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Development of Second-Generation Small-Molecule RhoA Inhibitors with Enhanced Water Solubility, Tissue Potency, and Significant in vivo Efficacy

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RhoA, a member of the Rho GTPases, is involved in a variety of cellular functions and could be a suitable therapeutic target for the treatment of cardiovascular diseases. However, few small-molecule RhoA inhibitors have been reported. Based on our previously reported lead compounds, 32 new 2-substituted quinoline (or quinoxaline) derivatives were synthesized and tested in biological assays. Six compounds showed high RhoA inhibitory activities, with IC_{50} values of 1.17–1.84 μM. Among these, (*E*)-3-(3-(ethyl(quinolin-2-yl)amino)phenyl)acrylic acid (**26b**) and (*E*)-3-(3-(butyl(quinolin-2-yl)amino)phenyl)acrylic acid (**26d**) demonstrated noticeable vasorelaxation effects against phenylephrine-induced contraction in thoracic aorta artery rings, and compound **26b** had good water solubility and showed significant in vivo efficacy, which was similar to that of 5-(1,4-diazepane-1-sulfonyl)isoquinoline (fasudil) in a subarachnoid hemorrhage–cardiovascular model. To the best of our knowledge, compound **26b** is the first example of a small-molecule RhoA inhibitor with potent in vivo efficacy, which could serve as a good lead for designing cardiovascular agents.

Introduction

RhoA, as one of the most well-characterized members of the Rho GTPases, plays important roles in multiple cellular processes, including cytoskeletal rearrangement, gene expression, and membrane trafficking, as well as cell adhesion, migration, differentiation, proliferation, and apoptosis.[1] Like all members of the GTPases, RhoA acts as a molecular switch, cycling between the active GTP-bound form and the inactive GDP-bound form.[2] Active GTP-bound RhoA interacts with downstream effector proteins, including the most well-characterized Rho-associated kinases (ROCK), to regular cellular functions.[3]

The RhoA/ROCK pathway is the main regulator in the pathogenesis of sustained smooth muscle cell contraction and vasospasm.[4,5] Activation of RhoA/ROCK inhibits myosin light chain phosphatase (MLCP), followed by direct phosphorylation of MLC and facilitation of contraction.[5] Thus, inhibition of the RhoA pathway has been considered an appealing target for pharmacological treatment of numerous cardiovascular (CVS) disorders.[6,7] Moreover, RhoA has been reported as a potential target for the treatment of cancer,[8] nervous system diseases,[9] and asthma.[10]

Subarachnoid hemorrhage (SAH), or bleeding into the subarachnoid space, is a devastating cerebrovascular disease that threatens the health of people throughout the world. Approximately 85% cases of spontaneous SAH are due to the rupture of an intracranial aneurysm, although it can also be caused by trauma.[11] Despite recent improvements in knowledge about SAH pathophysiology and angioplasty, the molecular mechanism of SAH is still unclear. New evidence has revealed that early brain injury (EBI), cortical spreading depolarization (CSD), and impaired microcirculatory function could be important players in SAH-induced injury.[12] However, cerebral vasospasm remains one of the primary poor outcomes after SAH.

As a ROCK inhibitor, fasudil (5-(1,4-diazepane-1-sulfonyl)isoquinoline) has been marketed in Japan for some time for the treatment of cerebral vasospasms occurring after SAH.[13] Fasudil significantly decreased vasospasm not only via an inhibition of smooth muscle contraction but also through up-regulation of endothelial nitric oxide synthase (eNOS), which was proven to be a safe and useful drug for patients with ruptured cerebral aneurysms.[14] In addition, some evidence suggests that statins could be ROCK inhibitors[15] and that some statins (e.g.,

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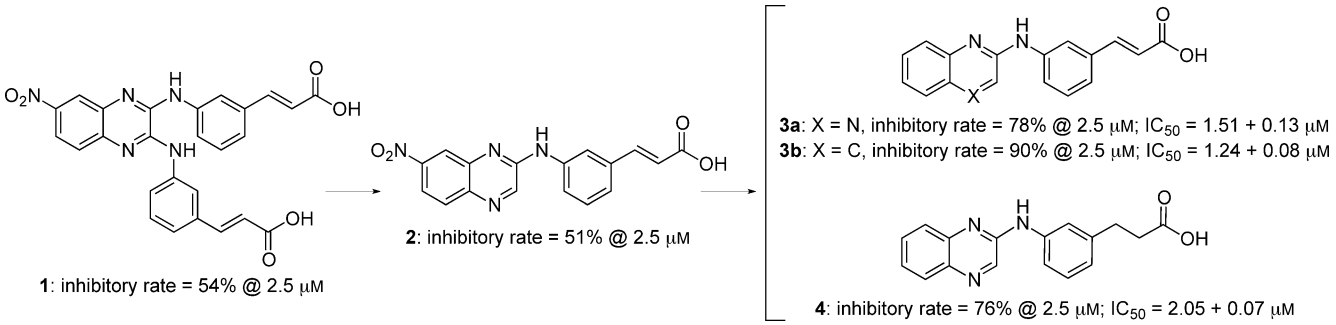


Figure 1. Discovery of first-generation small-molecule RhoA inhibitors.

pitavastatin and simvastatin) may decrease the risk of symptomatic vasospasm caused by SAH, especially in animal models, but no robust effect on clinical outcomes has been demonstrated.[16] These evidence support the idea that RhoA/ROCK inhibitors could be potential therapeutics for cerebral vasospasms after SAH. However, very limited efforts have been devoted toward the development of direct RhoA inhibitors.[17–24]

Toward this end, we previously reported the first-generation small-molecule RhoA inhibitors through structure-based virtual screening in conjunction with chemical synthesis and bioassays.[22] Starting from lead compound **1** (Figure 1), it was found that compound **2**, with a simplified structure (one amino substituent) retained the RhoA inhibitory activity with an inhibitory rate similar to that of lead compound **1**. After further structural modification, three compounds (**3a**,**b** and **4**) were found to have high RhoA inhibitory activities, with IC_{50} values of 1.24–2.05 μM. Compounds **3a**,**b** also had noticeable vasorelaxation effects against phenylephrine (PE)-induced contraction in thoracic aorta artery rings and therefore served as leads for the second-generation small-molecule RhoA inhibitors in this study.

In total, 32 new compounds were designed, synthesized, and tested with biological assays. Six compounds (**26b**, **26d**, **26i–j**, **26l**, and **26q**) were found to have high RhoA inhibitory activities, and two compounds (**26b** and **26d**) showed significant inhibitory effects against PE-induced contraction in the thoracic aorta artery, approximately twofold more potent than that of the first-generation small-molecule RhoA inhibitors discovered previously. Among these, compound **26b** showed good water solubility and promising therapeutic effects, similar to that of fasudil, in in vivo animal models.

Results and Discussion

Design of second-generation inhibitors

Through structure-based virtual screening and chemical modification, compounds **3a**,**b** and **4** were previously reported as first-generation small-molecule RhoA inhibitors from our group. These compounds were used as leads for designing second-generation small-molecule RhoA inhibitors. In this study, to further improve the inhibitory activity and physicochemical properties of the lead compounds, chemical modifications were performed, and two sets of analogues were prepared. Firstly, with the help of molecular modeling in the previous study, we obtained two-dimensional (Figure 2A) and three-dimensional (Figure 2B) interaction schemes of docked poses of **3b** in the GNP binding site of RhoA. We found that the aniline nitrogen atom was completely exposed to solvent. Please refer to ref. [22] for details regarding molecular docking. Thus, we designed and synthesized 22 analogues with various substitutions at the aniline nitrogen atom (compounds **26a–r**, Table 1; compounds **27a–d**, Table 2) to estimate if the substitution on the aniline nitrogen atom would affect physicochemical properties and inhibitory activity. Secondly, we replaced the functional group acid on the side chain with the bioisosteres and designed 10 analogues (**28a–b**, **29a–b**, **30a–b**, **31a–b**, and **32a–b**; Table 3).

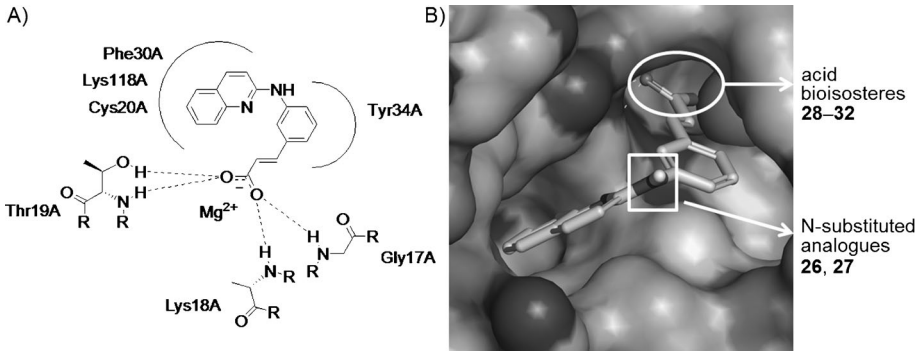
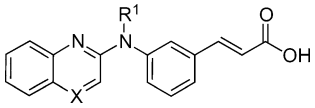
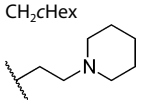
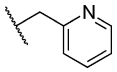


Figure 2. Predicted binding pose of **3b** in the GNP binding site of RhoA.[22]

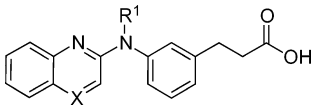
Table 1. Structure and RhoA inhibitory activity of compounds **3a**, **3b**, and **26a–r**.



Compd	X	R^1	Inhibition [%][a]
3a	N	H	78
3b	CH	H	90
26a	CH	Me	52
26b	CH	Et	86
26c	CH	*n*Pr	63
26d	CH	*n*Bu	92
26e	CH	*i*Pr	48
26f	CH	CH_2cPr	73
26g	CH	CH_2cPent	79
26h	CH	CH_2cHex	70
26i	CH		89
26j	CH	Bz	85
26k	CH		51
26l	CH	$(CH_2)_2OH$	90
26m	CH	$(CH_2)_3OH$	77
26n	CH	$(CH_2)_2OTBDMS$[b]	59
26o	CH	CH_2COOH	66
26p	CH	CH_2CONH_2	65
26q	N	Et	81
26r	N	CH_2COOH	58

[a] RhoA inhibition was determined by cell-based assay at 2.5 μM; data represent a single determination. [b] TBDMS = *tert*-butyldimethylsilyl.

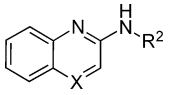
Table 2. Structure and RhoA inhibitory activity of compounds **4** and **27a–d**.

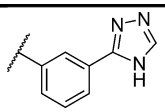
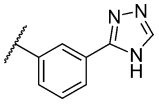
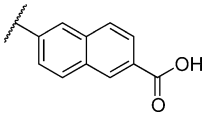
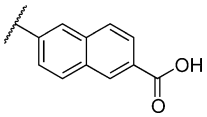
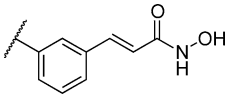
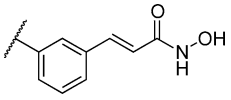
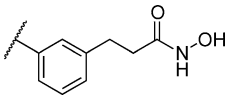
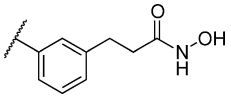
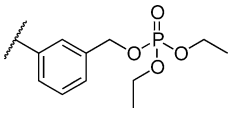
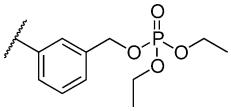


Compd	X	R^1	Inhibition [%][a]
4	N	H	76
27a	CH	Et	62
27b	CH	CH_2COOH	69
27c	N	Et	73
27d	N	CH_2COOH	62

[a] RhoA inhibition was determined at 2.5 μM; data represent a single determination.

Table 3. Structure and RhoA inhibitory activity of compounds **28a–b**, **29a–b**, **30a–b**, **31a–b**, and **32a–b**.



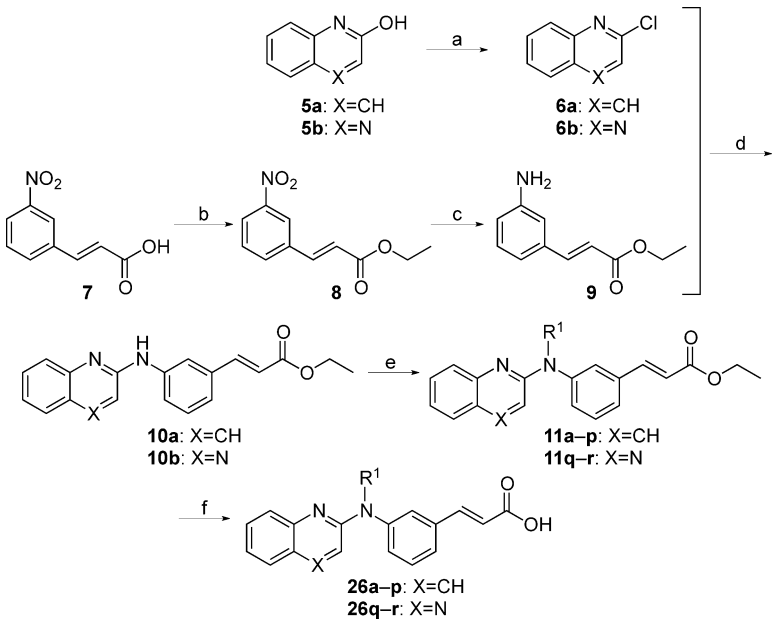
Compd	X	R^2	Inhibition [%][a]
28a	CH		42
28b	N		57
29a	CH		71
29b	N		76
30a	CH		71
30b	N		67
31a	CH		77
31b	N		65
32a	CH		55
32b	N		49

[a] RhoA inhibition was determined at 2.5 μM; data represent a single determination.

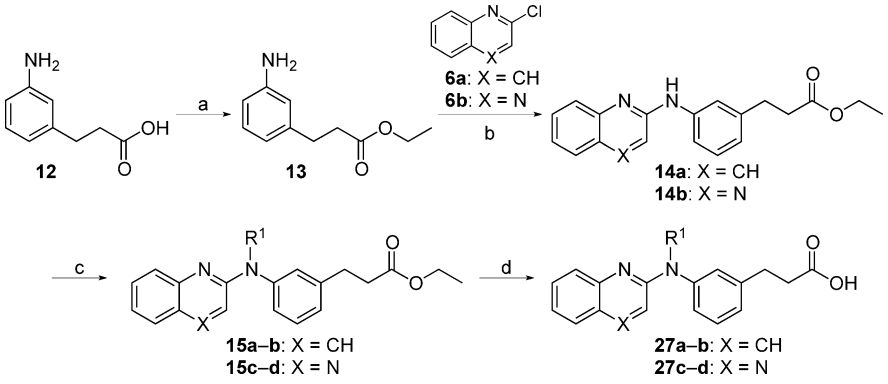
Synthesis

Scheme 1 depicts the synthetic route for the preparation of compounds **26a–r**. 2-Quinoxalinol **5a** or 2-quinoxalinone **5b** were chlorinated with phenyl phosphonic dichloride (BPOD) to yield the corresponding aryl chlorides, **6a** or **6b**. Esterification of 3-nitrocinnamic acid **7**, followed by reduction with $SnCl_2$ in ethanol, gave aniline **9**. Coupling of **6a–b** and aniline **9** afforded compounds **10a–b**, which were substituted with different haloalkanes, giving the esters of compounds **26a–r**. Hydrolysis of the esters with LiOH provided carboxylic acid products. Compounds **27a–d** were synthesized using a similar process as for compounds **26a–r** (Scheme 2).

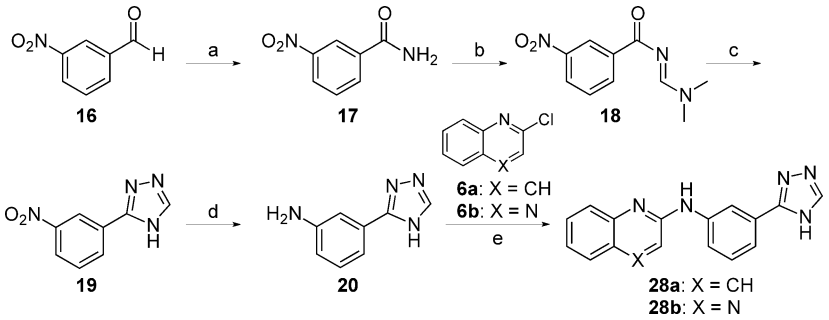
Compounds **28a–b** were synthesized using the approach outlined in Scheme 3. 3-Nitrobenzaldehyde **16** was treated with hydroxylamine hydrochloride using $Pd(OAc)_2$ as a catalyst in DMSO/H_2O to give amide **17**. Stirring at reflux with *N*,*N*-dimethylformamide dimethyl acetal (DMF-DMA) gave compound **18**. Cyclization of compound **18** with hydrazine hydrate afforded compound **19**, followed by reduction with $SnCl_2$ to give the desired aniline, **20**. Compound **20** was then coupled with **6a–b** in DMF to give products **28a–b**.



Scheme 1. *Reagents and conditions*: a) BPOD, reflux, 12 h, 81–85%; b) H_2SO_4, EtOH, reflux, 12 h, 81%; c) $SnCl_2$, EtOH, reflux, 12 h, 98%; d) DMF, reflux, 12 h, 51–81%; e) NaH, DMF, RT, 12 h; f) $LiOH{\cdot}H_2O$, MeOH, 40 °C, 16 h, 10–95% (over steps e and f).



Scheme 2. *Reagents and conditions*: a) H_2SO_4, EtOH, reflux, 12 h, 85%; b) DMF, reflux, 12 h, 58–62%; c) NaH, DMF, RT, 12 h; d) LiOH, MeOH, RT, 12 h, 4–71% (over steps c and d).



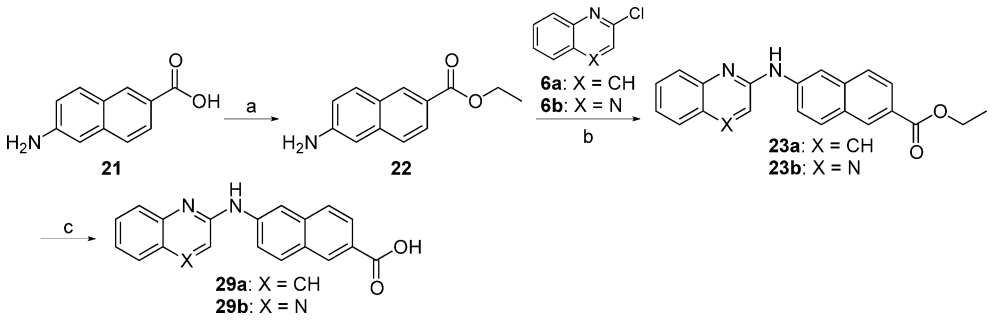
Scheme 3. *Reagents and conditions*: a) $NH_2OH{\cdot}HCl$, Cs_2CO_3, $Pd(OAc)_2$, DMSO, H_2O, reflux, 12 h, 57%; b) DMF-DMA, reflux, 5 h, 79%; c) AcOH, $N_2H_4{\cdot}H_2O$, reflux, 12 h, 36%; d) $SnCl_2$, EtOH, reflux, 12 h, 96%; e) DMF, reflux, 12 h, 49–59%.

Esterification of 6-amino-2-naphthoic acid **21**, followed by coupling with **6a**–**b** in DMF, gave esters **23a**–**b**, which were hydrolyzed using LiOH at room temperature to give carboxylic acid products **29a**–**b** (Scheme 4). Treatment of **10a**–**b** and **14a**–**b** with hydroxylamine and sodium methoxide in MeOH at 0 °C gave products **30a**–**b** and **31a**–**b** (Scheme 5). The coupling reaction of **6a**–**b** and **24** gave compounds **25a**–**b**, which were converted into phosphate products **32a**–**b** using diethyl chlorophosphate, triethylamine (TEA), and 4-dimethylaminopyridine (DMAP) in THF at room temperature (Scheme 6).

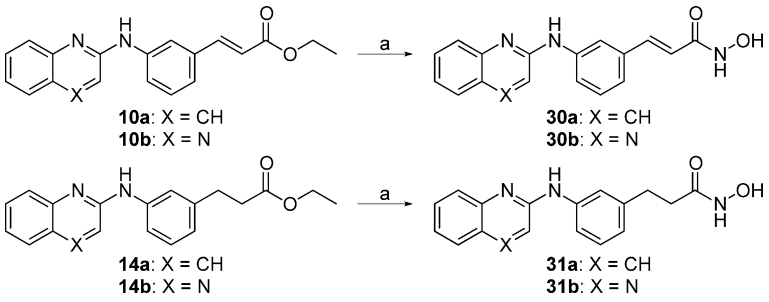
In total, 32 compounds (**26a**–**r**, **27a**–**d**, **28a**–**b**, **29a**–**b**, **30a**–**b**, **31a**–**b**, and **32a**–**b**) were designed and synthesized; their chemical structures are shown in Tables 1–3. These compounds were synthesized through the routes outlined in Schemes 1–6, and the details for synthetic procedures and structural characterizations are described in the Experimental Section. All compounds were confirmed to be ≥95% pure (Supporting Information Table 1S).

Water solubility

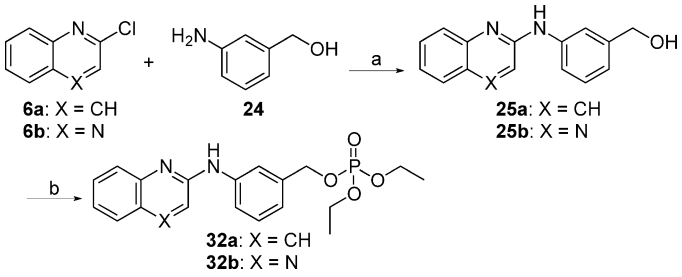
Attention was given to the low water solubility of compound **3a**. The crystal packing caused by the molecular planarity of the quinoxaline and aniline in compound **3a** was deemed likely to cause the low solubility of 43 μg mL^{-1}.[27] Therefore, we speculated that the solubility would probably be improved by introducing different substituents at the aniline nitrogen. The hypothesis was supported by melting point changes indicative of solubility changes. Introducing an ethyl substituent at the aniline nitrogen gave a dramatic decrease in the melting point from >300 °C (compound **3b**) to



Scheme 4. *Reagents and conditions*: a) H_2SO_4, EtOH, reflux, 12 h, 88%; b) DMF, reflux, 12 h, 58–74%; c) $LiOH{\cdot}H_2O$, MeOH, RT, 12 h, 74–84%.



Scheme 5. *Reagents and conditions*: a) MeONa, NH_2OH, MeOH, H_2O, 0 °C, 1 h, 34–98%.



Scheme 6. *Reagents and conditions*: a) DMF, reflux, 12 h, 58–64%; b) diethylchlorophosphate, TEA, DMAP, THF, RT, 16 h, 33–78%.

83–87 °C (compound **26b**). The solubility increased almost 14-fold, from 43 $\mu g\,mL^{-1}$ for **3a** to 639 $\mu g\,mL^{-1}$ for **26b**, by substitution at the aniline nitrogen (Table 4). The lower solubility of compound **26d** than that of **26b** may be due to the more lipophilic butyl group. In addition, introduction of the polar groups to the molecules would lower their log*P* values, which also could lead to improved solubility (compound **26i**). Compound **26i** could also be a salt form for both the acidic group (acid) and basic groups (tertiary amine) in the structure, which could explain its good water solubility.

Table 4. Isometric contraction measurements and water solubility of selected compounds.

Compd	IC_{50} [μM][a]	Solubility [$\mu g\,mL^{-1}$]
3a	123.2 ± 8.9	43
26b	70.9 ± 1.3	639
26d	73.4 ± 2.6	92
26i	26[b]	701
26j	20[b]	_[c]
26l	0[b]	_[c]
26q	20[b]	_[c]
fasudil	3	_[c]

[a] Data represent the mean ± SD of three independent experiments. [b] Percent inhibition at 150 μM. [c] Not determined.

Biological activities

Inhibition of RhoA

For the primary assay, the inhibitory activities of synthetic compounds against RhoA were evaluated by using the inhibitory rate at a single concentration. Using three first-generation small-molecule RhoA inhibitors (**3a–b** and **4**) developed in our group as positive controls, the percent inhibitions of compounds **26a–r**, **27a–d**, **28a–b**, **29a–b**, **30a–b**, **31a–b**, and **32a–b** were measured at 2.5 μM. The results are summarized in Tables 1–3. Of the synthetic derivatives tested, six compounds (**26b**, **26d**, **26i–j**, **26l**, and **26q**) displayed high inhibitory activities against RhoA (expressed as an inhibitory rate at 2.5 μM ≥ 80%) with half maximal inhibitory concentration (IC_{50}) values of 1.17 to 1.84 μM (Table 4), calculated by fitting the dose–response curves using a logistic derivative equation in Origin 8.0. To make a direct comparison, the methods used in this study were the same as previously reported.[22]

By analyzing the data in Tables 1–3 and Table 5, we found that all six good candidate inhibitors were from Table 1, and the inhibitory activities were comparable with those of the first-generation small-molecule RhoA inhibitors. The inhibitory activity of the most potent compound, **26d** (IC_{50} = 1.17 ± 0.19 μM, Table 5), was slightly increased over that of lead compound **3b** (IC_{50} = 1.24 ± 0.08 μM, Table 5), demonstrating that a substituent on the aniline nitrogen could be tolerated for cellular potency.

Table 5. RhoA inhibitory activity of selected compounds.

Compd	Inhibition [%][a]	IC_{50} [μM][b]
3a	78	1.51 ± 0.13
3b	90	1.24 ± 0.08
4	76	2.05 ± 0.07
26b	86	1.66 ± 0.28
26d	92	1.17 ± 0.19
26i	89	1.26 ± 0.13
26j	85	1.84 ± 0.24
26l	90	1.32 ± 0.17
26q	81	1.81 ± 0.19

[a] RhoA inhibition was determined at 2.5 μM; data represent a single determination. [b] Data represent the mean ± SD of three independent experiments.

Effect against PE-induced contraction in thoracic aorta artery rings

To test the vasorelaxation effects of the inhibitors against PE-induced contraction in thoracic aorta artery rings, lead compound **3a** and six other compounds displaying good inhibitory activities against RhoA (**26b**, **26d**, **26i–j**, **26l**, and **26q**) were evaluated. In all vessels submaximally contracted with 100 nM PE, compounds **3a**, **26b**, and **26d** (0–150 μM) strongly inhibited tension development in a dose-dependent fashion

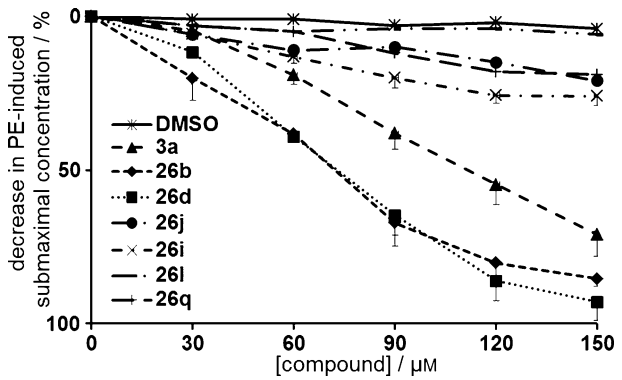


Figure 3. Effects of increasing concentrations of **3a**, **26b**, **26d**, **26j**, **26i**, **26l**, **26q**, and DMSO on PE-induced submaximal contraction in isolated arterial rings. Values are the means ± SD ($n = 3$).

(Figure 3), with maximal inhibitory rates of 71.0 ± 7.1%, 85.4 ± 2.4%, and 92.9 ± 6.1%, respectively (150 μM, $n = 3$, Figure 3), while compounds **26i–j**, **26l**, and **26q** showed weak vasorelaxation effects. The IC_{50} values of compounds **26b** and **26d** were 70.9 ± 1.3 μM and 73.4 ± 2.6 μM, respectively, which were approximately twofold more potent than that of the lead compound **3a** ($IC_{50} = 123.2 \pm 8.9$ μM, Table 4). These encouraging results may be the result of the better solubility of compounds **26b** and **26d**. The inconsistency between the water solubility and vasorelaxation effects of compound **26i** was complicated, and needs to be further investigated (Table 4). It indicated that chemical modification strategy focusing on water solubility in this study was efficient and valuable for improving pharmacological potencies.

General observation in SAH-CVS model

There were no significant differences in body weight and temperature among the experimental groups (data not shown). After induction of SAH, all animals stopped breathing for ~30 s. Fifteen rats died after blood injection, and the mortality in SAH animals was 15/46 (33%).

Morphometric vasospasm

Compound **26b**, the most potent and soluble compound, was selected to evaluate the relaxant effect on the cerebral vasospasm rat SAH-CVS model. The measured luminal cross-sectional perimeters of basilar arteries (BAs) in each group are shown in Table 6. Microscopic observation of the BA vasospasm of control rats and rats subjected to SAH with different treatments was shown in Figure 4. Moderate arterial narrowing and reduction of the intima in the SAH group and vehicle group were observed (Figure 4B,C). The inner perimeter of BAs in the above two groups became smaller than in the control group (386.25 ± 11.25 μm and 390.93 ± 20.60 μm vs. 472.50 ± 53.47 μm, $p < 0.05$). Compared with SAH and vehicle groups, the difference between the inner perimeter of the BAs in the fasudil group (468.83 ± 43.53 μm), the **26b** medium dose group (464.00 ± 50.46 μm) and the **26b** high dose group (475.60 ± 63.79 μm) was statistically significant ($p < 0.05$), but the slight increase observed in the **26b** low dose group (406.25 ± 16.97 μm) was not statistically significant ($p > 0.05$), indicating that in certain concentration ranges, **26b** showed relaxant effects in a dose-dependent fashion. Furthermore, even at a medium dose (6.25 equiv of fasudil), **26b** showed similar relaxant effects on cerebral vasospasm to fasudil ($p > 0.05$). To the best of our knowledge, compound **26b** is the first example of small-molecule RhoA inhibitor with significant in vivo efficacy, which could serve as a good lead for designing more potent therapeutics for vasospasms after SAH.

Table 6. Therapeutic effect of compound **26b** in the cerebral vasospasm rat SAH-CVS model.

Group	Dose [mM]	*N*	IP [μm][a]
control	–	6	472.50 ± 53.47
SAH	–	6	386.25 ± 11.25
SAH + vehicle	–	4	390.93 ± 20.60
fasudil	15.2 (1 equiv)	6	468.83 ± 43.53
26b (low dose)	23.7 (1.56 equiv)	4	406.25 ± 16.97
26b (medium dose)	95.0 (6.25 equiv)	6	464.00 ± 50.46
26b (high dose)	380 (25 equiv)	5	475.60 ± 63.79

[a] The inner perimeter (IP) of the basilar arteries was measured (see Experimental Section for details); data represent the mean ± SD of two independent experiments.

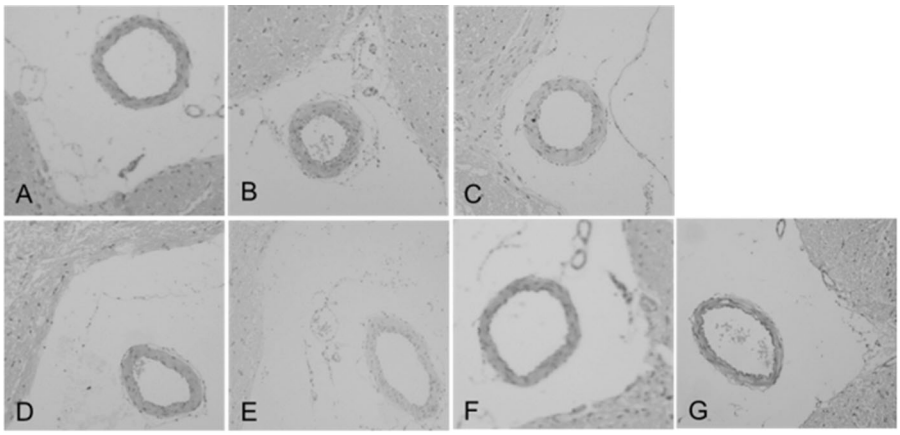


Figure 4. Microscopic observation of the BA vasospasm of control rats (A) and rats subjected to SAH with different treatments. Moderate vasospasm could be detected in the SAH group (B) and SAH + vehicle group (C), showing luminal narrowing, increased wall thickness, and corrugation of the tunica intima. The inner perimeter of BAs was significantly increased in the SAH + medium dose of **26b** group (E), the SAH + high dose of **26b** group (F) and SAH + fasudil group (G), but slightly increased in the low dose of **26b** group (D) (100× magnification).

Structure–activity relationships (SAR)

SAR analysis of a set of 32 compounds provided important insight into the essential structural requirements for effective RhoA inhibition. An analysis of the data shown in Tables 1–3 revealed some preliminary SARs for compounds **26a–r**, **27a–d**, **28a–b**, **29a–b**, **30a–b**, **31a–b**, and **32a–b**: 1) various substituents (including steric, electronic, and hydrophobic groups) on the aniline nitrogen had a limited influence on the inhibitory activity of compounds (Table 1, **3b** vs. **26a–r**); 2) substituents on the aniline nitrogen could significantly improve physicochemical properties of compounds (e.g., solubility) by breaking molecular planarity (Table 5, **3a** vs. **26b**); 3) the further exploration of an amino substituent on the quinoxaline/quinoline ring-like "acid bioisosteres" did not result in any improvements (Table 3) and carboxylic acid was still the best amino substituent for good activity (Table 3, **3b** vs. **28a** or **30a** or **32a**); and 4) between the quinoxaline (**3a**) and quinoline (**3b**) frameworks, the quinoline ring was optimal for improving biological activities (**26b** vs. **26q**).

Conclusions

We proceeded with research on RhoA inhibitors and discovered second-generation small-molecule RhoA inhibitors using chemical synthesis and bioassays. Based on the structures of lead compounds **3a–b** and **4**, chemical modifications were performed. In total, 32 new compounds were synthesized and tested with biological assays. Finally, six compounds (**26b**, **26d**, **26i–j**, **26l**, and **26q**) were found to show high RhoA inhibitory activities, and two compounds (**26b** and **26d**) showed significant inhibitory effects against PE-induced contraction in thoracic aorta artery rings. Compound **26b** showed promising therapeutic effects which were similar to those of fasudil in an SAH-CVS model. On the basis of in vitro and in vivo biological results, compound **26b** could serve as a good lead for designing more potent cardiovascular drug. The results in this study provide an experimental basis for the relationship between RhoA protein and vasospasms after SAH.

Experimental Section

Chemistry

General: Reagents (chemicals) were purchased from Lancaster, Alfa Aesar, J&K, Acros, and Shanghai Chemical Reagent Co. and were used without further purification. HSGF 254 (150–200 μm thickness; Yantai Huiyou Co., China) was used for analytical thin-layer chromatography (TLC). Yields were not optimized. Melting points were measured in capillary tubes on an SGW X-4 melting point apparatus without correction. Nuclear magnetic resonance (NMR) spectroscopy was performed on a Bruker AMX-400 NMR. Chemical shifts were reported in parts per million (ppm, δ) downfield from tetramethylsilane. Proton coupling patterns were described as singlet (s), doublet (d), triplet (t), quartet (q), and multiplet (m). Low- and high-resolution mass spectra (LRMS and HRMS) were given with electric and electrospray ionization (EI and ESI) produced by a Finnigan MAT-95 and LCQ-DECA spectrometer.

2-Chloroquinoline (6a): Quinolin-2-ol (2.0 g, 0.014 mol) was added to phenylphosphonic dichloride (20 mL). The mixture was stirred at reflux for 12 h at 110 °C under N_2. After the reaction was cooled to room temperature, it was slowly poured into ice water with continued stirring. After the product was re-dissolved in solvent, it was removed by filtration and washed with water to afford **6a** (yield: 1.911 g, 85%): ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 8.14$ (d, $J = 8.6$ Hz, 1H, ArH), 8.08 (d, $J = 8.5$ Hz, 1H, ArH), 7.83 (d, $J = 8.2$ Hz, 1H, ArH), 7.80–7.73 (m, 1H, ArH), 7.58 (m, 1H, ArH), 7.41 ppm (d, $J = 8.6$ Hz, 1H, ArH).

2-Chloroquinoxaline (6b): Compound **6b** was prepared from 2-quinoxalinone in the same manner as described for the preparation of **6a** (yield: 1.830 g, 81%): ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 8.54$ (s, 1H, ArH), 7.92–7.81 (m, 1H, ArH), 7.80–7.72 (m, 1H, ArH), 7.63–7.50 ppm (m, 2H, ArH).

Ethyl 3-nitrocinnamate (8): 3-Nitrocinnamic acid (2.0 g, 0.01 mol) was dissolved in 20 mL EtOH, and 1 mL concentrated sulfuric acid was added slowly to the reaction. The mixture was stirred at reflux for 12 h under N_2. After the reaction was cooled to room temperature, it was concentrated in vacuo, and saturated $NaHCO_3$ was added to make the reaction alkaline. The mixture was extracted with EtOAc (20 mL), and the organic layer was dried over $MgSO_4$, filtered, and concentrated in vacuo. Then, the residue was purified by flash chromatography on silica gel to afford compound **8** (yield: 1.84 g, 81%): ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.62$ (d, $J = 15.8$ Hz, 1H, ArH), 7.20 (t, $J = 7.6$ Hz, 1H, ArH), 6.98 (d, $J = 7.8$ Hz, 1H, ArH), 6.88 (s, 1H, ArH), 6.76 (d, $J = 8.1$ Hz, 1H, CH), 6.40 (d, $J = 16.2$ Hz, 1H, CH), 4.28 (q, $J = 7.2$ Hz, 2H, CH_2), 1.36 ppm (t, $J = 7.2$ Hz, 3H, CH_3).

(*E*)-Ethyl-3-(3-aminophenyl) acrylate (9): Ethyl 3-nitrocinnamate **8** (2.0 g, 0.009 mol) was added to EtOH (40 mL), followed by stannous chloride (10.35 g, 0.054 mol). The mixture was stirred at reflux

for 12 h. After the reaction was cooled to room temperature, it was concentrated in vacuo, and saturated $NaHCO_3$ was added to make the mixture alkaline, which was then extracted with EtOAc (20 mL). The organic layer was dried over $MgSO_4$, filtered, concentrated in vacuo, and purified by flash chromatography on silica gel to afford **9** (yield: 1.698 g, 98%): ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.62 (d, J = 16.0 Hz, 1H, ArH), 7.20 (t, J = 7.8 Hz, 1H, ArH), 6.98 (d, J = 7.6 Hz, 1H, ArH), 6.88 (s, 1H, ArH), 6.76 (d, J = 7.9 Hz, 1H, CH), 6.40 (d, J = 16.0 Hz, 1H, CH), 4.28 (q, J = 7.1 Hz, 2H, CH_2), 3.76 (s, 2H, NH_2), 1.36 ppm (t, J = 7.1 Hz, 3H, CH_3).

(*E*)-Ethyl 3-(3-(quinolin-2-ylamino)phenyl)acrylate (10a): 2-Chloroquinoline (**6a**, 200 mg, 0.001 mol) and (*E*)-ethyl-3-(3-aminophenyl) acrylate (**9**, 230 mg, 0.001 mol) were dissolved in 2 mL DMF. The mixture was stirred at reflux for 12 h at 110 °C under N_2. After the reaction was cooled to room temperature, it was poured into water and extracted with EtOAc (20 mL), and the combined organic layers were dried over $MgSO_4$, filtered, concentrated in vacuo, and purified by flash chromatography on silica gel to afford **10a** (yield: 194 mg, 51%): ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.95 (d, J = 8.9 Hz, 1H, ArH), 7.88 (s, 1H, ArH), 7.84 (d, J = 8.4 Hz, 1H, ArH), 7.76–7.60 (m, 4H, ArH), 7.35 (m, 2H, ArH), 7.24 (d, J = 7.7 Hz, 1H, ArH), 6.96 (d, J = 8.9 Hz, 1H, CH), 6.48 (d, J = 16.0 Hz, 1H, CH), 4.31 (q, J = 7.1 Hz, 2H, CH_2), 1.38 ppm (t, J = 7.1 Hz, 3H, CH_3).

(*E*)-Ethyl 3-(3-(quinoxalin-2-ylamino)phenyl)acrylate (10b): Compound **10b** was prepared from 2-chloroquinoxaline (**6b**) in the same manner as described for the preparation of **10a** (yield: 313 mg, 81%): ^{1}H NMR (400 Hz, $CDCl_3$): δ = 8.45 (s, 1H, ArH), 8.02 (s, 1H, ArH), 7.94 (d, J = 8.0 Hz, 1H, ArH), 7.80–7.84 (m, 2H, ArH), 7.71 (d, J = 16.0 Hz, 1H, ArH), 7.65 (t, J = 7.6 Hz, 1H, ArH), 7.49 (t, J = 8.0 Hz, 1H, ArH), 7.39 (t, J = 8.0 Hz, 1H, ArH), 7.25 (d, J = 7.6 Hz, 1H, CH), 6.48 (d, J = 16.0 Hz, 1H, CH), 4.30 (q, 2H, CH_2), 1.36 ppm (t, J = 7.2 Hz, 3H, CH_3).

(*E*)-3-(3-(Methyl(quinolin-2-yl)amino)phenyl)acrylic acid (26a): NaH was added portionwise to a solution of (*E*)-ethyl 3-(3-(quinoxalin-2-ylamino)phenyl)acrylate (**10a**, 0.7 mmol) in DMF (4 mL). The mixture was stirred for 15 min in an ice bath under N_2, then iodomethane (600 μL) was added dropwise, and stirring was continued for 12 h at room temperature. After the reaction was completed, the mixture was extracted with water (10 mL) and EtOAc (20 mL). The organic layer was dried over $MgSO_4$ and filtered. The filtrate was concentrated in vacuo and then purified by flash column chromatography on silica gel to afford **11a**, which was used directly in the next step without further purification.

Compound **11a** (199 mg, 0.0006 mol) and lithium hydroxide (43 mg) were dissolved in MeOH (4 mL), which was stirred overnight at 40 °C. After the reaction was completed, it was concentrated in vacuo, and aqueous HCl was added, which acidified the mixture to pH 5.5. The precipitate was collected to afford **26a** (yield: 60 mg, 14% over two steps): mp: 103–106 °C; HPLC: 100%; ^{1}H NMR (400 MHz, [D_6]acetone): δ = 7.94 (d, J = 9.2 Hz, 1H, ArH), 7.75 (dd, J = 17.3, 8.0 Hz, 4H, ArH), 7.66 (d, J = 7.6 Hz, 1H, ArH), 7.60 (dd, J = 16.5, 8.2 Hz, 2H, ArH), 7.48 (d, J = 7.6 Hz, 1H, ArH), 7.31 (t, J = 7.4 Hz, 1H, ArH), 6.85 (d, J = 9.3 Hz, 1H, CH), 6.64 (d, J = 16.0 Hz, 1H, CH), 3.68 ppm (s, 3H, CH_3); MS (ESI+): m/z = 305.1 $[M+H]^+$; HRMS (ESI+): m/z $[M+H]^+$ calcd for $C_{19}H_{17}N_2O_2$: 305.1290, found: 305.1285.

(*E*)-3-(3-(Ethyl(quinolin-2-yl)amino)phenyl)acrylic acid (26b): Compound **26b** was prepared from **10a** and iodoethane in the same manner as described for the preparation of **26a** (yield: 113 mg, 57% over two steps): mp: 83–87 °C; HPLC: 99%; ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 7.91 (s, 1H, ArH), 7.75–7.60 (m, 5H, ArH), 7.56–7.53 (m, 2H, ArH), 7.39–7.36 (m, 1H, ArH), 7.28–7.25 (m, 1H, ArH), 6.61 (d, J = 16.0 Hz, 2H, CH), 4.14 (dd, J = 14.1, 7.1 Hz, 2H, CH_2), 1.21 ppm (t, J = 6.9 Hz, 3H, CH_3); MS (ESI+): m/z = 319.1 $[M+H]^+$; HRMS (ESI+): m/z $[M+H]^+$ calcd for $C_{20}H_{19}N_2O_2$: 319.1447, found: 319.1441.

(*E*)-3-(3-(Propyl(quinolin-2-yl)amino)phenyl)acrylic acid (26c): Compound **26c** was prepared from **10a** and 1-iodopropane in the same manner as described for the preparation of **26a** (yield: 398 mg, 95% over two steps): mp: 73–76 °C; HPLC: 99%; ^{1}H NMR (400 MHz, [D_6]acetone): δ = 7.85 (d, J = 9.1 Hz, 1H, ArH), 7.75–7.62 (m, 5H, ArH), 7.56 (t, J = 7.7 Hz, 2H, ArH), 7.41 (d, J = 7.7 Hz, 1H, ArH), 7.24 (t, J = 7.0 Hz, 1H, ArH), 6.69 (d, J = 9.1 Hz, 1H, CH), 6.61 (d, J = 16.1 Hz, 1H, CH), 4.16–4.11 (m, 2H, CH_2), 1.84–1.69 (m, 2H, CH_2), 0.97 ppm (t, J = 7.4 Hz, 3H, CH_3); MS (ESI−): m/z = 331.2 $[M-H]^-$; HRMS (ESI−): m/z $[M-H]^-$ calcd for $C_{21}H_{19}N_2O_2$: 331.1447, found: 331.1452.

(*E*)-3-(3-(Butyl(quinolin-2-yl)amino)phenyl)acrylic acid (26d): Compound **26d** was prepared from **10a** and 1-iodobutane in the same manner as described for the preparation of **26a** (yield: 390 mg, 90% over two steps): mp: 69–72 °C; HPLC: 98%; ^{1}H NMR (400 MHz, [D_6]acetone): δ = 7.89 (d, J = 8.7 Hz, 1H, ArH), 7.70 (dd, J = 20.7, 10.4 Hz, 5H, ArH), 7.59 (t, J = 7.7 Hz, 2H, ArH), 7.45 (d, J = 7.7 Hz, 1H, ArH), 7.27 (t, J = 7.3 Hz, 1H, ArH), 6.70 (d, J = 9.2 Hz, 1H, CH), 6.63 (d, J = 16.0 Hz, 1H, CH), 4.24 (s, 2H, CH_2), 1.78–1.69 (m, 2H, CH_2), 1.51–1.38 (m, 2H, CH_2), 0.95 ppm (t, J = 7.4 Hz, 3H, CH_3); MS (ESI−): m/z = 345.2 $[M-H]^-$; HRMS (ESI−): m/z $[M-H]^-$ calcd for $C_{22}H_{21}N_2O_2$ 345.1603, found: 345.1609.

(*E*)-3-(3-(Isopropyl(quinolin-2-yl)amino)phenyl)acrylic acid (26e): Compound **26e** was prepared from **10a** and 2-iodopropane in the same manner as described for the preparation of **26a** (yield: 159 mg, 37% over two steps): mp: 100–103 °C; HPLC: 99%; ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 7.81 (t, J = 8.2 Hz, 2H, ArH), 7.63 (dd, J = 20.5, 11.3 Hz, 5H, ArH), 7.57–7.51 (m, 1H, ArH), 7.28 (d, J = 7.7 Hz, 1H, ArH), 7.21 (t, J = 7.6 Hz, 1H, ArH), 6.61 (d, J = 15.9 Hz, 1H, CH), 6.20 (d, J = 9.1 Hz, 1H, CH), 5.61–5.34 (m, 1H, CH), 1.15 ppm (d, J = 6.8 Hz, 6H, CH_3); MS (EI) m/z 332.2 (M^+), 317.1 (100%); HRMS (EI) m/z $[M]^+$ calcd for $C_{21}H_{20}N_2O_2$: 332.1525, found: 332.1525, 317.1294 (100%).

(*E*)-3-(3-((Cyclopropylmethyl)(quinolin-2-yl)amino)phenyl)acrylic acid (26f): Compound **26f** was prepared from **10a** and (bromomethyl)cyclopropane in the same manner as described in the preparation of **26a** (yield: 188 mg, 43% over two steps): mp: 96–99 °C; HPLC: 96%; ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 7.91 (d, J = 8.1 Hz, 1H, ArH), 7.78–7.60 (m, 5H, ArH), 7.55 (dt, J = 15.4, 7.5 Hz, 2H, ArH), 7.40 (d, J = 7.7 Hz, 1H, ArH), 7.27 (d, J = 6.5 Hz, 1H, ArH), 6.61 (t, J = 12.0 Hz, 2H, CH), 3.98 (d, J = 6.8 Hz, 2H, CH_2), 1.27–1.15 (m, 1H, CH), 0.40 (d, J = 6.7 Hz, 2H, CH_2), 0.17 ppm (q, J = 5.1 Hz, 2H, CH_2); MS (ESI−): m/z = 343.2 $[M-H]^-$; HRMS (ESI−): m/z $[M-H]^-$ calcd for $C_{22}H_{19}N_2O_2$: 343.1447, found: 343.1452.

(*E*)-3-(3-((Cyclopentylmethyl)(quinolin-2-yl)amino)phenyl)acrylic acid (26g): Compound **26g** was prepared from **10a** and iodomethylcyclopentane in the same manner as described for the preparation of **26a** (yield: 100 mg, 21% over two steps): mp: 102–105 °C; HPLC: 97%; ^{1}H NMR (400 MHz, CD_3OD): δ = 7.92 (d, J = 9.3 Hz, 1H, ArH), 7.72 (dd, J = 22.9, 7.1 Hz, 3H, ArH), 7.60 (dt, J = 15.3, 8.1 Hz, 4H, ArH), 7.42 (d, J = 7.8 Hz, 1H, ArH), 7.31 (t, J = 7.3 Hz, 1H, ArH), 6.74 (d, J = 9.3 Hz, 1H, CH), 6.55 (d, J = 16.0 Hz, 1H, CH), 4.17 (d, J = 7.6 Hz, 2H, CH_2), 2.35 (dt, J = 15.0, 7.6 Hz, 1H, CH), 1.74 (td, J = 12.4, 8.5 Hz, 4H, CH_2), 1.64–1.50 (m, 2H, CH_2), 0.91 ppm (d, J = 9.8 Hz, 2H, CH_2); MS (ESI−): m/z = 371.2 $[M-H]^-$; HRMS (ESI−): m/z $[M-H]^-$ calcd for $C_{24}H_{23}N_2O_2$: 371.1760, found: 371.1765.

(*E*)-3-(3-((Cyclohexylmethyl)(quinolin-2-yl)amino)phenyl)acrylic acid (26h): Compound **26h** was prepared from **10a** and cyclohexylmethyl bromide in the same manner as described for the preparation of **26a** (yield: 111 mg, 23% over two steps): mp: 87–90 °C; HPLC: 95%; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 7.87 (d, J = 9.2 Hz, 1H, ArH), 7.66 (d, J = 7.6 Hz, 1H, ArH), 7.60 (d, J = 8.2 Hz, 1H, ArH), 7.50 (dt, J = 15.2, 9.3 Hz, 4H, ArH), 7.31 (t, J = 13.3 Hz, 2H, ArH), 7.23 (t, J = 6.9 Hz, 1H, ArH), 6.64 (d, J = 9.1 Hz, 1H, CH), 6.52 (d, J = 15.9 Hz, 1H, CH), 4.02 (d, J = 6.6 Hz, 2H, CH$_2$), 1.67 (dd, J = 49.2, 25.9 Hz, 7H, CH$_2$, CH), 1.25 (d, J = 10.2 Hz, 1H, CH$_2$), 1.13 ppm (t, J = 9.4 Hz, 3H, CH$_2$); MS (ESI−): m/z = 385.2 [M−H]$^-$; HRMS (ESI−): m/z [M−H]$^-$ calcd for $C_{25}H_{25}N_2O_2$: 385.1916, found: 385.1922.

(*E*)-3-(3-((2-(Piperidin-1-yl)ethyl)(quinolin-2-yl)amino)phenyl)-acrylic acid (26i): Compound **26i** was prepared from **10a** and 2-piperidinoethylchloride hydrochloride in the same manner as described for the preparation of **26a** (yield: 239 mg, 63% over two steps): mp: 82–86 °C; HPLC: 98%; ^{1}H NMR (400 MHz, CD$_3$OD): δ = 7.83 (d, J = 9.1 Hz, 1H, ArH), 7.68 (d, J = 8.4 Hz, 1H, ArH), 7.61 (d, J = 7.7 Hz, 1H, ArH), 7.57–7.50 (m, 2H, ArH), 7.45 (dt, J = 15.4, 7.8 Hz, 2H, ArH), 7.36 (d, J = 16.0 Hz, 1H, ArH), 7.30–7.21 (m, 2H, ArH), 6.58 (d, J = 9.1 Hz, 1H, CH), 6.44 (d, J = 16.0 Hz, 1H, CH), 4.42 (t, J = 5.8 Hz, 2H, CH$_2$), 3.35 (t, J = 5.8 Hz, 2H, CH$_2$), 1.83 (dd, J = 12.4, 7.1 Hz, 4H, CH$_2$), 0.79 ppm (d, J = 9.5 Hz, 6H, CH$_2$); MS (ESI−): m/z = 400.2 [M−H]$^-$; HRMS (ESI−): m/z [M−H]$^-$ calcd for $C_{25}H_{26}N_3O_2$: 400.2025, found: 400.2031.

(*E*)-3-(3-(Benzyl(quinolin-2-yl)amino)phenyl)acrylic acid (26j): Compound **26j** was prepared from **10a** and benzyl bromide in the same manner as described for the preparation of **26a** (yield: 352 mg, 45% over two steps): mp: 98–102 °C; HPLC: 100%; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 7.93 (d, J = 9.0 Hz, 1H, ArH), 7.72–7.63 (m, 2H, ArH), 7.59 (d, J = 8.6 Hz, 1H, ArH), 7.51 (dd, J = 15.8, 8.0 Hz, 3H, ArH), 7.39 (t, J = 7.9 Hz, 1H, ArH), 7.31 (t, J = 6.8 Hz, 3H, ArH), 7.27–7.19 (m, 3H, ArH), 7.14 (t, J = 7.4 Hz, 1H, ArH), 6.77 (d, J = 9.1 Hz, 1H, CH$_2$), 6.48 (d, J = 16.0 Hz, 1H, CH$_2$), 5.39 ppm (s, 2H, CH$_2$); MS (EI) m/z 380.2 (M^+), 380.2(100%); HRMS (EI) m/z [M]$^+$ calcd for $C_{25}H_{20}N_2O_2$: 380.1525, found: 380.1525, 380.1524 (100%).

(*E*)-3-(3-((Pyridin-2-ylmethyl)(quinolin-2-yl)amino)phenyl)acrylic acid (26k): Compound **26k** was prepared from **10a** and 2-(bromomethyl)pyridine hydrochloride in the same manner as described in the preparation of **26a** (yield: 184 mg, 38% over two steps): mp: 116–119 °C; HPLC: 96%; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 8.52 (d, J = 4.4 Hz, 1H, ArH), 8.01 (d, J = 9.2 Hz, 1H, ArH), 7.84 (s, 1H, ArH), 7.75 (dd, J = 11.1, 8.0 Hz, 2H, ArH), 7.64–7.52 (m, 6H, ArH), 7.49 (d, J = 6.3 Hz, 2H, ArH), 7.28 (d, J = 6.3 Hz, 1H, ArH), 6.88 (d, J = 9.1 Hz, 1H, CH), 6.56 (d, J = 16.0 Hz, 1H, CH), 5.49 ppm (s, 2H, CH$_2$); MS (ESI−): m/z = 380.1 [M−H]$^-$; HRMS (ESI−): m/z [M−H]$^-$ calcd for $C_{24}H_{18}N_3O_2$: 380.1399, found: 380.1405.

(*E*)-3-(3-((2-Hydroxyethyl)(quinolin-2-yl)amino)phenyl)acrylic acid (26l): Tetrabutylammonium fluoride (1 mL) was added to a solution of **26n** (193 mg, 0.4 mmol) in dry THF (4 mL). The resulting reaction mixture was stirred at reflux overnight at room temperature. The solvent was removed under reduced pressure, then the residue was purified by flash chromatography on silica gel to afford **26l** (yield: 69 mg, 48%): mp: 195–198 °C; HPLC: 100%; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 7.91 (d, J = 9.1 Hz, 1H, ArH), 7.74 (s, 1H, ArH), 7.70 (d, J = 7.9 Hz, 1H, ArH), 7.61 (dd, J = 13.0, 7.9 Hz, 3H, ArH), 7.56 (d, J = 7.0 Hz, 1H, ArH), 7.51 (t, J = 7.7 Hz, 1H, ArH), 7.42 (d, J = 8.1 Hz, 1H, ArH), 7.26 (t, J = 7.2 Hz, 1H, ArH), 6.67 (d, J = 9.1 Hz, 1H, CH), 6.57 (d, J = 16.0 Hz, 1H, CH), 4.13 (t, J = 6.2 Hz, 2H, CH$_2$), 3.71 ppm (t, J = 6.2 Hz, 2H, CH$_2$); MS (ESI+): m/z = 335.1 [M+H]$^+$; HRMS (ESI+): m/z [M+H]$^+$ calcd for $C_{20}H_{19}N_2O_3$: 335.1396, found: 335.1390.

(*E*)-3-(3-((3-Hydroxypropyl)(quinolin-2-yl)amino)phenyl)acrylic acid (26m): Compound **26m** was prepared from **10a** and 3-iodopropanol in the same manner as described for the preparation of **26a** (yield: 216 mg, 49% over two steps): mp: 166–169 °C; HPLC: 97%; ^{1}H NMR (400 MHz, CD$_3$OD): δ = 7.81 (d, J = 9.1 Hz, 1H, ArH), 7.63 (d, J = 7.9 Hz, 1H, ArH), 7.59 (d, J = 7.8 Hz, 2H, ArH), 7.54 (d, J = 8.3 Hz, 3H, ArH), 7.48 (t, J = 7.7 Hz, 1H, ArH), 7.33 (d, J = 7.5 Hz, 1H, ArH), 7.23 (t, J = 7.4 Hz, 1H, ArH), 6.63 (d, J = 9.2 Hz, 1H, CH), 6.48 (d, J = 16.0 Hz, 1H, CH), 4.25 (t, J = 6.3 Hz, 2H, CH$_2$), 3.63 (t, J = 5.8 Hz, 2H, CH$_2$), 1.83–1.75 ppm (m, 2H, CH$_2$); MS (ESI−): m/z = 347.1 [M−H]$^-$; HRMS (ESI−): m/z [M−H]$^-$ calcd for $C_{21}H_{19}N_2O_3$: 347.1396, found: 347.1401.

(*E*)-3-(3-((2-((*tert*-Butyldimethylsilyl)oxy)ethyl)(quinolin-2-yl)amino)phenyl)acrylic acid (26n): Compound **26n** was prepared from **10a** and tert-butyl(2-iodoethoxy)dimethylsilane in the same manner as described for the preparation of **26a** (yield: 102 mg, 10% over two steps): mp: 124–127 °C; HPLC: 99%; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 7.92 (d, J = 9.2 Hz, 1H, ArH), 7.73 (s, 1H, ArH), 7.70 (d, J = 7.4 Hz, 1H, ArH), 7.67–7.54 (m, 4H, ArH), 7.50 (t, J = 7.7 Hz, 1H, ArH), 7.42 (d, J = 8.1 Hz, 1H, ArH), 7.30–7.23 (m, 1H, ArH), 6.70 (d, J = 9.1 Hz, 1H, CH), 6.57 (d, J = 16.0 Hz, 1H, CH), 4.17 (t, J = 6.3 Hz, 2H, CH$_2$), 3.93 (t, J = 6.3 Hz, 2H, CH$_2$), 0.82 (s, 9H, CH$_3$), 0.00 ppm (s, 6H, CH$_3$); MS (ESI+): m/z = 449.2 [M+H]$^+$; HRMS (ESI+): m/z [M+H]$^+$ calcd for $C_{26}H_{33}N_2O_3Si$: 449.2260, found: 449.2255.

(*E*)-3-(3-((Carboxymethyl)(quinolin-2-yl)amino)phenyl)acrylic acid (26o): Compound **26o** was prepared from **10a** and methyl bromoacetate in the same manner as described for the preparation of **26a** (yield: 31 mg, 10% over two steps): mp: 184–187 °C; HPLC: 100%; ^{1}H NMR (400 MHz, [D$_6$]acetone): δ = 7.99 (d, J = 9.1 Hz, 1H, ArH), 7.85 (s, 1H, ArH), 7.82–7.75 (m, 2H, ArH), 7.73–7.66 (m, 2H, ArH), 7.65–7.54 (m, 3H, ArH), 7.32 (t, J = 7.4 Hz, 1H, ArH), 6.87 (d, J = 9.1 Hz, 1H, CH), 6.61 (d, J = 16.0 Hz, 1H, CH), 4.92 ppm (s, 2H, CH$_2$); MS (ESI+): m/z = 349.1 [M+H]$^+$; HRMS (ESI+): m/z [M+H]$^+$ calcd for $C_{20}H_{17}N_2O_4$: 349.1188, found: 349.1183.

(*E*)-3-(3-((2-Amino-2-oxoethyl)(quinolin-2-yl)amino)phenyl)acrylic acid (26p): Compound **26p** was prepared from **10a** and 2-iodoacetamide in the same manner as described for the preparation of **26a** (yield: 136 mg, 26% over two steps): mp: 189–193 °C; HPLC: 100%; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 7.95 (d, J = 9.0 Hz, 1H, ArH), 7.76 (s, 1H, ArH), 7.72 (d, J = 7.8 Hz, 1H, ArH), 7.60 (dd, J = 15.2, 7.4 Hz, 3H, ArH), 7.53 (t, J = 6.9 Hz, 1H, ArH), 7.52–7.44 (m, 3H, ArH), 7.27 (t, J = 7.3 Hz, 1H, ArH), 7.07 (s, 1H, ArH), 6.77 (d, J = 9.2 Hz, 1H, CH), 6.54 (d, J = 16.0 Hz, 1H, CH), 4.63 ppm (s, 2H, CH$_2$); MS (ESI+): m/z = 348.1 [M+H]$^+$; HRMS (ESI+): m/z [M+H]$^+$ calcd for $C_{20}H_{18}N_3O_3$: 348.1348, found: 348.1343.

(*E*)-3-(3-(Ethyl(quinoxalin-2-yl)amino)phenyl)acrylic acid (26q): Compound **26q** was prepared from **10b** and methyl trifluoromethane sulfonate in the same manner as described for the preparation of **26a** (yield: 140 mg, 59% over two steps): mp: 157–160 °C; HPLC: 100%; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 8.15 (s, 1H, ArH), 7.82 (d, J = 8.1 Hz, 2H, ArH), 7.70 (t, J = 7.5 Hz, 2H, ArH), 7.67–7.54 (m, 3H, ArH), 7.46 (d, J = 8.2 Hz, 2H, ArH, CH), 6.64 (d, J = 16.0 Hz, 1H, CH), 4.10 (dd, J = 14.2, 7.1 Hz, 2H, CH$_2$), 1.22 ppm (t, J = 7.0 Hz, 3H, CH$_3$); MS (ESI+): m/z = 320.1 [M+H]$^+$; HRMS (ESI+): m/z [M+H]$^+$ calcd for $C_{19}H_{18}N_3O_2$: 320.1399, found: 320.1394.

(*E*)-3-(3-((Carboxymethyl)(quinoxalin-2-yl)amino)phenyl)acrylic acid (26r): Compound **26r** was prepared from **10b** and methyl

bromoacetate in the same manner as described for the preparation of **26a** (yield: 90 mg, 27% over two steps): mp: 200–203 °C; HPLC: 100%; ^{1}H NMR (400 MHz, [D$_6$]acetone): δ = 8.37 (s, 1H, ArH), 7.95 (s, 1H, ArH), 7.87 (d, J = 8.2 Hz, 1H, ArH), 7.75 (t, J = 10.9 Hz, 3H, ArH), 7.66 (dd, J = 14.1, 7.0 Hz, 3H, ArH), 7.49 (t, J = 7.5 Hz, 1H, CH), 6.64 (d, J = 16.1 Hz, 1H, CH), 4.86 ppm (s, 2H, CH$_2$); MS (ESI+): m/z = 372.1 $[M+Na]^+$; HRMS (ESI+): m/z $[M+Na]^+$ calcd for $C_{19}H_{15}N_3O_4$: 372.0960, found 372.0955.

Ethyl 3-(3-aminophenyl)propanoate (13): Compound **13** was prepared from 3-(3-aminophenyl)propionic acid in the same manner as described for the preparation of **8** (yield: 1.501 g, 85%): ^{1}H NMR (400 MHz, CDCl$_3$): δ = 7.18–7.04 (m, 1H, ArH), 6.65 (d, J = 7.7 Hz, 1H, ArH), 6.59 (d, J = 6.3 Hz, 2H, ArH), 4.15 (q, J = 7.1 Hz, 2H, CH$_2$), 3.52 (s, 2H, NH$_2$), 2.92–2.84 (m, 2H, CH$_2$), 2.70–2.52 (m, 2H, CH$_2$), 1.26 ppm (dd, J = 9.6, 4.7 Hz, 3H, CH$_3$).

Ethyl 3-(3-(quinolin-2-ylamino)phenyl)propanoate (14a): Compound **14a** was prepared from **13** in the same manner as described for the preparation of **10a** (yield: 294 mg, 58%): ^{1}H NMR (400 MHz, CDCl$_3$): δ = 7.92 (d, J = 8.9 Hz, 1H, ArH), 7.78 (d, J = 8.4 Hz, 1H, ArH), 7.64 (d, J = 8.0 Hz, 1H, ArH), 7.59 (dd, J = 11.2, 4.1 Hz, 1H, ArH), 7.44 (d, J = 8.0 Hz, 1H, ArH), 7.40 (s, 1H, ArH), 7.33–7.27 (m, 2H, ArH), 6.98 (d, J = 8.9 Hz, 1H, ArH), 6.93 (d, J = 7.5 Hz, 1H, ArH), 4.14 (q, J = 7.1 Hz, 2H, CH$_2$), 2.97 (t, J = 7.8 Hz, 2H, CH$_2$), 2.66 (t, J = 7.8 Hz, 2H, CH$_2$), 1.24 ppm (t, J = 7.1 Hz, 3H, CH$_3$).

Ethyl 3-(3-(quinoxalin-2-ylamino)phenyl)propanoate(14b): Compound **14b** was prepared from **13** in the same manner as described for the preparation of **10a** (yield: 313 mg, 62%): ^{1}H NMR (400 MHz, [D$_6$]acetone): δ = 8.41 (s, 1H, ArH), 7.91 (dd, J = 8.2, 0.9 Hz, 1H, ArH), 7.83 (s, 1H, ArH), 7.79 (dd, J = 8.2, 0.7 Hz, 1H, ArH), 7.66 (dd, J = 8.0, 1.6 Hz, 1H, ArH), 7.64–7.55 (m, 2H, ArH), 7.47–7.38 (m, 1H, ArH), 7.26 (t, J = 8.0 Hz, 1H, ArH), 6.92 (d, J = 7.6 Hz, 1H, ArH), 4.19–4.12 (m, 2H, CH$_2$), 2.97 (t, J = 7.7 Hz, 2H, CH$_2$), 2.68 (t, J = 7.7 Hz, 2H,CH$_2$), 1.27–1.21 ppm (m, 3H, CH$_3$).

3-(3-(Ethyl(quinolin-2-yl)amino)phenyl)propanoic acid (27a). Compound **27a** was prepared from **14a** and iodoethane in the same manner as described for the preparation of **26a** (yield: 204 mg, 49% over two steps): HPLC: 99%; ^{1}H NMR (400 MHz, CD$_3$OD): δ = 7.80 (d, J = 9.2 Hz, 1H, ArH), 7.73 (d, J = 8.5 Hz, 1H, ArH), 7.63 (d, J = 8.0 Hz, 1H, ArH), 7.60–7.52 (m, 1H, ArH), 7.43 (t, J = 7.7 Hz, 1H, ArH), 7.29–7.19 (m, 3H, ArH), 7.15 (d, J = 6.8 Hz, 1H, ArH), 6.62 (d, J = 9.2 Hz, 1H, ArH), 4.16 (q, J = 7.0 Hz, 2H, CH$_2$), 2.98 (t, J = 7.5 Hz, 2H, CH$_2$), 2.64 (t, J = 7.5 Hz, 2H, CH$_2$), 1.28 ppm (t, J = 7.1 Hz, 3H, CH$_3$); MS (ESI+): m/z = 321.2 $[M+H]^+$; HRMS (ESI+): m/z $[M+H]^+$ calcd for $C_{20}H_{21}N_2O_2$: 321.1603, found: 321.1598.

3-(3-((Carboxymethyl)(quinolin-2-yl)amino)phenyl)propanoic acid (27b): Compound **27b** was prepared from **14a** and methyl bromoacetate in the same manner as described for the preparation of **26a** (yield: 12 mg, 4% over two steps): mp: 139–142 °C; HPLC: 100%; ^{1}H NMR (400 MHz, [D$_6$]acetone): δ = 7.93 (d, J = 9.1 Hz, 1H, ArH), 7.78–7.65 (m, 2H, ArH), 7.59 (t, J = 7.6 Hz, 1H, ArH), 7.49–7.39 (m, 2H, ArH), 7.34 (d, J = 7.5 Hz, 1H, ArH), 7.29 (t, J = 7.3 Hz, 2H, ArH), 6.81 (d, J = 9.1 Hz, 1H, ArH), 4.80 (s, 2H, CH$_2$), 2.99 (t, J = 7.6 Hz, 2H, CH$_2$), 2.69 ppm (t, J = 7.6 Hz, 2H, CH$_2$); MS (ESI+): m/z = 351.1 $[M+H]^+$; HRMS (ESI+): m/z $[M+H]^+$ calcd for $C_{20}H_{19}N_2O_4$: 351.1345, found: 351.1339.

3-(3-(Ethyl(quinoxalin-2-yl)amino)phenyl)propanoic acid (27c): Compound **27c** was prepared from **14b** and iodoethane in the same manner as described in the preparation of **26a** (yield: 142 mg, 71% over two steps): mp: 155–157 °C; HPLC: 100%; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 8.07 (s, 1H, ArH), 7.81 (d, J = 7.0 Hz, 1H, ArH), 7.74–7.67 (m, 1H, ArH), 7.67–7.56 (m, 1H, ArH), 7.50–7.38 (m, 2H, ArH), 7.26 (dd, J = 17.2, 9.5 Hz, 3H, ArH), 4.06 (q, J = 7.0 Hz, 2H, CH$_2$), 2.88 (t, J = 7.5 Hz, 2H, CH$_2$), 2.58 (t, J = 7.6 Hz, 2H, CH$_2$), 1.20 ppm (t, J = 7.0 Hz, 3H, CH$_3$); MS (ESI+): m/z = 322.2 $[M+H]^+$; HRMS (ESI+): m/z $[M+H]^+$ calcd for $C_{19}H_{20}N_3O_2$: 322.1556, found: 322.1550.

3-(3-((Carboxymethyl)(quinoxalin-2-yl)amino)phenyl)propanoic acid (27d): Compound **27d** was prepared from **14b** and methyl bromoacetate in the same manner as described for the preparation of **26a** (yield: 28 mg, 9% over two steps): mp: 204–207 °C; HPLC: 100%; ^{1}H NMR (400 MHz, [D$_6$]acetone): δ = 8.29 (s, 1H, ArH), 7.86 (d, J = 8.1 Hz, 1H, ArH), 7.71 (d, J = 8.3 Hz, 1H, ArH), 7.65 (t, J = 7.6 Hz, 1H, ArH), 7.53–7.48 (m, 2H, ArH), 7.48–7.45 (m, 1H, ArH), 7.42 (d, J = 7.8 Hz, 1H, ArH), 7.35 (d, J = 7.4 Hz, 1H, ArH), 4.79 (s, 2H, CH$_2$), 3.01 (t, J = 7.6 Hz, 2H, CH$_2$), 2.71 ppm (t, J = 7.6 Hz, 2H, CH$_2$); MS (ESI+): m/z = 374.1 $[M+Na]^+$; HRMS (ESI+): m/z $[M+Na]^+$ calcd for $C_{19}H_{17}N_3O_4$: 374.1117, found: 374.1111.

3-Nitrobenzamide (17): 3-Nitrobenzaldehyde **(16**, 0.007 mol), hydroxylamine hydrochloride (56 mg, 0.008 mol), and cesium carbonate (259 mg, 0.008 mol) were added to thionyl chloride (1.5 mL) and water (0.5 mL). The mixture was stirred for 7 h at 100 °C, and then palladium diacetate (8 mg) was added to the reaction. The reaction was stirred for another 12 h. After the reaction was cooled to room temperature, it was poured into water and extracted with EtOAc (20 mL). The combined organic layers were dried over MgSO$_4$ and filtered. The filtrate was concentrated in vacuo and then purified by flash chromatography on silica gel to afford **17** (yield: 63 mg, 57%): ^{1}H NMR (400 MHz, CD$_3$OD): δ = 8.78–8.72 (m, 1H, ArH), 8.41 (m, J = 8.2, 2.2, 1.0 Hz, 1H, ArH), 8.30–8.25 (m, 1H, ArH), 7.74 ppm (t, J = 8.0 Hz, 1H, ArH).

(*E*)-*N*-((Dimethylamino)methylene)-3-nitrobenzamide (18): 3-Nitrobenzamide (**17**, 0.002 mol) was dissolved in *N*,*N*-dimethylformamide dimethyl acetal (DMF-DMA, 2 mL), and the mixtures was stirred at reflux for 5 h at 105 °C. After the reaction was cooled to room temperature, it was concentrated in vacuo and washed with pentane to afford **18** (yield: 391 mg, 79%): ^{1}H NMR (400 MHz, CDCl$_3$): δ = 9.17–9.09 (m, 1H, ArH), 8.72 (s, 1H, CH), 8.61 (d, J = 7.7 Hz, 1H, ArH), 8.37 (m, 1H, ArH), 7.63 (t, J = 7.9 Hz, 1H, ArH), 3.32 (s, 3H, CH$_3$), 3.28 ppm (s, 3H, CH$_3$).

3-(3-Nitrophenyl)-4*H*-1,2,4-triazole (19): Compound **18** (0.0014 mol) was added to acetic acid glacial (4 mL), followed by the addition hydrazine hydrate (150 mg). The mixture was stirred for 12 h at 90 °C under N$_2$. After the reaction was completed, it was cooled to room temperature, concentrated in vacuo, and filtered. The filter cake was dissolved in EtOAc and washed with saturated NaHCO$_3$ (2×10 mL) to afford **19** as a white crystalline solid (yield: 91 mg, 36%): ^{1}H NMR (400 MHz, CDCl$_3$): δ = 9.02 (s, 1H, ArH), 8.49 (d, J = 7.8 Hz, 1H, ArH), 8.40 (s, 1H, ArH), 8.31 (d, J = 8.0 Hz, 1H, ArH), 7.68 ppm (t, J = 8.0 Hz, 1H, ArH).

3-(4*H*-1,2,4-Triazol-3-yl)aniline (20): Compound **20** was prepared from **19** in the same manner as described for the preparation of **9** (yield: 310 mg, 96%): ^{1}H NMR (400 MHz, CDCl$_3$): δ = 9.34 (s, 1H, ArH), 8.63 (d, J = 7.7 Hz, 1H, ArH), 8.51 (s, 1H, ArH), 8.47 (d, J = 8.0 Hz, 1H, ArH), 7.71 ppm (t, J = 8.1 Hz, 1H, ArH).

***N*-(3-(4*H*-1,2,4-Triazol-3-yl)phenyl)quinolin-2-amine (28a)**: Compound **28a** was prepared from **20** in the same manner as described for the preparation of **10a** (yield: 185 mg, 59%): mp: 186–188 °C; HPLC: 98%; ^{1}H NMR (400 MHz, [D$_6$]acetone): δ = 8.76 (s, 1H, ArH), 8.38 (s, 1H, ArH), 8.29 (d, J = 8.0 Hz, 1H, ArH), 8.07 (d, J = 8.9 Hz, 1H, ArH), 7.83 (d, J = 8.3 Hz, 1H, ArH), 7.76 (t, J = 8.0 Hz, 2H,

ArH), 7.64–7.59 (m, 1H, ArH), 7.46 (t, J=7.8 Hz, 1H, ArH), 7.38–7.28 (m, 1H, ArH), 7.20–7.06 ppm (m, 1H, ArH); MS (ESI+): m/z=288.1 $[M+H]^+$; HRMS (ESI+): m/z $[M+H]^+$ calcd for $C_{17}H_{14}N_5$: 288.1249, found: 288.1244.

***N*-(3-(4*H*-1,2,4-Triazol-3-yl)phenyl)quinoxalin-2-amine (28b)**: Compound **28b** was prepared from **20** in the same manner as described for the preparation of **10a** (yield: 88 mg, 49%): mp: 261–264 °C; HPLC: 98%; ^{1}H NMR (400 MHz, [D$_6$]acetone): δ=9.28 (s, 1H, ArH), 8.76 (s, 1H, ArH), 8.61 (s, 1H, ArH), 8.28 (d, J=7.9 Hz, 1H, ArH), 7.91 (d, J=8.2 Hz, 1H, ArH), 7.83 (d, J=8.2 Hz, 2H, ArH), 7.72–7.66 (m, 1H, ArH), 7.54–7.47 ppm (m, 2H, ArH); MS (ESI+): m/z=289.1 $[M+H]^+$; HRMS (ESI+): m/z $[M+H]^+$ calcd for $C_{16}H_{13}N_6$: 289.1202, found: 289.1196.

Ethyl 6-amino-2-naphthoate (22): Compound **22** was prepared from 6-amino-2-naphthoic acid in the same manner as described for the preparation of **8** (yield: 1.042 g, 88%): ^{1}H NMR (400 MHz, $CDCl_3$): δ=8.45 (s, 1H, ArH), 7.95 (dd, J=8.6, 1.7 Hz, 1H, ArH), 7.78–7.74 (m, 1H, ArH), 7.59 (d, J=8.7 Hz, 1H, ArH), 7.03–6.93 (m, 2H, ArH), 4.41 (q, J=7.1 Hz, 2H, CH_2), 4.03 (s, 2H, NH_2), 1.43 ppm (t, J=7.1 Hz, 3H, CH_3).

Ethyl 6-(quinolin-2-ylamino)-2-naphthoate (23a): Compound **23a** was prepared from **22** and **6a** in the same manner as described in the preparation of **10a** (yield: 155 mg, 74%): ^{1}H NMR (400 MHz, $CDCl_3$): δ=8.55 (s, 1H, ArH), 8.38 (d, J=1.9 Hz, 1H, ArH), 8.05 (dd, J=8.6, 1.6 Hz, 1H, ArH), 8.02–7.98 (m, 1H, ArH), 7.91 (t, J=8.7 Hz, 2H, ArH), 7.82 (d, J=8.6 Hz, 1H, ArH), 7.69 (d, J=8.0 Hz, 1H, ArH), 7.68–7.58 (m, 2H, ArH), 7.39–7.34 (m, 1H, ArH), 7.07 (d, J=8.9 Hz, 1H, ArH), 4.44 (q, J=7.1 Hz, 2H, CH_2), 1.45 ppm (t, J=7.1 Hz, 3H, CH_3).

Ethyl 6-(quinoxalin-2-ylamino)-2-naphthoate (23b): Compound **23b** was prepared from **22** and **6b** in the same manner as described in the preparation of **10b** (yield: 120 mg, 58%): ^{1}H NMR (400 MHz, $CDCl_3$): δ=8.61 (s, 1H, ArH), 8.56 (d, J=8.8 Hz, 2H, ArH), 8.09 (dd, J=8.5, 1.6 Hz, 1H, ArH), 7.98 (dd, J=8.5, 3.6 Hz, 2H, ArH), 7.92 (d, J=8.3 Hz, 1H, ArH), 7.88 (d, J=8.7 Hz, 1H, ArH), 7.75–7.64 (m, 2H, ArH), 7.59–7.51 (m, 1H, ArH), 4.45 (q, J=7.1 Hz, 2H, CH_2), 1.46 ppm (t, J=7.1 Hz, 3H, CH_3).

6-(Quinolin-2-ylamino)-2-naphthoic acid (29a): LiOH·H_2O (30 mg) was added to a solution of **23a** (0.4 mmol) in MeOH (5 mL). The resulting reaction mixture was stirred at reflux for 12 h at 40 °C. Solvent was removed under reduced pressure and was acidified to pH 5.5 by 1 N HCl to dissolve the solid, which was filtered, washed with water, and air-dried to afford **29a** (yield: 106 mg, 84%): mp: 315–318 °C; HPLC: 97%; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ=9.90 (s, 1H, NH), 9.09 (s, 1H, ArH), 8.52 (s, 1H, ArH), 8.17 (d, J=8.8 Hz, 1H, ArH), 8.07 (d, J=8.9 Hz, 1H, ArH), 7.95 (s, 2H, ArH), 7.88 (t, J=9.0 Hz, 2H, ArH), 7.81 (d, J=8.1 Hz, 1H, ArH), 7.67 (t, J=7.7 Hz, 1H, ArH), 7.38 (t, J=7.4 Hz, 1H, ArH), 7.20 ppm (d, J=8.9 Hz, 1H, ArH); MS (ESI+): m/z=315.1 $[M+H]^+$; HRMS (ESI+): m/z $[M+H]^+$ calcd for $C_{20}H_{15}N_2O_2$: 315.1134, found: 315.1128.

6-(Quinoxalin-2-ylamino)-2-naphthoic acid (29b): Compound **29b** was prepared from **23b** in the same manner as described for the preparation of **29a** (yield: 81 mg, 74%): mp: 331–334 °C; HPLC: 100%; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ=10.46 (s, 1H, ArH), 9.10 (s, 1H, ArH), 8.75 (s, 1H, ArH), 8.60 (s, 1H, ArH), 8.17 (d, J=8.9 Hz, 1H, ArH), 8.09–7.90 (m, 4H, ArH), 7.84–7.77 (m, 1H, ArH), 7.61 ppm (t, J=7.6 Hz, 1H, ArH); MS (ESI+): m/z=316.1 $[M+H]^+$; HRMS (ESI+): m/z $[M+H]^+$ calcd for $C_{19}H_{14}N_3O_2$: 316.1086, found: 316.1086.

(*E*)-*N*-Hydroxy-3-(3-(quinolin-2-ylamino)phenyl)acrylamide (30a): Sodium methoxide (100 mg sodium, 8 mL MeOH) and hydroxylamine solution (50% in H_2O, 2 mL) was added to a solution of **10a** (0.63 mmol) in MeOH (5 mL). The resulting reaction mixture was stirred at 0 °C. The solvent was removed under reduced pressure and was then poured into water and acidified to pH 5.5 by 1 N HCl to dissolve the solid, which was filtered, washed with water, and air-dried to afford **30a** (yield: 107 mg, 56%): mp: 173–177 °C; HPLC: 98%; ^{1}H NMR (400 MHz, [D$_6$]acetone): δ=8.82 (s, 1H, NH), 8.56 (s, 1H, NH), 8.07 (d, J=8.9 Hz, 1H, ArH), 7.95 (d, J=7.7 Hz, 1H, ArH), 7.84 (d, J=8.3 Hz, 1H, ArH), 7.75 (d, J=7.9 Hz, 1H, ArH), 7.63 (dd, J=15.9, 8.1 Hz, 2H, ArH), 7.35 (dt, J=17.5, 7.4 Hz, 3H, ArH), 7.22 (d, J=7.5 Hz, 1H, ArH), 7.10 (d, J=8.9 Hz, 1H, CH), 6.65 ppm (d, J=15.6 Hz, 1H, CH); MS (ESI+): m/z=306.1 $[M+H]^+$; HRMS (ESI+): m/z $[M+H]^+$ calcd for $C_{18}H_{16}N_3O_2$: 306.1243, found: 306.1237.

(*E*)-*N*-Hydroxy-3-(3-(quinoxalin-2-ylamino)phenyl)acrylamide (30b): Compound **30b** was prepared from **10b** in the same manner as described for the preparation of **30a** (yield: 65 mg, 34%): mp: 228–232 °C; HPLC: 95%; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ=10.94 (s, 1H, OH), 10.11 (s, 1H, NH), 9.11 (s, 1H, NH), 8.60 (s, 1H, ArH), 8.44 (s, 1H, ArH), 7.88 (t, J=6.7 Hz, 3H, ArH), 7.69 (t, J=7.7 Hz, 1H, ArH), 7.50 (dd, J=12.0, 3.7 Hz, 2H, ArH), 7.43 (t, J=7.9 Hz, 1H, ArH), 7.23 (d, J=7.8 Hz, 1H, CH), 6.52 ppm (d, J=15.6 Hz, 1H, CH); MS (ESI+): m/z=329.1 $[M+Na]^+$; HRMS (ESI+): m/z $[M+Na]^+$ calcd for $C_{17}H_{14}N_4O_2$: 329.1014, found: 329.1009.

***N*-Hydroxy-3-(3-(quinolin-2-ylamino)phenyl)propanamide (31a)**: Compound **31a** was prepared from **14a** in the same manner as described for the preparation of **30a** (yield: 172 mg, 90%): mp: 85–89 °C; HPLC: 99%; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ=10.44 (s, 1H, OH), 9.38 (s, 1H, NH), 8.73 (s, 1H, NH), 8.05 (d, J=8.9 Hz, 1H, ArH), 7.89 (d, J=8.0 Hz, 1H, ArH), 7.80–7.67 (m, 3H, ArH), 7.58 (t, J=7.6 Hz, 1H, ArH), 7.29 (t, J=7.4 Hz, 1H, ArH), 7.24 (t, J=7.8 Hz, 1H, ArH), 7.06 (d, J=8.9 Hz, 1H, ArH), 6.81 (d, J=7.4 Hz, 1H, ArH), 2.83 (t, J=7.8 Hz, 2H, CH_2), 2.31 ppm (t, J=7.8 Hz, 2H, CH_2); MS (ESI+): m/z=308.1 $[M+H]^+$; HRMS (ESI+): m/z $[M+H]^+$ calcd for $C_{18}H_{18}N_3O_2$: 308.1399, found: 308.1394.

***N*-Hydroxy-3-(3-(quinoxalin-2-ylamino)phenyl)propanamide (31b)**: Compound **31b** was prepared from **14b** in the same manner as described for the preparation of **30a** (yield: 188 mg, 98%): mp: 213–217 °C; HPLC: 99%; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ=10.43 (s, 1H, OH), 9.91 (s, 1H, NH), 8.74 (s, 1H, NH), 8.56 (s, 1H, ArH), 7.88 (dd, J=16.7, 8.0 Hz, 2H, ArH), 7.76 (d, J=7.0 Hz, 2H, ArH), 7.66 (t, J=7.6 Hz, 1H, ArH), 7.48 (t, J=7.5 Hz, 1H, ArH), 7.29 (t, J=7.8 Hz, 1H, ArH), 6.89 (d, J=7.2 Hz, 1H, ArH), 2.85 (t, J=7.8 Hz, 2H, CH_2), 2.35–2.29 ppm (m, 2H, CH_2); MS (ESI+): m/z=331.1 $[M+Na]^+$; HRMS (ESI+): m/z $[M+Na]^+$ calcd for $C_{17}H_{16}N_4O_2$: 331.1171, found: 331.1165.

(3-(Quinolin-2-ylamino)phenyl)methanol (25a): Compound **25a** was prepared from 3-amino-benzenemethanol and **6a** in the same manner as described for the preparation of **10a** (yield: 646 mg, 64%): ^{1}H NMR (400 MHz, [D$_6$]acetone): δ=8.60 (s, 1H, NH), 8.01 (d, J=8.9 Hz, 2H, ArH), 7.97 (s, 1H, ArH), 7.76 (d, J=8.3 Hz, 1H, ArH), 7.71 (d, J=7.8 Hz, 1H, ArH), 7.59 (dd, J=11.2, 4.1 Hz, 1H, ArH), 7.29 (t, J=7.8 Hz, 2H, ArH), 7.07 (d, J=8.9 Hz, 1H, ArH), 6.99 (d, J=7.5 Hz, 1H, ArH), 4.66 ppm (s, 2H, CH_2).

(3-(Quinoxalin-2-ylamino)phenyl)methanol (25b): Compound **25b** was prepared from **24** and **6b** in the same manner as described in the preparation of **10b** (yield: 444 mg, 58%): ^{1}H NMR (400 MHz, [D$_6$]acetone): δ=8.66 (s, 1H, NH), 8.07 (d, J=8.6 Hz, 1H, ArH), 7.99 (s, 1H, ArH), 7.78 (d, J=8.5 Hz, 1H, ArH), 7.73 (d, J=

7.7 Hz, 1H, ArH), 7.61 (dd, $J=11.4$, 4.3 Hz, 1H, ArH), 7.32 (t, $J=$ 7.6 Hz, 2H, ArH), 7.10 (d, $J=8.8$ Hz, 1H, ArH), 7.03 (d, $J=7.6$ Hz, 1H, ArH), 4.68 ppm (s, 2H, CH_2).

Diethyl 3-(quinolin-2-ylamino)benzyl phosphate (32a): Diethyl chlorophosphate (232 μL) was added dropwise to a solution of **25a** (209 mg, 0.8 mmol), triethylamine (332 μL), and 4-dimethylaminopyridine (20 mg) in dry THF (4 mL) at room temperature under N_2. The mixture was stirred for 16 h at room temperature. After the reaction was completed, it was poured into saturated $KHSO_4$ solution, which was extracted with EtOAc (20 mL). The organic layer was washed twice with saturated. $NaHCO_3$ and twice with saturated NaCl, dried over $MgSO_4$, and filtered. The filtrate was concentrated in vacuo, then purified by flash chromatography on silica gel to afford **32a** (yield: 240 mg, 78%): HPLC: 97%; ^{1}H NMR (400 MHz, [D_6]acetone): $\delta=8.31$ (d, $J=9.0$ Hz, 1H, ArH), 7.89 (d, $J=8.0$ Hz, 2H, ArH), 7.86–7.74 (m, 2H, ArH), 7.70 (s, 1H, ArH), 7.48 (dd, $J=17.0$, 8.3 Hz, 2H, ArH), 7.30 (d, $J=9.0$ Hz, 2H, ArH), 5.12 (d, $J=8.2$ Hz, 2H, CH_2), 4.15–4.05 (m, 4H, CH_2), 1.28 ppm (t, $J=6.8$ Hz, 6H, CH_3); MS (ESI+): $m/z=387.1$ $[M+H]^+$; HRMS (ESI+): m/z $[M+H]^+$ calcd for $C_{20}H_{24}N_2O_4P$: 387.1474, found: 387.1468.

Diethyl 3-(quinoxalin-2-ylamino)benzyl phosphate (32b): Compound **32b** was prepared from **25b** in the same manner as described for the preparation of **32a** (yield: 205 mg, 33%): HPLC: 98%; ^{1}H NMR (400 MHz, [D_6]acetone): $\delta=9.28$ (s, 1H, NH), 8.56 (s, 1H, ArH), 8.20 (s, 1H, ArH), 8.07 (d, $J=8.1$ Hz, 1H, ArH), 7.88 (d, $J=$ 8.2 Hz, 1H, ArH), 7.83 (d, $J=8.3$ Hz, 1H, ArH), 7.67 (t, $J=7.6$ Hz, 1H, ArH), 7.49 (t, $J=7.5$ Hz, 1H, ArH), 7.40 (t, $J=7.8$ Hz, 1H, ArH), 7.12 (d, $J=7.5$ Hz, 1H, ArH), 5.11 (d, $J=8.1$ Hz, 2H, CH_2), 4.11 (q, $J=$ 7.3 Hz, 4H, CH_2), 1.29 ppm (t, $J=7.0$ Hz, 6H, CH_3); MS (ESI+): $m/z=$ 388.1 $[M+H]^+$; HRMS (ESI+): m/z $[M+H]^+$ calcd for $C_{19}H_{23}N_3O_4P$: 388.1426, found: 388.1421.

Water solubility

For each suspension, 2 mg of compound was weighed, followed by the addition of 1.5 mL ultra-pure water, then the mixture was sealed and shaken for 24 h. The suspensions were centrifuged at 12000 rpm (Eppendorf Centrifuge 5430) for 10 min to separate solid materials from the solution. The supernatant was not retained. After filtration through a filter membrane (0.45 μm), the concentration of the filtrate was determined by HPLC analysis.

An Agilent 1260 liquid chromatography system (Agilent Technologies, Germany) with a UV detector monitored at 280 nm was used. Compounds were separated by using a reversed-phase C_{18} analytical column (Zorbax Eclipse Plus, 4.6×150 mm, 5 μm particle size). Mobile phases A and B consist of water with 0.1% TFA and acetonitrile, respectively. An optimized gradient elution was used for HPLC separation: 0–3 min, 30% B; 3–13 min, 10% B; 13–15 min, 30% B. The flow rate used was 1.0 mL min^{-1}. The column temperature was kept at 25 °C. Samples were injected (injection volume: 10 μL) on to the column.

Biology

General: All solvents and reagents of reagent grade or ultra-pure quality were purchased commercially and used without further purification. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen. Human brain vascular smooth muscle cells (HBVSMCs) were purchased from ScienCell. Phenylephrine (PE), as well as oleoyl-L-α-lysophosphatidic acid (LPA) and dimethyl sulfoxide (DMSO), were purchased from Sigma. (*R*)-(−)-phenylephrine hydrochloride (PE) was purchased from Wako Chemicals. The RhoA G-LISA activation assay biochem kit was purchased from Cytoskeleton.

Animals: The animal use and care protocols, including all operation procedures, were approved by the Animal Care and Use Committee of Soochow University and conformed to the Guide for the Care and Use of Laboratory Animals by the National Institute of Health, China. Male Sprague–Dawley rats weighing 300–350 g were purchased from the Animal Center of the Chinese Academy of Sciences (Shanghai, China). They were acclimated in a humidified room and maintained on a standard pellet diet at the Animal Center of Soochow University for at least 7 days. The temperature in both the feeding room and the operation room was maintained at 25±1 °C.

RhoA activation assay: RhoA activity was measured by a RhoA activation assay as described previously.[22] Briefly, when HBVSMCs reached 70% confluence, the cells were starved overnight in serum-free DMEM (0% FBS). Then, 1.25 μL of chemical compound or DMSO (final concentration of compound: 2.5 μM) was added to the cells. After an additional 1 h of incubation at 37 °C, cells were treated with or without LPA (5 μg mL^{-1}) for 3 min. Following treatment, the cells were washed in ice-cold PBS and lysed. Thereafter, RhoA activity was measured from the cell lysates using the absorbance-based RhoA G-LISA activation assay biochem kit (Cytoskeleton) according to the manufacturer's protocol. Active RhoA was detected using indirect immune detection, followed by a colorimetric reaction, measured by absorbance at 490 nm. The inhibitory rate (IR) was calculated using Equation (1), in which L represents DMSO + LPA, C represents compound + LPA, and D is DMSO.

$$IR = \frac{OD_L - OD_C}{OD_L - OD_D} \quad (1)$$

Isometric contraction measurement: Isometric contraction was measured in rings from the thoracic aortic rings as previously described.[25] Briefly, isolated aortic rings, equilibrated with modified K-H and 95% O_2, 5% CO_2 at 37 °C, were initially placed under the optimal resting tension of 1.5 g for 60 min until a stable rest tension was achieved. The pharmacological effects of compounds were studied on rings submaximally contracted with 0.1 mM PE. Once a steady contraction was observed, one aorta ring was treated with compound (100 mM, dissolved in 100% DMSO) added in a cumulative manner (final concentration of 30 μM–150 μM), whereas another ring of each vessel received only the same volume of solvent (DMSO, 3 μL–15 μL) and was used as a paired time control. The concentration of compound was increased once the maximal relaxant effect of the preceding concentration was recorded. Isometric tension was recorded by an analogue-to-digital system (ALCB10 MPA2000, Shanghai Alcott Biotech, Shanghai, China) connected to a desktop computer.

Subarachnoid hemorrhage (SAH) model: SAH was induced by the double-hemorrhage pre-chiasmatic injection model in rats as previously described.[26] Briefly, after the rats were anesthetized with 4% chloral hydrate (400 mg kg^{-1} body weight), they were positioned prone in a stereotactic frame. After careful disinfection, a midline scalp incision was made, and a 1 mm hole was drilled 7.5 mm anterior to the bregma in the midline, at angle of 30° caudally. Non-heparinized, freshly autologous blood (0.2 mL) from the femoral artery was then injected aseptically into the pre-chiasmatic cistern through this burr hole over a period of 30 s. Immediately after the injection of blood, the hole was sealed with wax to prevent leak-

age. The animals were tilted at a 30° for 30 min with their heads down, in a prone position, to permit pooling of blood around the BA. Afterward, the rats were returned to their cages, the room temperature was kept at 25 ± 1 °C, and 20 mL of 0.9% NaCl was injected subcutaneously to prevent dehydration. A second blood injection was administered 48 h after the first injection by the same procedure.

Fifty-two rats were assigned randomly to seven groups: control group ($n=6$), SAH group ($n=8$), SAH + vehicle (DMSO) group ($n=6$), SAH + fasudil group ($n=8$), SAH + low dose of **26b** group ($n=8$), SAH + medium dose of **26b** group ($n=8$), and SAH + high dose of **26b** group ($n=8$). In our pilot trial, we found that fasudil at a dose of 0.08 mg kg^{-1} (15.2 mM, 5 μL) significantly prevented vasoconstriction in the rat BA via intrathecal injection. Therefore, we chose this dose for fasudil as positive control in SAH-CVS model. Considering the IC_{50} value of compound **26b** was ~25-fold greater than that of fasudil in an isolated arterial rings assay (Table 4); the high dose of compound **26b** was set at 380 mM in the same proportion. Both fasudil and **26b** were dissolved in DMSO, and the final concentrations were 15.2 mM (fasudil), 23.7 mM (low dose), 95 mM (medium dose), and 380 mM (high dose), respectively. The first SAH induction was defined as day 1. A volume of 5 μL of the fasudil or **26b** was administered directly into the cisterna magna on day 4 and day 5, while vehicle animals received an equal volume of DMSO.

The rats were re-anesthetized and euthanized on day 6. They were perfused with 250 mL of phosphate-buffered saline solution under a pressure of 100 cm H_2O. The BAs in each group were immediately removed and placed in the fixative solution (a mixture of 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4) for 24 h for morphometric analysis.

Morphometric measurements: The luminal perimeter of BAs for each specimen was measured using a digitized image analysis system (BX40, Olympus, Japan) with Image-pro Plus v6.0 software. Specimens for the light microscopy study were dehydrated in graded ethanol, embedded in paraffin, sectioned at a thickness of 3 μm using a microtome, and stained with hematoxylin and eosin. Light microscopic sections of arteries were projected as digitized video images. The inner perimeters of the vessels were measured by tracing the luminal surface of the intima. The two measurements were averaged.

Statistical analysis

All data were presented as means ± SD. SPSS 16.0 was used for statistical analysis. Differences between the two groups were analyzed using a two-tailed unpaired Student's t test. The differences among multiple groups were assessed using a one-way analysis of variance. Statistical significance was accepted at $p < 0.05$.

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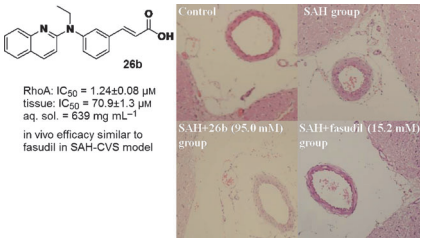
Whoa RhoA! Second-generation small-molecule RhoA inhibitors were obtained by structural modifications to the aniline nitrogen and acid side chain of lead compounds. Among 32 compounds synthesized and tested in biological assays, one compound was identified as the most potent and water soluble, showing good in vitro and in vivo efficacy.



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Development of Second-Generation Small-Molecule RhoA Inhibitors with Enhanced Water Solubility, Tissue Potency, and Significant in vivo Efficacy