, 130.63, 129.15, 129.05, 127.97, 126.55, 123.74, 121.05, 42.02, 39.53, 39.35, 34.71, 31.37, 27.66, 22.09, 14.34. HR-MS (ESI): calcd for $C_{24}H_{28}ClN_6$ $[M + H]^+$, 435.2058; found 435.2060.

N-(4-fluorobenzyl)-1-methyl-3-propyl-5-(2-(pyridin-3-yl)*ethyl)-1H-pyrazolo[4,3-d]pyrimidin-7-amine* (**8g**). White solid, yield: 72.1%, mp: 99−100 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.32 (dd, J = 4.8, 1.7 Hz, 1H), 8.28 (dd, J = 2.3, 0.9 Hz, 1H), 7.81 (t, J = 5.9 Hz, 1H), 7.51−7.42 (m, 3H), 7.21−7.17 (m, 1H), 7.16−7.09 (m, 2H), 4.70 (d, J = 5.8 Hz, 2H), 4.19 (s, 3H), 3.01−2.92 (m, 4H), 2.72 (t, J = 7.5 Hz, 2H), 1.73−1.63 (m, 2H), 0.89 (t, J = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 161.54 (d, J = 243.41 Hz), 161.02, 150.04, 149.51, 147.37, 143.51, 142.24, 137.59, 136.59 (d, J = 3.03 Hz), 136.23, 129.85 (d, J = 8.08 Hz, 2C), 123.66, 121.02, 115.29 (d, J = 21.21 Hz, 2C), 43.30, 39.89, 39.53, 31.11, 27.64, 22.10, 14.31. HR-MS (ESI): calcd for $C_{23}H_{26}FN_6$ $[M+H]^+$, 405.2197; found 405.2196.

4.6. General procedure for synthesis of compounds ***11a~c***

Compound **9a** (200 mg, 1.34 mmol), isopropylamine (0.115 mL, 1.34 mmol) and TEA (0.556 mL, 4.02 mmol) were added to isopropanol (10 mL) and then refluxed for 5−9 h. After the reaction is finished, the isopropanol was concentrated to give compound **10a**. Compound **10a** (77 mg, 0.51 mmol) and 3,4,5-trimethoxybenzaldehyde (101 mg, 0.51 mmol) were added to dilute hydrochloric acid (6 mL) and keep refluxing for 6−8 h. After that time, the mixture was evaporated under vacuum to dry. The crude was purified by silica gel column, eluting with EtOAc/PE (1/1 v/v) to give compound **11a**.

Compounds **11b ~ c** were obtained using the similar procedures as compound **11a**. *(E)-N-isopropyl-2-(3,4,5-trimethoxystyryl)pyrimidin-4-amine* (**11a**). Yellow solid, yield: 66.1%, mp: 88−90 °C. ^{1}H NMR (400 MHz, DMSO- d_6) δ 8.02 (s, 1H), 7.70 (d, J = 15.9 Hz, 1H), 7.18 (d, J = 7.5 Hz, 1H), 6.98 (s, 2H), 6.97 (d, J = 15.9 Hz, 1H), 6.27 (d, J = 5.9 Hz, 1H), 4.27 (s, 1H), 3.84 (s, 6H), 3.68 (s, 3H), 1.18 (d, J = 6.5 Hz, 6H). HR-MS (ESI): calcd for C_{18}H24N3O3 $[M + H]^+$, 330.1812; found 330.1814.

(E)-N-phenethyl-2-(3,4,5-trimethoxystyryl)pyrimidin-4-amine (**11b**). Brown solid, yield: 63.2%, mp: 105−106 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.05 (s, 1H), 7.74 (d, J = 15.9 Hz, 1H), 7.42 (**s**, 1H), 7.34−7.28 (m, 4H), 7.22 (s, 1H), 7.03 (d, J = 15.9 Hz, 1H), 6.98 (s, 2H), 6.33 (d, J = 5.8 Hz, 1H), 3.85 (s, 6H), 3.69 (s, 3H), 3.61 (m, 2H), 2.89 (t, J = 7.5 Hz, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 163.81, 162.02, 154.37, 153.57 (2C), 140.11, 138.57, 136.35, 132.09, 129.24 (2C), 128.82 (2C), 128.71, 126.60, 105.17 (2C), 104.52, 60.54, 56.39 (2C),41.96, 35.30. HR-MS (ESI): calcd for $C_{23}H_{26}N_3O_3$ $[M + H]^+$, 392.1969; found 392.1962.

(E)-N-(4-fluorophenethyl)-2-(3,4,5-trimethoxystyryl)pyrimidin-4-amine **(11c)**. Brown solid, yield: 63.2%, mp: 130−131 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.04 (s, 1H), 7.72 (d, J = 15.9 Hz, 1H), 7.40 (s, 1H), 7.33 (dd, J = 8.5, 5.7 Hz, 2H), 7.13 (t, J = 8.9 Hz, 2H), 7.01 (d, J = 15.9 Hz, 1H), 6.98 (s, 2H), 6.32 (d, J = 6.0 Hz, 1H), 3.84 (s, 6H), 3.68 (s, 3H), 3.59 (m, 2H), 2.88 (t, J = 7.3 Hz, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 163.73, 162.53, 161.15 (d, J = 205.03 Hz), 154.23, 153.55 (2C), 138.58, 136.31, 136.09, 132.06, 130.84 (d, J = 8.08 Hz, 2C), 128.42, 115.27 (d, J = 21.21 Hz, 2C), 104.93 (2C), 104.46, 60.31, 56.14 (2C), 41.90, 34.38. HR-MS (ESI): calcd for $C_{23}H_{25}FN_3O_3$ $[M + H]^+$, 410.1874; found 410.1876.

4.7. General procedure for synthesis of compounds ***12a~c***

Compounds **12a~c** were synthesized from 4,6-dichloro-2-methylpyrimidine (**9b**) as starting material, according to the similar procedures as compound **11a~c**.

(E)-6-chloro-N-isopropyl-2-(3,4,5-trimethoxystyryl)pyrimidin-4-amine (**12a**). White solid, yield: 71.6%, mp: 137−138 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.72 (d, J = 15.8 Hz, 1H), 7.53 (d, J = 7.9 Hz, 1H), 7.03 (s, 2H), 6.99 (d, J = 15.8 Hz, 1H), 6.30 (s, 1H), 4.30 (s, 1H), 3.84 (s, 6H), 3.69 (s, 3H), 1.18 (d, J = 6.6 Hz, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 164.59, 162.98, 157.45, 153.56 (2C), 138.91, 138.17, 131.59, 127.13, 105.52 (2C), 101.52, 60.53, 56.42 (2C), 42.10, 22.81 (2C). HR-MS (ESI): calcd for $C_{18}H_{23}ClN_3O_3$ $[M + H]^+$, 364.1422; found 364.1422.

(E)-6-chloro-N-phenethyl-2-(3,4,5-trimethoxystyryl)pyrimidin-4-amine (**12b**). White solid, yield: 76.1%, mp: 130−131 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.75 (d, J = 16.0 Hz, 1H), 7.73 (s, 1H), 7.36−7.26 (m, 4H), 7.27−7.17 (m, 1H), 7.03 (s, 2H), 7.00 (d, J = 16.7 Hz, 1H), 6.37 (s, 1H), 3.85 (s, 6H), 3.69 (s, 3H), 3.65 (m, 2H), 2.88 (t, J = 7.3 Hz, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 164.61, 163.68, 157.50, 153.58 (2C), 139.87, 138.99, 138.28, 131.57, 129.26 (2C), 128.82 (2C), 127.11, 126.65, 105.57, 101.65, 60.54 (2C), 56.44, 42.24, 35.23. HR-MS (ESI): calcd for $C_{23}H_{25}ClN_3O_3$ $[M + H]^+$, 426.1579; found 426.1578.

(E)-6-chloro-N-(4-fluorophenethyl)-2-(3,4,5-trimethoxystyryl) pyrimidin-4-amine (**12c**). White solid, yield: 69.3%, mp: 162−163 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.73 (d, J = 16.5 Hz, 1H), 7.71 (s, 1H), 7.36−7.27 (m, 2H), 7.18−7.07 (m, 2H), 7.03 (s, 2H), 6.99 (s, 1H), 6.36 (s, 1H), 3.84 (s, 6H), 3.69 (s, 3H), 3.67−3.59 (m, 2H), 2.86 (t, J = 7.3 Hz, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 164.59, 163.66, 161.36 (d, J = 242.40 Hz), 157.49, 153.57 (2C), 138.96, 138.29, 136.02, 131.56, 131.06 (d, J = 8.08 Hz, 2C), 127.09, 115.46 (d, J = 20.20 Hz, 2C), 105.55 (2C), 101.64, 60.54, 56.42 (2C), 42.20, 34.34. HR-MS (ESI): calcd for $C_{23}H_{24}ClFN_3O_3$ $[M + H]^+$, 444.1485; found 444.1504.

4.8. General procedure for synthesis of compounds ***13a~c***

Compounds **13a~c** were synthesized from 2,4-dichloro-6-methyl-1,3,5-triazine (**9c**) as starting material, according to the similar procedures as compound **11a~c**.

(E)-4-(isopropylamino)-6-(3,4,5-trimethoxystyryl)-1,3,5-triazin-2-ol (**13a**). Yellow solid, yield: 61.9%, mp: 139−140 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.43 (s, 1H), δ 7.84 (d, J = 16.0 Hz, 1H), 7.66 (d, J = 8.1 Hz, 1H), 7.00 (s, 1H), 6.90 (s, 2H), 6.71 (d, J = 15.9 Hz, 1H), 4.03 (m, 1H), 3.84 (s, 6H), 3.71 (s, 3H), 1.13 (d, J = 6.6 Hz, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 164.64, 162.03, 156.38, 153.67 (2C), 141.61, 140.05, 130.33, 119.10, 105.90 (2C), 60.63, 56.44 (2C), 42.22, 22.47 (2C). HR-MS (ESI): calcd for $C_{17}H_{23}N_4O_4$ $[M + H]^+$, 347.1714; found 347.1747.

(E)-4-(phenethylamino)-6-(3,4,5-trimethoxystyryl)-1,3,5-triazin-2-ol (**13b**). Yellow solid, yield: 63.9%, mp: 137−138 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.93−7.76 (m, 2H), 7.36−7.16 (m, 5H), 6.96 (d, J = 22.8 Hz, 2H), 6.72 (d, J = 16.0 Hz, 1H), 4.17−4.07 (m, 1H), 3.84 (s, 6H), 3.71 (s, 3H), 3.55−3.43 (m, 2H), 2.84 (t, J = 7.4 Hz, 2H). HR-MS (ESI): calcd for $C_{22}H_{25}N_4O_4$ $[M + H]^+$, 409.1870; found 409.1864.

(E)-4-((4-fluorophenethyl)amino)-6-(3,4,5-trimethoxystyryl)-1,3,5-triazin-2-ol (**13c**). Yellow solid, yield: 66.8%, mp: 138−139 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.51 (s, 1H), 7.94−7.79 (m, 2H), 7.32−7.26 (m, 2H), 7.17−7.06 (m, 2H), 6.95 (d, J = 16.0 Hz, 1H), 6.92 (s, 1H), 6.72 (d, J = 16.0 Hz, 1H), 3.84 (s, 6H), 3.71 (s, 3H), 3.51−3.41 (m, 2H), 2.83 (t, J = 7.3 Hz, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 165.49,162.26, 160.30 (d, J = 242.40 Hz), 156.31, 153.64 (2C), 141.77, 140.02, 136.02 (d, J = 3.03 Hz), 130.90 (d, J = 8.08 Hz, 2C), 130.32, 119.17, 115.46 (d, J = 21.21 Hz, 2C), 105.92 (2C), 60.62, 56.42 (2C), 42.21, 33.98. HR-MS (ESI): calcd for $C_{22}H_{24}FN_4O_4$ $[M + H]^+$, 427.1776; found 427.1779.

4.9. General procedure for synthesis of intermediates ***19a~c***

Cinnamic acid **14a** (1.05 g, 6.76 mmol) and oxalyl chloride (1.16 mL, 14.01 mmol), were dissolved in dry DCM (20 mL) at 0 °C using DMF as catalyst. Stirring was kept for 2−3 h at 25−30 °C. After that time, the DCM was evaporated off under vacuum. The oil crude was directly used in next step with no further purification. The DCM (1 mL) solution of corresponding acyl chloride was added to

the solution of methyl 3-amino-2-thiophenecarboxylate (1.06 g, 6.75 mmol), TEA (0.94 mL, 6.8 mmol) in DCM (10 mL). Stirring was kept for 6–7 h at about 25 °C. After that time, the organic layer was washed with saturated citric acid, water. At last, the organic layer was dried by anhydrous Na_2SO_4.The DCM was evaporated to obtain crude. The crude was purified by recrystallization using ethanol as solution to obtain title compound **15a**.

Compound **15a** (1.00 g, 3.48 mmol) was dissolved in methanol (15 mL). 5 N NaOH solution (2 mL) was added to the mixture. Then stirring was kept for 2–3 h at 60 °C. While the reaction was finished, 2 N HCl solution was added until the value at pH 1. And then methanol was removed under reduced pressure. The precipitate was filtered to give compound **16a**.

Compound **16a** (1.00 g, 3.66 mmol) were dissolved in dry DCM and oxalyl chloride (0.62 mL, 7.32 mmol) was added slowly in ice bath. Then DMF was added as catalyst. The mixture was warmed to 25–30 °C and stirred for 3 h. After that time, the solution was removed and directly used without further purification. The crude was dissolved in acetone (15 mL) and was slowly added $NH_3 \cdot H_2O$ (8 mL) in ice bath. After the operation, stirring was kept for 3–4 h at 25–30 °C. While the reaction was finished, acetone was remove. The crude was added 2 N HCl solution until the value at pH 7. The precipitate was filtered to afford compound **17a**.

Compound **17a** (1.00 g, 3.67 mmol) was suspended in methanol and cooled to 0 °C. Sodium methoxide (30%, 5.5 mL) solution was added slowly drop-wise. And then, the mixture was refluxed for 2–3 h. After that time, it was cooled to room temperature. The solvent was removed and acidified to pH 6–7 with 2 N HCl. The precipitate was produced and filtered, washed with water. The crude was recrystallized from acetone to obtain compound **18a**.

Compound **18a** (1.11 g, 3.93 mmol) was added to phosphorus oxychloride ($POCl_3$) (5 mL, 53.75 mmol) in ice bath. The suspension was heated and stirred at about 100 °C for 6–12 h under N_2 atmosphere. After that time, the mixture was cooled to room temperature and poured into ice/water (50 g). The suspension was extracted with ethyl acetate (150 mL × 3). The ethyl acetate layer was washed with saturated $NaHCO_3$, dried through anhydrous Na_2SO_4. Ethyl acetate was removed in vacuum to get intermediate **19a**. Compounds **19b ~ c** were prepared according to the same procedure as compound **19a**.

*(E)-N-isopropyl-2-styrylthieno[3,2-d]pyrimidin-4-amine***(20a)**. Compound **19a** (121 mg, 0.41 mmol) and propan-2-amine (26.5 mg, 0.45 mmol) were added to isopropanol (16 mL). The solution was stirred and refluxed for 12–15 h. After that time, the isopropanol was removed under vacuum. The residue was purified by silica gel column, eluting with EtOAc/PE (15/85 v/v) to give compound **20a**. Compounds **20b ~ s** were prepared using the same procedure.

Title compound **20a** was isolated as a white solid in 81.1% yield, mp: 188–189 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.95 (d, J = 15.9 Hz, 1H), 7.67 (d, J = 5.3 Hz, 1H), 7.63 (d, J = 7.6 Hz, 2H), 7.42–7.36 (m, 3H), 7.30 (t, J = 7.3 Hz, 1H), 7.21 (d, J = 15.9 Hz, 1H), 4.73 (d, J = 7.1 Hz, 1H), 4.70–4.62 (m, 1H), 1.38 (d, J = 6.4 Hz, 6H).^{13}C NMR (101 MHz, $CDCl_3$) δ 161.53, 160.81, 156.46, 136.81, 136.48, 130.65, 128.91, 128.81 (2C), 128.59, 127.58 (2C), 125.57, 113.27, 43.12, 23.25 (2C). HR-MS (ESI): calcd for $C_{17}H_{18}N_3S$ $[M + H]^+$, 296.1216; found 296.1191.

(E)-N-methyl-2-styrylthieno[3,2-d]pyrimidin-4-amine **(20b)**. White solid, yield: 69.3%, mp: 159–160 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.99 (d, J = 15.9 Hz, 1H), 7.67 (d, J = 5.3 Hz, 1H), 7.63 (d, J = 7.4 Hz, 2H), 7.41 (d, J = 5.3 Hz, 1H), 7.38 (t, J = 7.6 Hz, 2H), 7.31 (t, J = 7.3 Hz, 1H), 7.22 (d, J = 15.9 Hz, 1H), 3.29 (d, J = 4.9 Hz, 3H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 161.36, 160.41, 157.54, 136.58, 136.46, 130.67, 128.67 (2C), 128.57, 128.48, 127.43 (2C), 125.27, 113.41, 28.01. HR-MS (ESI): calcd for $C_{15}H_{14}N_3S$ $[M + H]^+$, 268.0903; found 268.0904.

(E)-N,N-dimethyl-2-styrylthieno[3,2-d]pyrimidin-4-amine **(20c)**. White solid, yield: 70.1%, mp: 139–140 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.93 (d, J = 15.9 Hz, 1H), 7.70 (d, J = 5.5 Hz, 1H), 7.62 (d, J = 7.7 Hz, 2H), 7.42–7.35 (m, 3H), 7.30 (t, J = 7.3 Hz, 1H), 7.18 (d, J = 15.9 Hz, 1H), 3.48 (s, 6H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 161.56, 160.47, 158.33, 136.66, 136.09, 131.53, 128.71, 128.65 (2C), 128.39, 127.39 (2C), 124.90, 112.83, 38.86. HR-MS (ESI): calcd for $C_{16}H_{16}N_3S$ $[M + H]^+$, 282.1059; found 282.1064.

(E)-N-butyl-2-styrylthieno[3,2-d]pyrimidin-4-amine (**20d**). White solid, yield: 72.5%, mp: 116–117 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.96 (d, J = 15.9 Hz, 1H), 7.67 (d, J = 5.3 Hz, 1H), 7.63 (d, J = 7.5 Hz, 2H), 7.41 (d, J = 5.3 Hz, 1H), 7.38 (t, J = 7.6 Hz, 2H), 7.30 (t, J = 7.3 Hz, 1H), 7.21 (d, J = 15.9 Hz, 1H), 4.90 (s, 1H), 3.76 (dd, J = 13.0, 7.1 Hz, 2H), 1.76–1.71 (m, 2H), 1.54–1.46 (m, 2H), 1.02 (t, J = 7.4 Hz, 3H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 161.34, 160.57, 157.06, 136.61, 136.39, 130.61, 128.66 (2C), 128.64, 128.46, 127.42 (2C), 125.34, 113.15, 40.94, 31.86, 20.16, 13.88. HR-MS (ESI): calcd for $C_{18}H_{20}N_3S$ $[M+H]^+$, 310.1372; found 310.1373.

(E)-N-isobutyl-2-styrylthieno[3,2-d]pyrimidin-4-amine (**20e**). White solid, yield: 56.1%, mp: 131–132 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.95 (d, J = 15.9 Hz, 1H), 7.68 (d, J = 5.3 Hz, 1H), 7.63 (d, J = 7.5 Hz, 2H), 7.42 (d, J = 5.3 Hz, 1H), 7.38 (t, J = 7.6 Hz, 2H), 7.31 (t, J = 7.3 Hz, 1H), 7.20 (d, J = 15.9 Hz, 1H), 4.96 (s, 1H), 3.58 (t, J = 6.4 Hz, 2H), 2.06–2.04 (m, 1H), 1.06 (d, J = 6.7 Hz, 6H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 161.30, 160.62, 157.22, 136.59, 136.40, 130.63, 128.66 (2C), 128.61, 128.46, 127.43 (2C), 125.36, 113.10, 48.66, 28.69, 20.32 (2C). HR-MS (ESI): calcd for $C_{18}H_{20}N_3S$ $[M + H]^+$, 310.1372; found 310.1376.

*(E)-4-(2-styrylthieno[3,2-d]pyrimidin-*4-yl*)morpholine* (**20f**). White solid, yield: 52.3%, mp: 139–140 °C. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.55 (d, J = 5.5 Hz, 1H), 8.23 (d, J = 15.9 Hz, 1H), 7.75 (d, J = 7.0 Hz, 2H), 7.63 (d, J = 5.5 Hz, 1H), 7.53–7.45 (m, 3H), 7.28 (d, J = 15.9 Hz, 1H), 4.19 (t, J = 4.2 Hz, 4H), 3.86 (t, J = 4.8 Hz,4H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 156.93, 154.75, 150.11, 142.82, 139.01, 134.92, 131.09, 129.64 (2C), 128.68 (2C), 120.02, 119.08, 113.13, 66.27 (2C), 47.19 (2C). HR-MS (ESI): calcd for $C_{18}H_{18}N_3OS$ $[M + H]^+$, 324.1655; found 324.1170.

*(E)-4-(4-methylpiperazin-*1-yl*)-2-styrylthieno[3,2-d]pyrimidine* (**20g**). White solid, yield: 46.8%, mp: 139–140 °C. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.28 (d, J = 5.4 Hz, 1H), 7.92 (d, J = 15.9 Hz, 1H), 7.72 (d, J = 7.9 Hz, 2H), 7.50 (d, J = 5.4, 1H), 7.42 (t, J = 7.6 Hz, 2H), 7.35 (t, J = 7.3 Hz, 1H), 7.18 (d, J = 15.9 Hz, 1H), 3.35 (Brs, 8H), 2.74 (s, 3H). HR-MS (ESI): calcd for $C_{19}H_{21}N_4S$ $[M + H]^+$, 337.1481; found 337.1479.

(E)-N-phenyl-2-styrylthieno[3,2-d]pyrimidin-4-amine (**20h**). White solid, yield: 72.9%, mp: 142–144 °C. ^{1}H NMR (600 MHz, DMSO-d_6) δ 11.31 (s, 1H), 8.48 (d, J = 4.5 Hz, 1H), 8.04 (d, J = 15.8 Hz, 1H), 7.80 (d, J = 4.4 Hz, 2H), 7.69 (d, J = 7.0 Hz, 2H), 7.60 (d, J = 5.4 Hz, 1H), 7.46–7.54 (m, 5H), 7.35 (d, J = 15.8 Hz, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 156.06, 155.69, 149.53, 142.72, 140.10, 137.42, 134.69, 131.15, 129.70, 129.36, 128.66, 126.51, 124.26, 120.80, 118.88, 115.65. HR-MS (ESI): calcd for $C_{20}H_{16}N_3S$ $[M + H]^+$, 330.1059; found 330.1062.

(E)-N-methyl-N-phenyl-2-styrylthieno[3,2-d]pyrimidin-4-amine (**20i**). White solid, yield: 46.8%, mp: 179–180 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.32 (d, J = 15.8 Hz, 1H), 7.87 (d, J = 5.5 Hz, 1H), 7.76–7.73 (m, 2H), 7.65–7.78 (m, 2H), 7.63–7.58 (m, 3H), 7.46–7.41 (m, 5H), 3.84 (s, 3H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 157.13, 155.60, 148.97, 143.86, 141.35, 137.65, 134.42, 130.69, 130.48, 130.45 (2C), 128.96, 128.93 (2C), 128.76 (2C), 119.07, 119.01 (2C), 114.26, 40.70. HR-MS (ESI): calcd for $C_{21}H_{18}N_3S$ $[M + H]^+$, 344.1216; found 344.1220.

(E)-N-(4-bromophenyl)-2-styrylthieno[3,2-d]pyrimidin-4-amine (**20j**). White solid, yield: 79.2%, mp: 170–172 °C. ^{1}H NMR (600 MHz, DMSO-d_6) δ 11.17 (s, 1H), 8.48 (d, J = 5.3 Hz, 1H), 8.01 (d, J = 15.9 Hz, 1H), 7.84 (d, J = 7.7 Hz, 2H), 7.71–7.74 (m, 4H), 7.60 (d, J = 5.4 Hz,

1H), 7.46−7.51 (m, 3H), 7.32 (d, J = 15.9 Hz, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 161.78, 160.47, 154.99, 139.46, 136.48, 136.40, 135.00, 131.76 (2C), 129.27 (2C), 129.22, 128.97, 127.90 (2C), 124.86, 123.86 (2C), 115.29, 114.57. HR-MS (ESI): calcd for $C_{20}H_{15}BrN_3S$ $[M + H]^+$, 408.0165; found 408.0169.

(E)-N-(3-bromophenyl)-2-styrylthieno[3,2-d]pyrimidin-4-amine (**20k**). White solid, yield: 59.4%, mp: 138−140 °C. ^{1}H NMR (600 MHz, DMSO-d_6) δ 11.14 (s, 1H, NH), 8.48 (d, J = 5.4 Hz, 1H), 8.35 (s, 1H), 8.01 (d, J = 15.8 Hz, 1H), 7.79 (s, 1H), 7.71 (d, J = 7.7 Hz, 2H), 7.59 (d, J = 5.4 Hz, 1H), 7.46−7.52 (m, 5H), 7.33 (d, J = 15.8 Hz, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 161.86, 160.35, 154.81, 141.81, 136.60, 136.35, 135.14, 130.83, 129.38 (2C), 129.30, 128.85, 127.81 (2C), 125.77, 124.88, 124.23, 121.81, 120.23, 114.71. HR-MS (ESI): calcd for $C_{20}H_{15}BrN_3S$ $[M + H]^+$, 408.0165; found 408.0177.

(E)-N-(4-methoxyphenyl)-2-styrylthieno[3,2-d]pyrimidin-4-amine (**20l**). White solid, yield: 72.1%, mp: 133.8−135 °C. ^{1}H NMR (600 MHz, DMSO-d_6) δ 11.21 (s, 1H), 8.43(s, 1H), 8.05 (d, J = 15.7 Hz, 1H), 7.74−7.60(m, 4H), 7.57 (d, J = 4.5 Hz, 1H), 7.47−7.50 (m, 3H), 7.32 (d, J = 15.8 Hz, 1H), 7.09 (d, J = 8.6 Hz, 2H), 3.82 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 161.26, 160.48, 156.54, 155.79, 136.46, 134.66, 132.44, 129.27 (3C), 129.149 (2C), 128.98, 127.80 (2C), 125.04, 124.56, 114.21 (2C), 113.85, 55.61. HR-MS (ESI): calcd for $C_{21}H_{18}N_3OS$ $[M + H]^+$, 360.1165; found 360.1166.

(E)-2-styryl-N-(4-(trifluoromethyl)phenyl)thieno[3,2-d]pyrimidin-4-amine (**20m**). White solid, yield: 47.1%, mp: 127−128 °C. ^{1}H NMR (600 MHz, DMSO-d_6) δ 11.04 (s, 1H, NH), 8.48 (d, J = 5.4 Hz, 1H), 8.17 (d, J = 8.4 Hz, 2H), 8.01 (d, J = 15.9 Hz, 1H), 7.88 (d, J = 8.6 Hz, 2H), 7.76 (d, J = 7.3 Hz, 2H), 7.60 (d, J = 5.4 Hz, 1H), 7.44−7.50 (m, 3H), 7.33 (d, J = 15.9 Hz, 1H). HR-MS (ESI): calcd for $C_{21}H_{16}F_3N_3S$ $[M + H]^+$, 398.0914; found 398.0917.

(E)-N-(4-fluorobenzyl)-2-styrylthieno[3,2-d]pyrimidin-4-amine (**20n**). White solid, yield: 63.8%, mp: 158−160 °C. ^{1}H NMR (600 MHz, DMSO-d_6) δ 10.27 (s, 1H), 8.43 (d, J = 5.4 Hz, 1H), 8.21 (d, J = 15.8 Hz, 1H), 7.75 (d, J = 6.9 Hz, 2H), 7.56−7.46 (m, 6H), 7.27 (dd, J = 15.8, 2.0 Hz, 1H), 7.23−7.19 (m, 2H), 4.94 (d, J = 5.8 Hz, 2H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 162.92 (d, J = 243.11 Hz), 156.93, 155.67, 143.05, 139.01, 134.79, 134.74 (d, J = 3.02 Hz), 131.12, 130.47 (d, J = 7.55 Hz, 2C), 129.64 (3C), 128.69 (2C), 119.90, 118.80, 115.69 (d, J = 21.65 Hz, 2C), 114.80, 44.15. HR-MS (ESI): calcd for $C_{21}H_{17}FN_3S$ $[M + H]^+$, 362.1122; found 362.1127.

(E)-N-(4-bromobenzyl)-2-styrylthieno[3,2-d]pyrimidin-4-amine (**20o**). White solid, yield: 73.1%, mp: 125−126 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.93 (d, J = 15.9 Hz, 1H), 7.70 (d, J = 5.3 Hz, 1H), 7.61 (d, J = 7.5 Hz, 2H), 7.47−7.36 (m, 5H), 7.36−7.29 (m, 3H), 7.21 (d, J = 15.9 Hz, 1H), 5.24 (t, J = 4.4 Hz, 1H), 4.94 (d, J = 5.7 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 161.39, 160.90, 156.65, 137.23, 136.75, 136.52, 133.45, 131.00, 129.32 (2C), 128.92 (2C), 128.74 (2C), 128.62, 128.44, 127.47 (2C), 125.44, 113.30, 44.37. HR-MS (ESI): calcd for $C_{21}H_{17}BrN_3S$ $[M + H]^+$, 423.0321; found 423.0427.

(E)-N-(2-chlorobenzyl)-2-styrylthieno[3,2-d]pyrimidin-4-amine (**20p**). White solid, yield: 63.1%, mp: 171−172 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.97 (d, J = 15.9 Hz, 1H), 7.69 (d, J = 5.3 Hz, 1H), 7.63 (d, J = 7.5 Hz, 2H), 7.57 (dd, J = 5.6, 3.7 Hz, 1H), 7.43−7.37 (m, 4H), 7.32 (t, J = 7.3 Hz, 1H), 7.26−7.20 (m, 3H), 5.38 (s, 1H), 5.06 (d, J = 6.0 Hz, 2H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 161.30, 160.80, 156.59, 136.64, 136.56, 135.98, 133.70, 130.91, 130.23, 129.62, 128.95, 128.69 (2C), 128.53, 128.48, 127.44 (2C), 127.03, 125.34, 113.39, 42.76. HR-MS (ESI): calcd for $C_{21}H_{17}ClN_3S$ $[M + H]^+$, 378.0826; found 378.0824.

(E)-N-(3-methoxybenzyl)-2-styrylthieno[3,2-d]pyrimidin-4-amine (**20q**). White solid, yield: 57.2%, mp: 165−167 °C. ^{1}H NMR (600 MHz, DMSO-d_6) δ 10.17 (s, 1H), 8.42 (d, J = 5.4 Hz, 1H), 8.19 (d, J = 15.8 Hz, 1H), 7.75 (d, J = 7.0 Hz, 2H), 7.56−7.45 (m, 4H), 7.29 (t, J = 7.9 Hz, 1H), 7.24 (d, J = 15.8 Hz, 1H), 7.10 (s, 1H), 7.05 (d, J = 7.7 Hz, 1H), 6.86 (dd, J = 8.2, 2.4 Hz, 1H), 4.92 (d, J = 5.8 Hz, 2H), 3.73 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 160.17, 159.79, 159.22, 156.98, 141.93, 136.81, 136.44, 134.03, 129.77, 129.27 (2C), 129.20, 128.32, 127.80 (2C), 124.20, 120.32, 114.03, 113.90, 112.55, 55.27, 44.14. HR-MS (ESI): calcd for $C_{22}H_{20}N_3OS$ $[M + H]^+$, 374.1322; found 374.1306.

(E)-N-(4-fluorophenethyl)-2-styrylthieno[3,2-d]pyrimidin-4-amine (**20r**). White solid, yield: 58.9%, mp: 130−131 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.99 (d, J = 15.9 Hz, 1H), 7.67 (d, J = 5.3 Hz, 1H), 7.63 (d, J = 7.7 Hz, 2H), 7.45−7.37 (m, 3H), 7.32 (t, J = 7.3 Hz, 1H), 7.27−7.20 (m, 3H), 7.03 (t, J = 8.6 Hz, 2H), 4.91 (s, 1H), 3.98 (q, J = 6.7 Hz, 2H), 3.05 (t, J = 7.1 Hz, 2H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 161.41 (d, J = 244.62 Hz), 161.37, 160.63, 156.76, 136.62 (d, J = 1.51 Hz), 131.27 (d, J = 6.02 Hz, 2C), 130.68, 128.71 (3C), 128.52, 127.46 (2C), 125.37, 124.26, 124.24, 115.48 (d, J = 22.65 Hz, 2C), 113.39, 41.28, 29.54. HR-MS (ESI): calcd for $C_{22}H_{19}FN_3S$ $[M + H]^+$, 376.1278; found 376.1278.

(E)-N-(2-fluorophenethyl)-2-styrylthieno[3,2-d]pyrimidin-4-amine (**20s**). White solid, yield: 58.9%, mp: 131−132 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.01 (d, J = 15.9 Hz, 1H), 7.67 (d, J = 5.3 Hz, 1H), 7.64 (d, J = 7.3 Hz, 2H), 7.42−7.37 (m, 3H), 7.31 (t, J = 7.3 Hz, 1H), 7.28−7.19 (m, 3H), 7.12−7.05 (m, 2H), 5.00 (s, 1H), 4.01 (q, J = 6.8 Hz, 2H), 3.13 (t, J = 7.1 Hz, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 160.69, 161.36 (d, J = 243.41 Hz), 160.57, 156.93, 136.61, 135.80, 133.34, 131.82 (d, J = 5.05 Hz), 129.45, 129.37 (2C), 129.07, 128.77 (d, J = 8.08 Hz), 127.71 (2C), 126.66 (d, J = 16.16 Hz), 124.91, 124.85 (d, J = 3.03 Hz), 115.63 (d, J = 21.21 Hz), 113.62, 40.99, 29.09. HR-MS (ESI): calcd for $C_{22}H_{19}FN_3S$ $[M + H]^+$, 376.1278; found 376.1265.

Compound **19b** (105 mg, 0.4 mmol) and 25% methanamine solution (62 mg, 0.5 mmol) were added to isopropanol (20 mL). The suspension was stirred and refluxed for 13−15 h. While complete the reaction, isopropanol was removed under vacuum and the crude was purified by silica gel column, eluting with EtOAc/PE (15/85 v/v) to obtain

compound **21a**. Compounds **21b ~ i** were prepared using the same procedure.

(E)-2-(2-(furan-2-yl)vinyl)-N-methylthieno[3,2-d]pyrimidin-4-amine (**21a**). White solid, yield: 64.6%, mp: 216−217 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.77 (d, J = 15.7 Hz, 1H), 7.65 (d, J = 5.3 Hz, 1H), 7.46 (d, J = 1.3 Hz, 1H), 7.39 (d, J = 5.3 Hz, 1H), 7.11 (d, J = 15.7 Hz, 1H), 6.52 (d, J = 3.2 Hz, 1H), 6.45 (dd, J = 3.3, 1.8 Hz, 1H), 4.93 (s, 1H), 3.26 (d, J = 4.9 Hz, 3H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 161.80, 159.79, 158.25, 152.42, 144.53, 133.98, 126.76, 124.73, 123.51, 112.76, 112.54, 112.28, 38.89. HR-MS (ESI): calcd for $C_{13}H_{12}N_3OS$ $[M + H]^+$, 358.0754; found 358.0750.

(E)-N-cyclopropyl-2-(2-(furan-2-yl)vinyl)thieno[3,2-d]pyrimidin-4-amine (**21b**). White solid, yield: 61.7%, mp: 126−127 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.76 (d, J = 5.4 Hz, 1H), 7.70 (d, J = 15.7 Hz, 1H), 7.45 (s, 1H), 7.40 (d, J = 5.4 Hz, 1H), 7.06 (d, J = 15.7 Hz, 1H), 6.50 (d, J = 3.2 Hz, 1H), 6.44 (dd, J = 3.2, 1.8 Hz, 1H), 5.48 (s, 1H), 3.11−3.04 (m, 1H), 1.01−0.98 (m 2H), 0.81−0.78 (m, 2H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 157.02, 156.18, 154.31, 148.16, 138.39, 128.01, 121.77, 119.96, 119.08, 107.45, 106.99, 106.14, 19.45 (2C), 4.74. HR-MS (ESI): calcd for $C_{15}H_{14}N_3OS$ $[M + H]^+$, 284.0852; found 284.0854.

(E)-2-(2-(furan-2-yl)vinyl)-4-(4-methylpiperazin-1-yl)thieno[3,2-d]pyrimidine (**21c**). White solid, yield: 51.5%, mp: 129−130 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.70 (d, J = 1.4 Hz, 1H), 7.68 (d, J = 8.7 Hz, 1H), 7.45 (d, J = 1.3 Hz, 3H), 7.41 (d, J = 5.5 Hz, 3H), 7.07 (d, J = 15.7 Hz, 3H), 6.52 (d, J = 3.3 Hz, 3H), 6.45 (dd, J = 3.3, 1.8 Hz, 3H), 4.10−4.02 (m, 4H), 2.61−2.55 (m, 4H), 2.37 (s, 3H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 162.37, 160.36, 157.64, 152.88, 143.15, 131.26, 126.77, 125.22, 123.69, 112.44, 111.80, 110.86, 55.00 (2C), 46.11, 45.86 (2C). HR-MS (ESI): calcd for $C_{17}H_{19}N_4OS$ $[M + H]^+$, 327.1274; found 327.1280.

(E)-4-(2-(2-(furan-2-yl)vinyl)thieno[3,2-d]pyrimidin-4-yl)morpholine (**21d**).

White solid, yield: 51.5%, mp: 124−125 °C. ^{1}H NMR (600 MHz,

$CDCl_3$) δ 7.71 (d, *J* = 5.5 Hz, 1H), 7.69 (d, *J* = 15.7 Hz, 1H), 7.46 (d, *J* = 1.1 Hz, 1H), 7.42 (d, *J* = 5.5 Hz, 1H), 7.08 (d, *J* = 15.7 Hz, 1H), 6.52 (d, *J* = 3.2 Hz, 1H), 6.45 (dd, *J* = 3.2, 1.8 Hz, 1H), 4.04–4.02 (m, 4H), 3.89–3.87 (m, 4H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 162.50, 160.36, 157.90, 152.80, 143.22, 131.47, 126.60, 125.23, 123.81, 112.46, 111.84, 111.00, 66.81 (2C), 46.29 (2C). HR-MS (ESI): calcd for $C_{16}H_{16}N_3O_2S$ $[M + H]^+$, 314.0958; found 314.0953.

(E)-2-(2-(furan-2-yl*)vinyl)-N-(4-isopropylphenyl)thieno[3,2-d] pyrimidin-4-amine* (**21e**). White solid, yield: 66.2%, mp: 145–146 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.74 (d, *J* = 15.7 Hz, 1H), 7.68 (d, *J* = 5.4 Hz, 1H), 7.55 (d, *J* = 8.3 Hz, 2H), 7.47 (s, 1H), 7.40 (d, *J* = 5.3 Hz, 1H), 7.29 (d, *J* = 8.4 Hz, 2H), 7.13 (d, *J* = 15.7 Hz, 1H), 6.79 (s, 1H), 6.53 (d, *J* = 3.2 Hz, 1H), 6.46–6.44 (m, 1H), 3.01–2.94 (m, 1H), 1.31 (s, 3H), 1.30 (s, 3H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 157.25, 156.40, 150.96, 148.12, 141.73, 138.47, 130.51, 127.50, 122.19 (2C), 121.77, 120.21, 119.35, 119.33 (2C), 108.18, 107.04, 106.36, 28.95, 19.27 (2C). HR-MS (ESI): calcd for $C_{21}H_{20}N_3OS$ $[M + H]^+$, 362.1322; found 362.1316.

(E)-N-benzyl-2-(2-(furan-2-yl*)vinyl)thieno[3,2-d]pyrimidin-4-amine* (**21f**). White solid, yield: 67.5%, mp: 177–178 °C. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.37 (t, *J* = 5.8 Hz, 1H), 8.04 (d, *J* = 5.3 Hz, 1H), 7.71 (d, *J* = 1.0 Hz, 1H), 7.62 (d, *J* = 15.8 Hz, 1H), 7.42 (d, *J* = 7.4 Hz, 2H), 7.34 (d, *J* = 5.3 Hz, 1H), 7.31 (t, *J* = 7.6 Hz, 2H), 7.22 (t, *J* = 7.3 Hz, 1H), 6.84 (d, *J* = 15.8 Hz, 1H), 6.71 (d, *J* = 3.3 Hz, 1H), 6.55 (dd, *J* = 3.2, 1.8 Hz, 1H), 4.79 (d, *J* = 5.9 Hz, 2H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 160.10, 159.86, 156.29, 151.99, 143.75, 139.83, 132.72, 128.10 (2C), 127.43 (2C), 126.60, 126.58, 124.26, 122.88, 112.97, 112.07, 111.42, 43.38. HR-MS (ESI): calcd for $C_{19}H_{16}ON_3S$ $[M + H]^+$, 334.1009; found 334.1008.

(E)-N-(4-chlorobenzyl)-2-(2-(furan-2-yl*)vinyl)thieno[3,2-d]pyrimidin-4-amine* (**21g**). White solid, yield: 52.8%, mp: 141–142 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.71 (d, *J* = 15.7 Hz, 1H), 7.68 (d, *J* = 5.4 Hz, 1H), 7.46 (s, 1H), 7.41 (d, *J* = 5.3 Hz, 1H), 7.37 (d, *J* = 8.5 Hz, 2H), 7.33 (d, *J* = 8.4 Hz, 2H), 7.10 (d, *J* = 15.7 Hz, 1H), 6.51 (d, *J* = 3.2 Hz, 1H), 6.45 (dd, *J* = 3.2, 1.8 Hz, 1H), 5.17 (t, *J* = 5.5 Hz, 1H), 4.91 (d, *J* = 5.7 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 161.28, 160.95, 156.57, 152.86, 143.28, 137.26, 133.41, 130.91, 129.31 (2C), 128.89 (2C), 126.71, 125.44, 124.03, 113.16, 111.88, 111.12, 44.28. HR-MS (ESI): calcd for $C_{19}H_{15}ClN_3OS$ $[M + H]^+$, 368.0619; found 368.0622.

(E)-N-(4-fluorobenzyl)-2-(2-(furan-2-yl*)vinyl)thieno[3,2-d]pyrimidin-4-amine* (**21h**). White solid, yield: 61.7%, mp: 184–185 °C. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.40 (t, *J* = 5.8 Hz, 1H), 8.09 (d, *J* = 5.3 Hz, 1H), 7.76 (s, 1H), 7.63 (d, *J* = 15.8 Hz, 1H), 7.46 (dd, *J* = 8.3, 5.8 Hz, 2H), 7.37 (d, *J* = 5.3 Hz, 1H), 7.15 (t, *J* = 8.9 Hz, 2H), 6.83 (d, *J* = 15.8 Hz, 1H), 6.77 (d, *J* = 3.2 Hz, 1H), 6.59 (dd, *J* = 3.0, 1.8 Hz, 1H), 4.76 (d, *J* = 5.8 Hz, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 161.65 (d, *J* = 242.40 Hz), 160.71, 160.39, 156.75, 152.47, 144.57, 136.61 (d, *J* = 3.03 Hz), 133.64, 130.97 (d, *J* = 8.08 Hz, 2C), 126.98, 124.88, 123.54, 115.48 (d, *J* = 21.21 Hz, 2C), 113.51, 112.79, 112.32, 43.18. HR-MS (ESI): calcd for $C_{19}H_{15}FON_3S$ $[M + H]^+$, 352.0925; found 352.0919.

(E)-N-(2-fluorobenzyl)-2-(2-(furan-2-yl*)vinyl)thieno[3,2-d]pyrimidin-4-amine* (**21i**). White solid, yield: 52.9%, mp: 184–185 °C. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.38 (s, 1H), 8.08 (d, *J* = 3.5 Hz, 1H), 7.75 (s, 1H), 7.63 (d, *J* = 15.8 Hz, 1H), 7.46 (t, *J* = 6.4 Hz, 2H), 7.36 (d, *J* = 5.2 Hz, 1H), 7.15 (t, *J* = 8.2 Hz, 2H), 6.85 (d, *J* = 15.8 Hz, 1H), 6.76 (s, 1H), 6.58 (d, *J* = 1.2 Hz, 1H), 4.77 (d, *J* = 5.5 Hz, 2H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 161.60 (d, *J* = 241.60 Hz), 160.66, 160.34, 156.71, 152.43, 144.53, 136.56 (d, *J* = 3.02 Hz), 133.60, 129.93 (d, *J* = 9.06 Hz), 126.93, 124.84 (2C), 123.50 (2C), 115.44 (d, *J* = 21.14 Hz), 113.46, 112.75, 112.28, 43.14. HR-MS (ESI): calcd for $C_{19}H_{15}FN_3OS$ $[M + H]^+$, 352.0914; found 352.0916.

(E)-N-methyl-2-(3,4,5-trimethoxystyryl)thieno[3,2-d]pyrimidin-4-amine (**22a**). Compound **19c** (145 mg, 0.4 mmol) and 25% methanamine solution (62 mg, 0.5 mmol) were dissolved in isopropanol (16 mL) and refluxed for 13–15 h. After completion of the reaction, the solvent was removed, and the residue was purified by column chromatography (gradient elution of EtOAc/PE 15/85 v/v) to obtain compound **22a**. Compounds **22b ~ x** were obtained using the same procedure.

Title compound **22a** was isolated as a white solid in 77.2% yield, mp: 239–240 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.89 (d, *J* = 15.8, 1H), 7.67 (d, *J* = 5.2 Hz, 1H), 7.39 (d, *J* = 5.2, 1H), 7.13 (d, *J* = 15.8 Hz, 1H), 6.86 (d, *J* = 2.8 Hz, 2H), 5.01 (s, 1H), 3.90 (s, 6H), 3.87 (s, 3H), 3.26 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 161.33, 160.49, 157.59, 153.35 (2C), 138.61, 136.34, 132.28, 130.71, 128.13, 125.36, 113.37, 104.44 (2C), 60.98, 56.10 (2C), 28.09. HR-MS (ESI): calcd for $C_{18}H_{20}N_3O_3S$ $[M + H]^+$, 358.1220; found 358.1210.

(E)-N,N-dimethyl-2-(3,4,5-trimethoxystyryl)thieno[3,2-d]pyrimidin-4-amine (**22b**). White solid, yield: 73.6%, mp: 216–217 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.82 (d, *J* = 15.8 Hz, 1H), 7.69 (d, *J* = 5.5 Hz, 1H), 7.38 (d, *J* = 5.5 Hz, 1H), 7.08 (d, *J* = 15.8 Hz, 1H), 6.85 (s, 2H), 3.90 (s, 6H), 3.87 (s, 3H), 3.47 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 161.57, 160.42, 158.43, 153.34 (2C), 138.54, 135.97, 132.35, 131.63, 128.27, 124.93, 112.85, 104.39 (2C), 60.97, 56.09 (2C), 38.94 (2C). HR-MS (ESI): calcd for $C_{19}H_{22}N_3O_3S$ $[M + H]^+$, 372.1376; found 372.1381.

(E)-N-cyclopropyl-2-(3,4,5-trimethoxystyryl)thieno[3,2-d]pyrimidin-4-amine (**22c**). White solid, yield: 51.5%, mp: 184–185 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.83 (d, *J* = 15.8 Hz, 1H), 7.79 (d, *J* = 5.4 Hz, 1H), 7.41 (d, *J* = 5.3 Hz, 1H), 7.08 (d, *J* = 15.8 Hz, 1H), 6.85 (s, 2H), 5.81–5.70 (m, 1H), 3.89 (s, 6H), 3.87 (s, 3H), 3.11–3.06 (m, 1H), 1.01 (q, *J* = 6.4 Hz, 2H), 0.83–0.79 (m, 2H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 161.66, 160.80, 159.29, 153.48 (2C), 138.93, 136.70, 133.46, 132.22, 127.46, 124.69, 112.41, 104.69 (2C), 61.06, 56.22 (2C), 24.42, 9.78 (2C). HR-MS (ESI): calcd for $C_{20}H_{22}O_3N_3S$ $[M + H]^+$, 384.1376; found 384.1377.

(E)-4-(pyrrolidin-1-yl*)-2-(3,4,5-trimethoxystyryl)thieno[3,2-d] pyrimidine* (**22d**). White solid, yield: 70.2%, mp: 114–115 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.85 (d, *J* = 15.7 Hz, 1H), 7.71 (d, *J* = 5.4 Hz, 1H), 7.41 (d, *J* = 5.4 Hz, 1H), 7.10 (d, *J* = 15.8 Hz, 1H), 6.86 (s, 2H), 3.98 (s, 4H), 3.91 (s, 6H), 3.88 (s, 3H), 2.09 (s, 4H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 160.52, 160.31, 156.28, 153.32 (2C), 138.70, 136.15, 132.30, 131.66, 127.93, 124.59, 113.50, 104.57 (2C), 60.90, 56.10 (2C), 47.77 (2C), 25.39 (2C). HR-MS (ESI): calcd for $C_{21}H_{24}O_3N_3S$ $[M + H]^+$, 398.1533; found 398.1534.

(E)-4-(4-methylpiperazin-1-yl*)-2-(3,4,5-trimethoxystyryl)thieno [3,2-d]pyrimidine* (**22e**). White solid, yield: 55.7%, mp: 145–146 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.79 (d, *J* = 15.8 Hz, 1H), 7.69 (d, *J* = 5.5 Hz, 1H), 7.39 (d, *J* = 5.5 Hz, 1H), 7.07 (d, *J* = 15.8 Hz, 1H), 6.84 (s, 2H), 4.09–4.04 (m, 4H), 3.88 (s, 6H), 3.85 (s, 3H), 2.60–2.55 (m, 4H), 2.35 (s, 3H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 162.31, 160.37, 157.72, 153.32 (2C), 138.65, 136.15, 132.19, 131.40, 128.02, 125.18, 112.54, 104.44 (2C), 60.93, 56.09 (2C), 54.97 (2C), 46.03, 45.84 (2C). HR-MS (ESI): calcd for $C_{22}H_{27}N_4O_3S$ $[M + H]^+$, 427.1798; found 427.1797.

(E)-4-(4-ethylpiperazin-1-yl*)-2-(3,4,5-trimethoxystyryl)thieno [3,2-d]pyrimidine* (**22f**). White solid, yield: 52.1%, mp: 155–156 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.81 (d, *J* = 15.8 Hz, 1H), 7.70 (d, *J* = 5.5 Hz, 1H), 7.40 (d, *J* = 5.5 Hz, 1H), 7.09 (d, *J* = 15.7 Hz, 1H), 6.86 (s, 2H), 4.12–4.08 (m, 4H), 3.90 (s, 6H), 3.88 (s, 3H), 2.67–2.62 (m, 4H), 2.50 (q, *J* = 7.2 Hz, 2H), 1.15 (t, *J* = 7.2 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 162.34, 160.41, 157.71, 153.36 (2C), 138.61, 136.12, 132.25, 131.40, 128.14, 125.23, 112.51, 104.41 (2C), 60.98, 56.10 (2C), 52.86, 52.37 (2C), 45.97 (2C), 11.98. HR-MS (ESI): calcd for $C_{23}H_{29}O_3N_4S$ $[M + H]^+$, 441.1955; found 441.1954.

(E)-4-(2-(3,4,5-trimethoxystyryl)thieno[3,2-d]pyrimidin-4-yl*) morpholine* (**22g**). White solid, yield: 66.7%, mp: 179–180 °C. ^{1}H NMR (600 MHz, DMSO-d_6) δ 7.80 (d, *J* = 15.8 Hz, 1H), 7.71 (d, *J* = 5.4 Hz, 1H), 7.41 (d, *J* = 5.5 Hz, 1H), 7.09 (d, *J* = 15.8 Hz, 1H), 6.85 (s, 2H), 4.06–4.03 (m, 4H), 3.91–3.86 (m, 13H). ^{13}C NMR (101 MHz,

DMSO-d_6) δ 162.73, 160.22, 158.00, 153.57 (2C), 138.57, 136.49, 134.19, 132.15, 128.25, 124.97, 112.21, 105.21 (2C), 66.51 (2C), 60.54, 56.42 (2C), 46.27 (2C). HR-MS (ESI): calcd for $C_{21}H_{24}N_3O_4S$ [M + H]$^+$, 414.1482; found 414.1480.

(E)-N-(p-tolyl)-2-(3,4,5-trimethoxystyryl)thieno[3,2-d]pyrimidin-4-amine (**22h**). White solid, yield: 71.4%, mp: 137−138 °C. ^{1}H NMR (600 MHz, DMSO-d_6) δ 10.49 (s, 1H), 8.34 (d, J = 4.9 Hz, 1H), 7.89 (d, J = 15.8 Hz, 1H), 7.68 (d, J = 7.1 Hz, 2H), 7.49 (d, J = 5.3 Hz, 1H), 7.30 (d, J = 8.0 Hz, 2H), 7.19 (d, J = 15.8 Hz, 1H), 7.02 (s, 2H), 3.87 (s, 6H), 3.71 (s, 3H), 2.36 (s, 3H). HR-MS (ESI): calcd for $C_{24}H_{24}O_3N_3S$ [M + H]$^+$, 434.1542; found 434.1536.

(E)-N-(4-isopropylphenyl)-2-(3,4,5-trimethoxystyryl)thieno[3,2-d]pyrimidin-4-amine (**22i**). White solid, yield: 67.6%, mp: 214−215 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.88 (d, J = 15.8 Hz, 1H), 7.70 (d, J = 5.4 Hz, 1H), 7.53 (d, J = 8.3 Hz, 2H), 7.40 (d, J = 5.4 Hz, 1H), 7.30 (d, J = 8.3 Hz, 2H), 7.15 (d, J = 15.8 Hz, 1H), 6.87 (s, 2H), 6.85 (s, 1H), 3.91 (s, 6H), 3.89 (s, 3H), 1.31 (s, 3H), 1.30 (s, 3H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 157.29, 156.39, 151.20, 148.61 (2C), 142.10, 134.14, 131.93 (2C), 130.34, 127.83, 127.38, 122.96, 122.24 (2C), 120.09, 119.82, 108.19, 99.92 (2C), 56.16 (2C), 51.37, 28.97, 19.27 (2C). HR-MS (ESI): calcd for $C_{26}H_{28}O_3N_3S$ [M + H]$^+$, 462.1846; found 462.1845.

(E)-N-(4-methoxyphenyl)-2-(3,4,5-trimethoxystyryl)thieno[3,2-d]pyrimidin-4-amine (**22j**). White solid, yield: 59.3%, mp: 106−107 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.86 (d, J = 15.8 Hz, 1H), 7.65 (d, J = 5.4 Hz, 1H), 7.47−7.43 (m, 2H), 7.37 (d, J = 5.4 Hz, 1H), 7.13 (d, J = 15.8 Hz, 1H), 7.03 (s, 1H), 6.96 (d, J = 8.7 Hz, 2H), 6.85 (s, 2H), 3.90 (s, 6H), 3.88 (s, 3H), 3.86 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 162.02, 160.93, 158.60, 156.79, 153.37 (2C), 138.77, 136.71, 133.41, 132.10, 129.74, 128.14, 127.48, 124.48 (2C), 114.28 (2C), 112.47, 104.52 (2C), 60.99, 56.12 (2C), 55.58. HR-MS (ESI): calcd for $C_{24}H_{24}O_4N_3S$ [M + H]$^+$, 450.1482; found 450.1483.

(E)-N-(4-(trifluoromethyl)phenyl)-2-(3,4,5-trimethoxystyryl)thieno[3,2-d]pyrimidin-4-amine (**22k**). White solid, yield: 47.2%, mp: 145−146 °C. ^{1}H NMR (600 MHz, DMSO-d_6) δ 11.49 (s, 1H), 8.49 (d, J = 5.3 Hz, 1H), 8.19 (d, J = 8.4 Hz, 2H), 8.00 (d, J = 15.9 Hz, 1H), 7.85 (d, J = 8.6 Hz, 2H), 7.62 (d, J = 5.4 Hz, 1H), 7.34 (d, J = 15.9 Hz, 1H), 7.03 (s, 2H), 3.85 (s, 6H), 3.72 (s, 3H). HR-MS (ESI): calcd for $C_{24}H_{21}F_3O_3N_3S$ [M + H]$^+$, 488.1257; found 488.1251.

(E)-4-((2-(3,4,5-trimethoxystyryl)thieno[3,2-d]pyrimidin-4-yl)amino)phenol (**22l**). White solid, yield: 53.7%, mp: 154−155 °C. ^{1}H NMR (600 MHz, DMSO-d_6) δ 10.63 (s, 1H, OH), 9.72 (s, 1H, NH), 8.32 (s, 1H, ArH), 7.93 (d, J = 15.6 Hz, 1H, =CH), 7.47 (d, J = 5.0 Hz, 3H, ArH), 7.18 (d, J = 15.8 Hz, 1H, =CH), 7.01 (s, 2H, ArH), 6.90 (d, J = 8.6 Hz, 2H, ArH), 3.87 (s, 6H, CH_3), 3.72 (s, 3H, CH_3). HR-MS (ESI): calcd for $C_{23}H_{22}O_4N_3S$ [M + H]$^+$, 436.1334; found 436.1328.

(E)-N-methyl-N-phenyl-2-(3,4,5-trimethoxystyryl)thieno[3,2-d]pyrimidin-4-amine (**22m**). White solid, yield: 50.7%, mp: 187−188 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.94 (d, J = 15.8 Hz, 1H), 7.51−7.46 (m, 4H), 7.41−7.38 (m, 2H), 7.27 (d, J = 5.4 Hz, 1H), 7.18 (d, J = 15.8 Hz, 1H), 6.90 (s, 2H), 3.93 (s, 6H), 3.89 (s, 3H), 3.74 (s, 3H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 161.80, 160.74, 157.44, 153.54 (2C), 143.89, 138.88, 136.31, 133.29, 132.50, 129.78, 129.68 (2C), 128.65, 128.43, 124.17 (2C), 114.20 (2C), 104.71, 61.11, 56.29 (2C), 39.66. HR-MS (ESI): calcd for $C_{24}H_{24}N_3O_3S$ [M + H]$^+$, 434.1533; found 434.1527.

(E)-N-benzyl-2-(3,4,5-trimethoxystyryl)thieno[3,2-d]pyrimidin-4-amine (**22n**). White solid, yield: 68.3%, mp: 140−141 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.88 (d, J = 15.7 Hz, 1H), 7.70 (d, J = 5.3 Hz, 1H), 7.46 (d, J = 8.4 Hz, 2H), 7.43 (s, 1H), 7.39 (t, J = 7.5 Hz, 2H), 7.33 (t, J = 7.4 Hz, 1H), 7.14 (d, J = 15.7 Hz, 1H), 6.87 (s, 2H), 5.16 (s, 1H), 4.99 (d, J = 5.5 Hz, 2H), 3.91 (s, 6H), 3.88 (s, 3H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 161.31, 160.79, 156.75, 153.35 (2C), 138.75, 138.60, 136.50, 132.20, 130.86, 128.79 (2C), 127.98 (2C), 127.69, 125.35, 113.21, 104.55 (2C), 60.91, 56.09 (2C), 45.11. HR-MS (ESI): calcd for $C_{24}H_{24}O_3N_3S$ [M + H]$^+$, 434.1533; found 434.1531.

(E)-N-(4-methylbenzyl)-2-(3,4,5-trimethoxystyryl)thieno[3,2-d]pyrimidin-4-amine (**22o**). White solid, yield: 68.1%, mp: 179−180 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.89 (d, J = 15.8 Hz, 1H), 7.69 (d, J = 5.3 Hz, 1H), 7.41 (d, J = 5.3 Hz, 1H), 7.35 (d, J = 7.9 Hz, 2H), 7.20 (d, J = 7.8 Hz, 2H), 7.14 (d, J = 15.8 Hz, 1H), 6.87 (s, 2H), 5.12 (t, J = 5.2 Hz, 1H), 4.94 (d, J = 5.5 Hz, 2H), 3.91 (s, 6H), 3.88 (s, 3H), 2.37 (s, 3H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 161.47, 160.93, 156.90, 153.50 (2C), 138.87, 137.63, 136.59, 135.68, 132.39, 130.98, 129.62 (2C), 128.22, 128.18 (2C), 125.50, 113.39, 104.68 (2C), 61.09, 56.25 (2C), 45.05, 21.27. HR-MS (ESI): calcd for $C_{25}H_{26}O_3N_3S$ [M + H]$^+$, 448.1689; found 448.1690.

(E)-N-(4-methoxybenzyl)-2-(3,4,5-trimethoxystyryl)thieno[3,2-d]pyrimidin-4-amine (**22p**). White solid, yield: 51.5%, mp: 138−139 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.89 (d, J = 15.8 Hz, 1H), 7.67 (dd, J = 5.3, 2.3 Hz, 1H), 7.41−7.39 (m, 1H), 7.37 (d, J = 8.4 Hz, 2H), 7.14 (d, J = 15.8 Hz, 1H), 6.92−6.88 (m, 2H), 6.86 (s, 2H), 5.19 (d, J = 4.7 Hz, 1H), 4.90 (d, J = 5.3 Hz, 2H), 3.90 (d, J = 1.9 Hz, 6H), 3.88 (s, 3H), 3.80 (d, J = 2.3 Hz, 3H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 161.26, 160.71, 159.20, 156.65, 153.31 (2C), 138.67, 136.40, 132.21, 130.86, 130.53, 129.41 (2C), 128.05, 125.33, 114.17 (2C), 113.23, 104.38 (2C), 60.96, 56.07 (2C), 55.33, 44.59. HR-MS (ESI): calcd for $C_{25}H_{26}N_3O_4S$ [M + H]$^+$, 464.1649; found 464.1643.

(E)-N-(4-chlorobenzyl)-2-(3,4,5-trimethoxystyryl)thieno[3,2-d]pyrimidin-4-amine (**22q**). White solid, yield: 71.5%, mp: 136−137 °C.^{1}H NMR (600 MHz, DMSO-d_6) δ 10.28 (s, 1H), 8.42 (d, J = 5.3 Hz, 1H), 8.09 (d, J = 15.7 Hz, 1H), 7.54 (d, J = 5.2 Hz, 1H), 7.52 (d, J = 8.2 Hz, 2H), 7.43 (d, J = 8.2 Hz, 2H), 7.22 (dd, J = 15.7, 2.6 Hz, 1H), 7.04 (s, 2H), 4.96 (d, J = 5.5 Hz, 3H), 3.87 (s, 6H), 3.73 (s, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 157.02, 155.87, 153.67 (2C), 147.82, 143.28, 140.27, 139.05, 137.60, 132.40, 130.44, 130.31 (2C), 128.93 (2C), 119.33, 118.87, 114.67, 106.21 (2C), 60.68, 56.50 (2C), 44.17. HR-MS (ESI): calcd for $C_{24}H_{23}ClO_3N_3S$ [M + H]$^+$, 468.1145; found 468.1139.

(E)-N-(2-chlorobenzyl)-2-(3,4,5-trimethoxystyryl)thieno[3,2-d]pyrimidin-4-amine (**22r**). White solid, yield: 51.5%, mp: 167−168 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.87 (d, J = 15.8 Hz, 1H), 7.69 (d, J = 5.3 Hz, 1H), 7.56−7.52 (m, 1H), 7.43−7.41 (m, 1H), 7.40 (s, 1H), 7.25−7.22 (m, 2H), 7.12 (d, J = 15.8 Hz, 1H), 6.86 (s, 2H), 5.39 (t, J = 5.7 Hz, 1H), 5.07 (d, J = 5.9 Hz, 2H), 3.91 (s, 6H), 3.88 (s, 3H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 161.19, 160.76, 156.61, 153.33 (2C), 138.67, 136.53, 135.99, 133.65, 132.19, 131.00, 130.07, 129.61, 128.94, 127.92, 127.05, 125.28, 113.34, 104.46 (2C), 60.95, 56.09 (2C), 42.69. HR-MS (ESI): calcd for $C_{24}H_{23}ClN_3O_3S$ [M + H]$^+$, 468.1134; found 468.1148.

(E)-N-(furan-2-ylmethyl)-2-(3,4,5-trimethoxystyryl)thieno[3,2-d]pyrimidin-4-amine (**22s**). White solid, yield: 63.4%, mp: 172−173 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.90 (d, J = 15.8 Hz, 1H), 7.68 (d, J = 5.3 Hz, 1H), 7.43−7.37 (m, 2H), 7.14 (d, J = 15.7 Hz, 1H), 6.87 (s, 2H), 6.40−6.32 (m, 2H), 5.26 (s, 1H), 4.97 (d, J = 5.4 Hz, 2H), 3.90 (s, 6H), 3.88 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 161.20, 160.85, 156.41, 153.38 (2C), 151.55, 142.39, 138.68, 136.51, 132.23, 131.03, 128.04, 125.34, 113.44, 110.61, 107.91, 104.46 (2C), 60.99, 56.11 (2C), 37.98. HR-MS (ESI): calcd for $C_{22}H_{22}N_3O_4S$ [M + H]$^+$, 424.1326; found 424.1337.

(E)-N-(pyridin-4-ylmethyl)-2-(3,4,5-trimethoxystyryl)thieno[3,2-d]pyrimidin-4-amine (**22t**). White solid, yield: 55.2%, mp: 191−192 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.54 (d, J = 4.8 Hz, 2H), 7.72 (d, J = 15.8 Hz, 1H), 7.70 (s, 1H), 7.41 (d, J = 5.2 Hz, 1H), 7.33 (d, J = 4.8 Hz, 2H), 7.08 (d, J = 15.8 Hz, 1H), 6.79 (s, 2H), 5.84 (t, J = 5.2 Hz, 1H), 4.97 (d, J = 5.6 Hz, 2H), 3.87 (s, 6H), 3.86 (s, 3H). HR-MS (ESI): calcd for $C_{23}H_{23}O_3N_4S$ [M + H]$^+$, 435.1485; found 435.1484.

(E)-4-(benzyloxy)-2-(3,4,5-trimethoxystyryl)thieno[3,2-d]pyrimidine (**22u**). Benzyl alcohol (86.4 mg, 0.8 mmol) and anhydrous K_2CO_3 (132 mg, 0.96 mmol) were added to anhydrous DMF (5 mL)

under N_2 atmosphere. The mixture was stirred for 0.5 h and then added compound **19c** (271 mg, 0.75 mmol). The mixture was stirred at 80 °C for 8 h. After completion of the reaction, EtOAc were added and washed with saturated brine solution. The combined organic layer was dried over anhydrous Na_2SO_4, and concentrated in vacuum to afford residue. The residue was further recrystallized in ethanol to give compound **22u** as a white solid in 57.4% yield, mp: 127–128 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.93 (d, J = 15.8 Hz, 1H), 7.83 (d, J = 5.4 Hz, 1H), 7.56 (d, J = 6.6 Hz, 2H), 7.47–7.36 (m, 4H), 7.19 (d, J = 15.8 Hz, 1H), 6.89 (s, 2H), 5.75 (s, 2H), 3.93 (s, 6H), 3.90 (s, 3H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 163.54, 162.83, 161.17, 153.44 (2C), 138.94, 137.29, 136.26, 134.17, 131.93, 128.63 (2C), 128.35, 128.33 (2C), 127.31, 124.46, 115.61, 104.55 (2C), 68.22, 61.01, 56.14 (2C). HR-MS (ESI): calcd for $C_{24}H_{23}N_2O_4S$ $[M + H]^+$, 435.6218; found 435.6221.

(E)-N-isopropyl-2-(3,4,5-trimethoxystyryl)thieno[3,2-d]pyrimidin-4-amine (**22v**). Light yellow solid, yield: 81.7%, mp: 208–209 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.06 (d, J = 5.3 Hz, 1H), 7.78 (d, J = 15.8 Hz, 1H), 7.58 (d, J = 7.8 Hz, 1H), 7.33 (d, J = 5.3 Hz, 1H), 7.12 (d, J = 15.9 Hz, 1H), 7.00 (s, 2H), 4.69–4.65 (m, 1H), 3.86 (s, 6H), 3.69 (s, 3H), 1.28 (d, J = 6.6 Hz, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 160.82, 160.51, 156.35, 153.55 (2C), 138.40, 135.77, 133.23, 132.32, 129.00, 124.76, 113.33, 105.02 (2C), 60.53, 56.38 (2C), 41.97, 22.85 (2C). HR-MS (ESI): calcd for $C_{20}H_{23}N_3O_3S$ $[M + H]^+$, 386.1533; found 386.1518.

(E)-N-phenethyl-2-(3,4,5-trimethoxystyryl)thieno[3,2-d]pyrimidin-4-amine (**22w**).

White solid, yield: 83.6%, mp: 170–172 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.08 (d, J = 5.4 Hz, 1H), 7.93 (t, J = 5.4 Hz, 1H), 7.83 (d, J = 15.8 Hz, 1H), 7.38–7.27 (m, 5H), 7.26–7.17 (m, 1H), 7.16 (d, J = 15.9 Hz, 1H), 7.00 (s, 2H), 3.86 (s, 6H), 3.84–3.76 (m, 2H), 3.70 (s, 3H), 3.01 (t, J = 7.6 Hz, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 160.86, 160.54, 156.96, 153.59 (2C), 140.15, 138.48, 135.95, 133.36, 132.28, 129.25 (2C), 128.95, 128.83 (2C), 126.60, 124.84, 113.45, 105.06 (2C), 60.55, 56.39 (2C), 42.44, 35.37. HR-MS (ESI): calcd for $C_{25}H_{25}N_3O_3S$ $[M + H]^+$, 448.1689; found 448.1690.

(E)-N-(4-fluorophenethyl)-2-(3,4,5-trimethoxystyryl)thieno [3,2-d]pyrimidin-4-

amine (**22x**). White solid, yield: 80.3%, mp: 179–180 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.08 (d, J = 5.4 Hz, 1H), 7.90 (t, J = 5.4 Hz, 1H), 7.81 (d, J = 15.8 Hz, 1H), 7.41–7.32 (m, 3H), 7.19–7.07 (m, 3H), 7.00 (s, 2H), 3.86 (s, 6H), 3.85–3.75 (m, 2H), 3.70 (s, 3H), 3.00 (t, J = 7.4 Hz, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 161.31 (d, J = 242.4 Hz), 160.85, 160.54, 156.98, 153.59 (2C), 138.50, 136.31 (d, J = 3.03 Hz), 135.95, 133.37, 132.28, 131.01 (d, J = 7.07 Hz, 2C), 128.93, 124.84, 115.74 (d, J = 21.21 Hz, 2C), 113.42, 105.09 (2C), 60.55, 56.39 (2C), 42.35, 34.46. HR-MS (ESI): calcd for $C_{25}H_{24}FN_3O_3S$ $[M + H]^+$, 446.1595; found 466.1596.

4.10. Cell culture

RAW 264.7 macrophages and LO2 cells were bought from BeNa Culture Collection (China). The cell culture medium DMEM (Hyclone, USA) contains 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin solution (100X, Beyotime Biotechnology, China). The cells were cultured in a humidified incubator containing 5% CO_2 at 37 °C.

4.11. Assay for NO production

Raw 264.7 cells in logarithmic growth phase were inoculated into 48 wells plate with the density of 7×10^4 cells per well and 300 μL medium, and incubated for 24 h. The cells were treated with compound for 1 h, and then stimulated with LPS (0.5 μg/mL) for 24 h. The cell supernatant was extracted and centrifuged. The 50 μL supernatant was mixed with equal volume of Griess reagent (Beyotime biotechnology, China). Nitrite production was determined by measuring the optical density at 540 nm, and the concentration of nitrite in cell supernatant was calculated using the standard curve of $NaNO_2$ production.

$$Inhibition\ rate = \frac{model\ group\ NO\ concentration - Compound\ group\ NO\ concentration}{model\ group\ NO\ concentration - control\ group\ NO\ concentration} * 100\%$$

4.12. Assay of iNOS activity

The iNOS enzyme activity assay has been described in the previous article [15].

4.13. Western blot assay [44,45]

RAW 264.7 cells (2×10^6 cells) in the logarithmic growth phase were cultured in a petri dish with a diameter of 6 cm and incubated for 24 h. The cells were treated with compound **22o** for 1 h, and then stimulated with LPS (0.5 μg/mL) for 24 h. The cells were lysed in 300–400 μL RIPA cell lysis buffer (Beyotime Biotechnology, China) containing PMSF, and incubated on ice for 30 min. After high-speed centrifugation, the cell supernatant was extracted. Cells lysed mixed with Native Gel Sample Loading Buffer (Beyotime Biotechnology, China), nest approximate proteins were run on 8% SDS-PAGE and then transferred to PVDF membrane (Millipore Immobilon-P, USA). The blotted membrane was incubated with iNOS antibody (Cell Signaling Technology, USA) overnight in a refrigerator at 4 °C, rinsed three times, and then incubated with the HRP-conjugated secondary antibody for 1 h. Finally, use chemiluminescence to analyze the results.

4.14. Induction and treatment of rat arthritis in vivo

Male SD rats, 160–180 g, were purchased from the Experimental Animal Center of Anhui Medical University. The rats kept a light-dark cycle for 12 h in a room controlled by humidity (30–60%) and temperature (23 ± 2 °C). After acclimatizing to the environment for one week, the rats were randomly divided into five groups (10 rats per group): Normal group, AIA (Adjuvant-Induced Arthritis), sinomenine (60 mg/kg), **22o** L group (40 mg/kg) and **22o** H group (80 mg/kg). A single intradermal injection of 0.1 mL of CFA (10 mg/mL, Chondrex, USA) on the right hind paw induced the AIA model. The Normal group was injected with the same volume of normal saline. On the 14th to 30th days after CFA induction, the weight change and paw swelling degree of the rats were measured every three days. Compound **22o** was dissolved in 0.5% carboxymethyl cellulose sodium (CMC-Na) solution. The compound concentration is set to 8, 16 mg/mL, respectively. Rats were administered intragastrically once a day. The concentration of the positive drug sinomenine is 12 mg/mL. The Normal group was given the same volume of 0.5% carboxymethyl cellulose sodium

(CMC-Na) solution. The animals were sacrificed on the 30th day after AIA induction. Take rat serum and use ELISA kit to detect the content of inflammatory factors in serum. Remove the knee joint, trim, and fix the ankle joint in 4% formaldehyde solution. Then decalcify in 10% EDTA. Then proceed to histopathological analysis.

4.15. Induction and treatment of rat arthritis in vivo

Take out the rat tissue block after decalcification, perform a series of dehydration, and then embed it in paraffin wax. The samples were serially sectioned at 4 μm and treated routinely for hematoxylin and eosin (H&E), safranin O (SO), and iNOS tains. Check histological changes under a microscope. For the SO staining process, the first step, the specimens were dewaxed and hydrated. Then the slides were washed and treated with Mayer's hematoxylin solution for 5 min (Beyotime Biotechnology, China). Then wash again for 20 min. The stained tissue was developed with 0.002% fast green solution (Solarbio, China) for 30 s, and finally washed with 1% glacial acetic acid. The slides were placed in 0.1% SO solution (Solarbio, China) for 6 min, and then fixed with mounting medium (Muto Pure Chemicals) for subsequent research and analysis.

For the iNOS immunohistochemistry staining process, the sample undergoes the same treatment in the early stage, and then the slide incubate for 10 min at 120–130 °C and a 10×10^{-3} M sodium citrate buffer solution. The sodium citrate buffer solution is made of anhydrous citric acid (Solarbio, China) and anhydrous trisodium citrate (Solarbio, China). The slides were blocked in PBS containing 10% BSA at 37 °C for 60 min, and then incubated with the primary antibody (anti-rat iNOS (1:100); Affinity Biosciences, China). Next, the specimen was treated with a secondary antibody for 2 h. Then wash the slides and fix them with ProLong Gold antifading reagent (Solarbio, China). Use 3DHISTECH's Slide Converter (Hungary 3DHISTECH) to obtain histological images.

4.16. Microsome stability

In hepatocytes metabolism test, pre-incubate the hepatocyte suspensions in the 37 °C CO_2 incubator for 20 min. Add 400 μL of positive control/test compound solution into wells on a 24-well plate. Start the reaction by adding 400 μL of hepatocytes (2×10^6 cells/mL) into wells on a 24-well plate. Gently shake the 24-well plate to mix the cell suspension. At the specified time points (0, 15, 30, 60, 90 and 120 min), remove 30 μL of the reaction mixture from the plate and place it in a 1.5 mL EP tube. Add 300 μL IS to the EP tube and vortex for 1 min. Ensure adequate mixing with reactants to terminate the reaction. Centrifuge the supernatant at 4000 rpm for 15 min at a low temperature of 4 °C. Transfer 100 μL of the supernatant to a 96-well plate for LC-MS/MS analysis. Use the following equation to calculate the microsome clearance rate: Following equations were applied to calculate the microsome clearance:

elimination rate constant (k) = -gradient;

Half life ($T_{1/2}$) (min) = 0.693/k.

$CL_{int(mic)}$ = 0.693/half-life/mg microsome protein per mL. Liver wt: 25.7, 30, 32, 40, 87.5, 30.8 and 16.7 g/kg for human, monkey, dog, rat, mouse, rabbit and mini-pig.

4.17. CYP enzymatic inhibitory activity assay

Compound **22o** was diluted to 8 different concentrations (0, 0.05, 0.15, 0.5, 1.5, 5.0, 15, 50 μM), and then combine with human liver microsomes (0.1 mg/mL) and cofactor NADPH (1 mM). Incubate. β-Naphthoflavone, Sulfaphenazole, Tranylcypromine, quinidine, and ketoconazole were used as positive controls. Phenacetin, diclofenac, Mephenytoin, dextromethorphan, and midazolam were mixed and were used as substrates of CYP1A2, 2C9, 2C19, 2D6, and 3A4, respectively. The mixture of test compound **22o** or positive control was preheated with human liver microsomes at 37 °C for 10 min, respectively. After adding a certain amount of substrate mixture and cofactor NADPH, the solution was incubated for another 10 min. The samples were then quenched with stop solution containing internal standard. The reaction was terminated with a 400 μL cold acetonitrile solution containing 200 ng/mL tolbutamide and 200 ng/mL labetalol. The sample solution was centrifuged at 4000 rpm for 20 min, and the supernatant was extracted. Dilute 200 μL of the supernatant with 100 μL of HPLC water and shake it for 10 min. Finally, the samples were analyzed by LC-MS/MS. IC_{50} are calculated using Graph Pad Prism.

4.18. In vivo PK study

Before the in *vivo* study, all rats were confirmed as healthy. Each animal has a corresponding number, and the number is nailed to the mouse's ear through ear studs. Compound **22o** was evaluated in Sprague-Dawley rats (6–7 weeks old, 230–250 g). The rats had been fasted overnight before taking the drug and returned to food 4 h after taking the compound **22o**. Give SD rats 1 mg/kg or 10 mg/kg by intravenous injection (i.v.) or oral administration (p.o.), respectively. For intravenous injection doses, the compounds were dissolved in 10% DMSO, 50% PEG400, and 40% water. For oral administration doses, the compounds were dissolved in 0.5% CMC. The time points of blood sampling for intravenous injection were 0.08, 0.25, 0.5, 1, 2, 4, 8 and 24 h after administration (n = 3). The time points of blood collection for oral administration were 0.25, 0.5, 1, 2, 4, 8 and 24 h after administration (n = 3). The 300 μL blood collected at each point and transferred to a new EP tube. The tube contains 10 μL of heparin as an anticoagulant and centrifuged at 4000 rpm for 10 min. Take out 50 μL of plasma and transfer to another EP tube, and then add 200 μL of methanol containing a predetermined internal standard (IS) to the plasma to precipitate plasma proteins. After the mixture was vortexed for 1 min, the mixture was centrifuged at 12,000 g for 10 min, and the supernatant was extracted. Finally, the supernatant was analyzed by LC-MS/MS.

4.19. Ethics approval and consent to participate

The study was approved by the Ethics Committee of Anhui Medical University, and all animals received humane care under the guidance of the National Institutes of health(USA).

4.20. Statistical analysis

All data are expressed as mean ± SEM, and were analyzed by SPSS software. Student's *t*-test and one-way ANOVA test were used for statistical analyses of differences groups. P values were <0.05 or <0.01 or <0.001were considered statistically significant.

Declaration of competing interest

All authors have contributed to the work, have read the manuscript and have agreed to be listed as authors. The submitted manuscript has not been published elsewhere and nor is it currently under review by another publication. No potential conflict of interest relevant to this article..

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Abbreviations

AIA	adjuvant-induced arthritis
LPS	lipopolysaccharide
LO2	human normal liver cell
RAW264.7	Mouse peritoneal macrophages
IL-6	interleukin-6
TNF-α	tumor necrosis factor alpha
NO	nitric oxide
eNOS	endothelial nitric oxide synthase
nNOS	neuronal nitric oxide synthase
iNOS	inducible nitric oxide synthase
IL-1β	interleukin-1β
IR	inhibitory rate
IC_{50}	half maximal inhibitory concentration
ELISA	enzyme linked immunosorbent assay
SARs	structure-activity relationships
CYP	cytochrome P450
HE	hematoxylin-eosin
EDTA	Ethylene Diamine Tetraacetic Acid
SO	safranine O\
SD	Sprague-Dawley
IFA	Imcomplete Freund's Adjuvant
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide
^{1}H NMR	proton nuclear magnetic resonance
^{13}C NMR	carbon nuclear magnetic resonance
HR-MS	high-resolution electron impact mass spectra
DMF	*N,N*-dimethylformamide
THF	tetrahydrofuran
MeOH	methanol
EtOAc	ethyl acetate
HCl	hydrochloric acid
HPLC	high-performance liquid chromatography
DMSO	dimethyl sulfoxide
Pd/C	palladium/carbon
DCM	dichloromethane
DMA	dimethyl adipate
TBTU	tributylthiourea
DIPEA	*N,N*-diisopropylethylamine
$POCl_3$	phosphorus oxychloride
TEA	trithylamine
EDCI	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejmech.2021.113174.

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