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Novel amidrazone derivatives: Design, synthesis and activity evaluation

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ABSTRACT

A series of new 6-styryl-naphthalene-2-amidrazone derivatives were synthesized and evaluated as potential ASIC1a inhibitors. Among them, compound **5e** showed the most activity to inhibit $[Ca^{2+}]_i$. elevation in acid-induced articular chondrocytes. Together with the important role of ASIC1a in the pathogenesis of tissue acidification diseases including rheumatoid arthritis, these results might provide a meaningful hint or inspiration in developing drugs targeting at tissue acidification diseases.

1. Introduction

Rheumatoid arthritis is a systemic autoimmune disease, which is characterized by inflammation synovitis and progressive joint damage mainly in the small diarthrodial joints of the hands and feet.[1,2] Oxygen consumption and the metabolic rate are increased in inflamed areas, thus promoting lactic acid secretion leading to a drop in pH.[3] Chronic inflammatory conditions such as RA exhibit a degree of acidosis in the articular synovial fluid (SF), and the pH of the SF may drop to < 6.0 in patients with active RA.[4,5] Importantly, a low SF pH was shown to correlate with radiological joint destruction in RA patients.[6] And cartilage homeostasis is affected by the local pH.[7,8]

Local tissue acidosis subsequently activates some ion channels such as acid sensing ion channels (ASICs), further leads to the occurrence of diseases. ASICs, belonging to the degenerin/epithelial sodium channel (DEG/ENaC) superfamily, can be activated by proton under acid condition. To date, at least seven different ASIC subunit proteins (ASIC1a, ASIC1b, ASIC1b2, ASIC2a, ASIC2b, ASIC3, ASIC4) have been cloned and identified in mammalian.[9] They share the same topology structures of their subunits (two transmembrane domains, a large extracellular loop, and short intracellular N and C-termini), and the majority of ASIC subunit can form amiloride-sensitive cation channels.[10] Among them, ASIC1a is special under acid condition on account of elevation intracellular $[Ca^{2+}]$.[11] Intracellular Ca^{2+} ($[Ca^{2+}]_i$) is a ubiquitous second messenger in signal transduction pathways that modulates diverse physiological functions and excessive $[Ca^{2+}]_i$ can cause a series of pathological reaction. Our and other labs have recently reported that extracellular acidification could induce articular chondrocyte injury through acid-sensing ion channel 1a (ASIC1a)-mediated intracellular calcium ($[Ca^{2+}]_i$) overload, which suggested that ASIC1a in chondrocytes may be a potential therapeutic target for articular cartilage destruction in RA.[12,13] In support of this, pharmacological blockade of ASIC1a or deletion of the ASIC1 gene has a protective effect for tissue acidification disease.

As we all know, amiloride **1** is a weak, non-selective ASIC1 inhibitor with the IC_{50} 30.2 μM.[14,15] Based on this scaffold, focused on ASIC1, many analogues have been designed and synthesized. Among them, amidine A-317567 **2** was a potent blocker of ASIC1a than amiloride with IC_{50} 2.0 μM which was determined by the patch clamp using dissociated adult rat dorsal root ganglion neurons.[16] Compounds **3** and **4** also showed the potent activity against ASIC1a[17](Fig. 1). Besides, there are already several venom peptides with much greater selectivity than small molecules, such as PcTx1,[18] which was isolated from the venom of the South American tarantula *Psalmopoeus cambridgei*, of psalmotoxin 1. And there is also a small molecule ASIC inhibitor NS383 that is claimed to be selective for ASIC1a and ASIC3.[19] These compounds are not selective for the ASIC1a except PcTx1.

However, these active compounds above give us preliminary structure-activity relationships. We found that there was a common amidine group with a large polarity, which was easier to form hydrogen bond with the ion channel protein. Therefore, based on this, target compounds were designed by adding an amino group to form another larger polarity group amidrazone. Moiety of pyrazine-2,6-diamine or 1,2,3,4-tetrahydroisoquinoline (compounds **1**,

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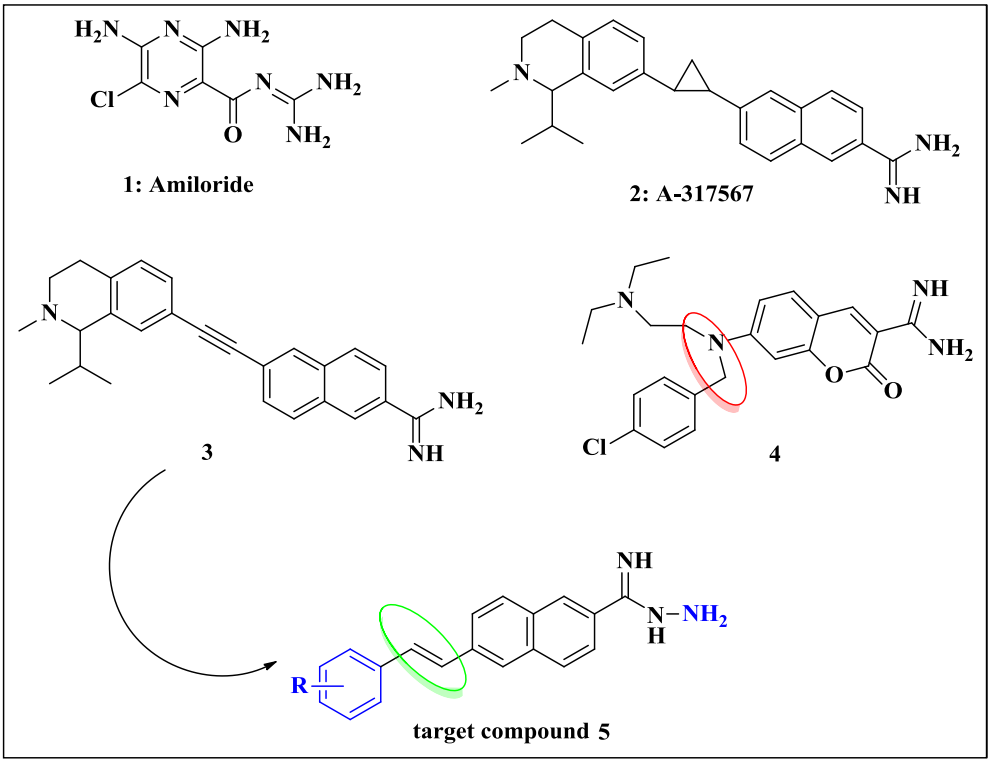


Fig. 1. Preliminary structure-activity relationships as for target compounds.

2, **3**, Fig. 1) containing nitrogen may be detrimental to selectivity, more nitrogen of the six membered ring, the more weak to the activity (compounds **1**, **2**), therefore a simple benzene ring was used in this study. The authors reported that the isomeric *Z*-olefin compound was ~10-fold less potent than the *E*-olefin compound, and the reduction of the double bond to alkane compound also led to a marked decrease in ASIC-3 inhibition. From this point of view, we designed and synthesized E-olefin compound for ASIC1a for the reason that ASIC1a and ASIC-3 belong to the same super family.

Based on all above, to find efficient lead compound with ASIC1a inhibition activity, we designed and synthesized a series of 6-styryl-naphthalene-2-amidrazone derivatives. In order to evaluate whether the compounds had ASIC1a inhibition activity, we used the laser scanning confocal microscope to observe the effect of target compounds on $[Ca^{2+}]_i$ in rat articular chondrocytes. We hope that the designed lead compounds may shed light on ASIC1a inhibitors for medicinal chemistry in the field.

2. Results and discussion

2.1. Chemistry

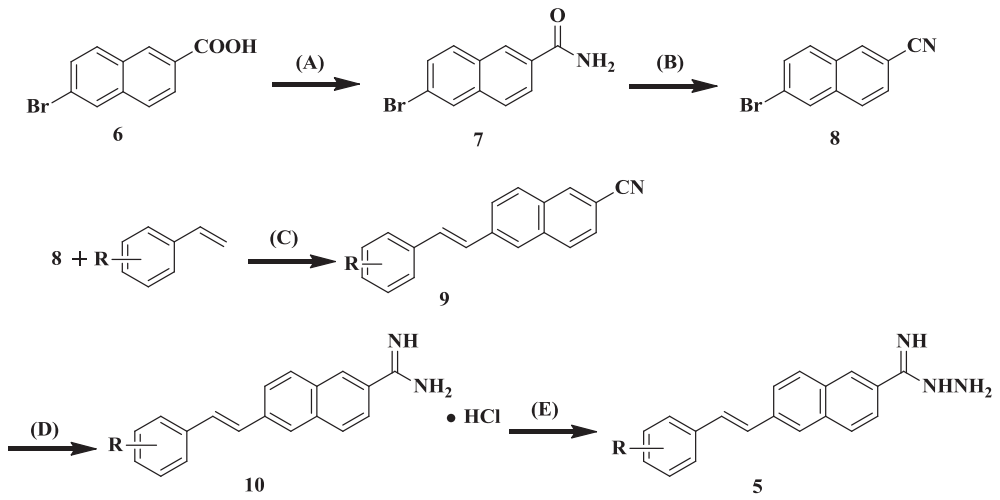
6-Styryl-naphthalene-2-amidrazones (target compound **5**) were synthesized according to the protocol outlined in Scheme 1. Among them, compound **7** (6-bromo-naphthalene-2-carboxylic acid amide) was prepared starting from a condensation of 6-bromo-2-naphthoic acid with the ammonia solution. Then compound **9** was synthesized from a Heck reaction of compound **8** with styrene or substituted styrene. Subsequently, compound **9** was treated with $LiN(TMS)_2$ to produce compound **10** and then compound **10** was reacted with hydrazine hydrate to gain the target compound **5**. Details of the synthesis process and structural characterization were shown in support information.

2.2. Activity evaluation of blocking ASIC1a

Whether direct Ca^{2+} entry through ASIC1a channels makes a substantial and direct contribution to $[Ca^{2+}]_i$ homeostasis is still debatable in the some cells now.[20,21] However, under appropriate acid condition, activation of ASIC1a receptors can potentially cause elevation intracellular Ca^{2+} in rat articular chondrocytes. Therefore, if ASIC1a is blocked, the level of Ca^{2+} is lower than non-blocking group. The potential effects of the synthesized compounds on blocking ASIC1a activity were investigated by observing $[Ca^{2+}]_i$ in acid-induced rat articular chondrocytes, which were measured by laser scanning confocal microscopy. For this purpose, we initially observed the effects of compound **5** on chondrocytes viability to determine concentration for the subsequent experiments. MTT assays showed that apart from compound **5h** and compound **5e** with the concentration of 50 μM, all other compound **5** had no significant influence on the viability of chondrocytes with the concentration of 1.563–25 μM (compounds **5a**, **5b**, **5d**, **5f**, **5g**) and 3.125–25 μM (compounds **5c**, **5e**) by a 24 h treatment (Fig. 2). Therefore, we selected those concentrations to observe their effects on ASIC1a function in rat articular chondrocytes.

2.2.1. Compound *5* decrease acid-induced protein expression of ASIC1a

In order to test whether compound **5** could inhibit acid-induced protein expression of ASIC1a, we observed the effect of different pH and time on ASIC1a protein expression. Western blotting results showed that the ASIC1a protein expression in articular chondrocytes was remarkably increased as the medium pH decreased, with no significant difference between pH 6.0 and pH 5.5. The expression of ASIC1a protein increased within 3 h, but gradually decreased after 3 h (Fig. 3). Exposured to acidic medium (pH 6.0) for 3 h, the acidosis condition was selected as the following western blotting experiments. Then, cultured chondrocytes were pretreated with compound **5** for 24 h followed by stimulation with acidic medium (pH 6.0) for 3 h. Western blotting results



Scheme 1. Synthesis of compound **5**. **R** = H (**5a**); **R** = 4-F (**5b**); **R** = 4-Cl (**5c**); **R** = 2-F (**5d**); **R** = 4-OCH_3 (**5e**); R = 3-F (**5f**); **R** = 3-Cl (**5g**); **R** = 4-CH_3 (**5h**). Reagent and Conditions: (**A**) $(COCl)_2$, CH_2Cl_2, rt; $NH_3{\cdot}H_2O$, ice bath; (**B**) $POCl_3$, 80 °C; (**C**) $Pd(OAc)_2$, 1,1′-Bis(diphenylphosphino) ferrocene, Cs_2CO_3, DMF, 90 °C; (**D**) $LiN(TMS)_2$, THF, rt, overnight; 2 M EtOH/HCl, rt, 12 h; (**E**) $N_2H_4{\cdot}H_2O$, MeOH, rt.

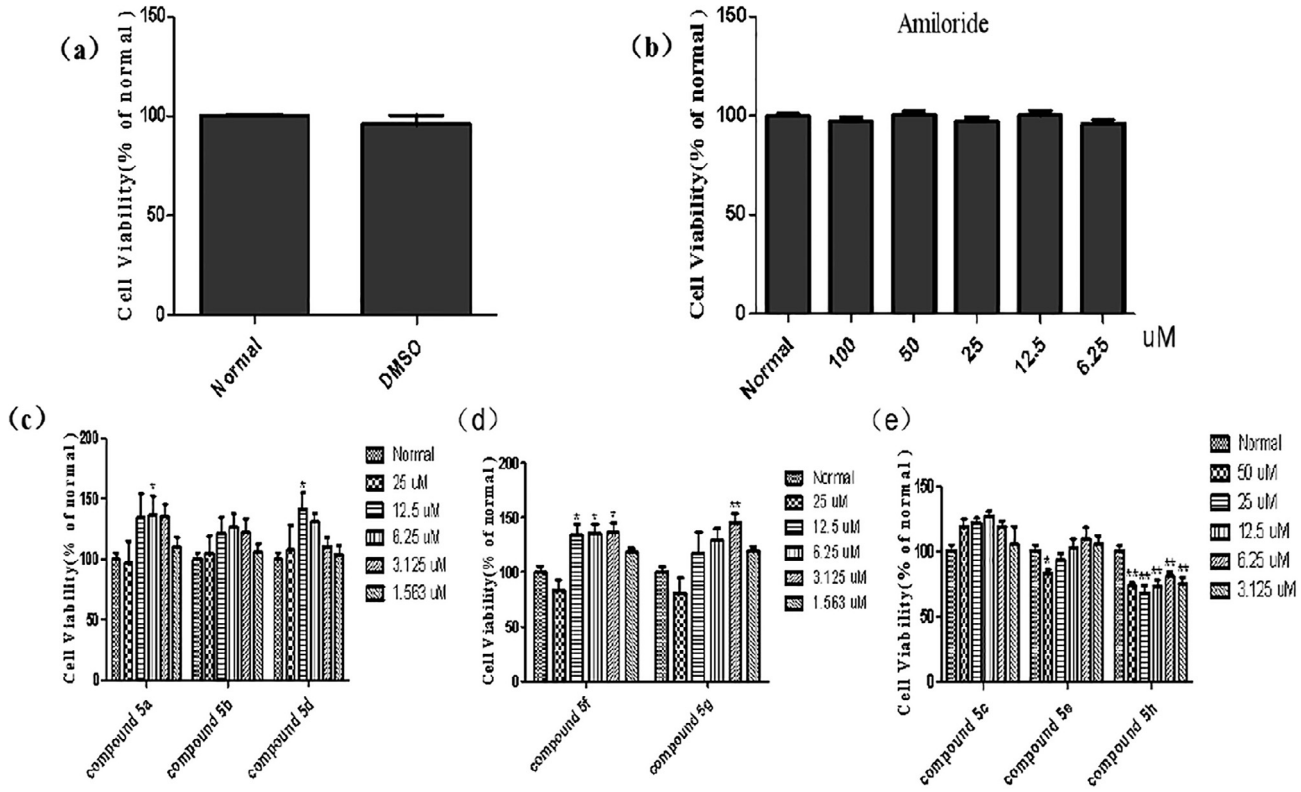


Fig. 2. Effect of target compounds **5** on articular chondrocytes viability. (**a**) DMSO group (5‰). (b) Amiloride (100, 50, 25, 12.5 and 6.25 μM). (**c**) Compounds **5a**, **5b** and **5d** (25, 12.5, 6.25, 3.125 and 1.563 μM). (**d**) Compounds **5f** and **5g** (25, 12.5, 6.25, 3.125 and 1.563 μM). (**e**) Compounds **5c**, **5e** and **5h** (50, 25, 12.5, 6.25 and 3.125 μM). Cell viability was determined by MTT assay. Data were expressed as means ± SE obtained in three independent experiments.$^{*}p < 0.05$, $^{**}p < 0.01$ versus normal group.

showed that with addition compound **5b**, most of the compounds could observably decreased the acid-induced protein expression of ASIC1a and the highest concentration group had similar effect on inhibition of ASIC1a protein expression compared with positive drug amiloride group (Fig. 4). *Para*-F, the strong electron-withdrawing group in compound **5b** may be the reason that the compound had no effect on ASIC1a expression compared with the other compound **5**. The strong electron-withdrawing group significantly decrease cloud density of benzene ring. This should be affect the pi-pi interaction between the title compound and the target.

*2.2.2. Compound **5** inhibit $[Ca^{2+}]_i$ elevation in acid-induced articular chondrocytes*

In order to further investigate the roles of compound **5** in influencing ASIC1a function, we tested the effect of compound **5** and amiloride, the ASIC1a non-specific inhibitor, on the acid-induced articular chondrocytes. In our all experiments, 5 μM nimodipine

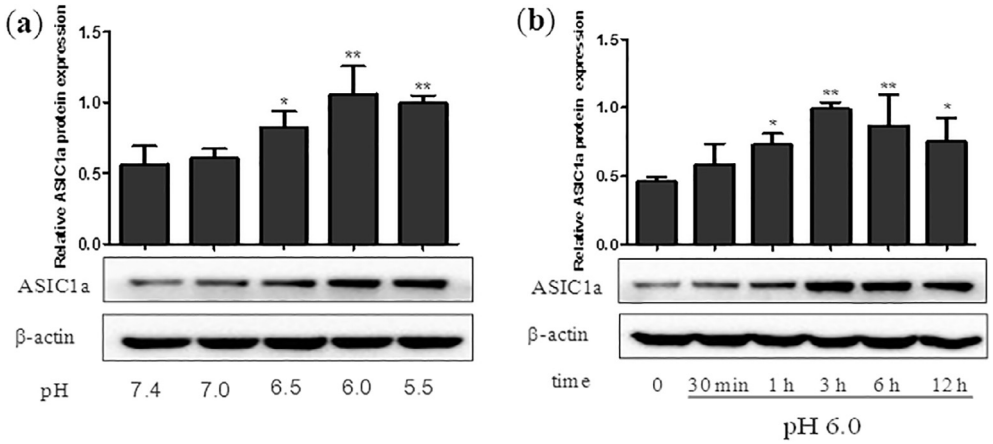


Fig. 3. Effect of different pH and time point on the protein expression of ASIC1a. (**a**) Different pH group. (**b**) Different time point under pH 6.0. Cultured chondrocytes were treated with different pH medium or different time point under pH 6.0. The level of ASIC1a protein was assessed by Western blotting. β-Actin was chosen as an internal control. Data were presented as mean ± SD obtained in three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ versus normal group (pH 7.4).

was added to all solutions as a blocker of voltage-gated Ca^{2+}-channels to inhibit possible secondary activation of channels and release of the internal Ca^{2+} stores. From the laser scanning confocal micrographs and the representative traces, the results showed that pH 6.0 solution significantly increased $[Ca^{2+}]_i$ in articular chondrocytes. Furthermore, pretreated with compound **5** or blockade of ASIC1a with amiloride could significantly reduce the fluorescence intensity of intracellular calcium by acid-induced (Fig. 5). Among the tested compounds and amiloride, inhibition rate of Ca^{2+} fluorescence intensity was 20.83% (**5c**, 50 μM), 33.37% (**5a**, 25 μM), 52.07% (**5d**, 25 μM), 59.51% (**5g**, 25 μM), 63.23% (**5h**, 50 μM) 68.92% (**5e**, 25 μM), and 23.49% (amiloride, 100 μM). Results showed that most compounds were superior to amiloride and compound **5e** was the most potent.

3. Conclusion

In summary, we designed and synthesized some 6-styryl-naphthalene-2- amidrazone derivatives as potential ASIC1a inhibitors, followed by chemical synthesis and biological evaluated for them. Among them, compound **5e** exhibited strong inhibition effect on ASIC1a, whose inhibition rate was 68.92% at 25 μM. These results preliminarily showed that some title compounds had potential inhibition effect on function, which suggested that 6-styryl- naphthalene-2-amidrazone derivatives may have a potential blocking effect of ASIC1a, but this still need further experiments to confirm through patch clamp and their selectivity on ASICs also require further study.

Together with the important role of ASIC1a in the pathogenesis of tissue acidification diseases including rheumatoid arthritis, we hope that these results might provide a meaningful hint or inspiration in developing drugs targeting at tissue acidification diseases.

4. Experimental section

4.1. Chemistry

The degree of reactions was monitored by thin layer chromatography (TLC) on Merck pre-coated silica GF254 plates. Melting points (approximately) were confirmed by a XT4MP apparatus (Taike Corp., Beijing, China). ^{1}H NMR and ^{13}C NMR spectra were collected on PX400 spectrometer at room temperature with TMS and solvent signals allotted as internal standards. Chemical shifts were reported in ppm (d). Mass spectra were performed on an Agilent 1260–6221 TOF mass spectrometer.

4.1.1. General procedure for the synthesis of target compounds *5a–h*

To a solution of the compound **10** (0.3 mmol) in methanol (10 mL) was added to 80% hydrazine hydrate (0.9 mmol). The reaction mixture was stirred at room temperature overnight monitored by TLC (methylene chloride, methanol, V/V = 10:1). As the reaction progressed, solid was continuously produced. The product was collected by filtration to give compound **5a**–**h** as colorless solids.

5a: 6-Styryl-naphthalene-2-amidrazone, light yellow solid, yield, 28%, mp 221–222 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.15 (s, 1H), 8.01–7.77 (m, 5H), 7.66 (d, J = 7.5 Hz, 2H), 7.49–7.37 (m, 4H), 7.29 (dd, J = 14.7, 7.3 Hz, 1H), 5.76 + 5.23 (2brs, 4H, —C(=NH)NHNH$_2$). ESI-MS: 288.1 ($C_{19}H_{17}N_3$, $[M+H]^+$).

5b: 6-[2-(4-Fluoro-phenyl)-vinyl]-naphthalene-2-amidrazone, light yellow solid, yield, 31%; mp 235–236 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.15 (s, 1H), 7.98–7.77 (m, 5H), 7.71 (dd, J = 8.0, 5.9 Hz, 2H), 7.42 (d, J = 16.6 Hz, 1H, —CH=CH—), 7.36 (d, J = 16.6 Hz, 1H, —CH=CH—), 7.24 (t, J = 8.7 Hz, 2H), 5.75 + 5.25 (2brs, 4H, —C(=NH)NHNH$_2$). ESI-MS: 306.1 ($C_{19}H_{16}FN_3$, $[M+H]^+$).

5c: 6-[2-(4-Chloro-phenyl)-vinyl]-naphthalene-2-amidrazone, light yellow solid, yield, 25%, mp 249–250 °C; ^{1}H NMR (600 MHz, DMSO-d_6) δ: 8.14 (s, 1H), 7.99–7.77 (m, 5H), 7.67 (t, J = 8.6 Hz, 2H), 7.48–7.35 (m, 4H), 5.95 + 5.74 (2brs, 4H, —C(=NH)NHNH$_2$). HR-ESI-MS: m/z $[M+H]^+$ calcd for $C_{19}H_{16}ClN_3$: 322.1106; found: 322.1105.

5d: 6-[2-(2-Fluoro-phenyl)-vinyl]-naphthalene-2-amidrazone, light yellow solid, yield, 33%; mp 239–240 °C; ^{1}H NMR (600 MHz, DMSO-d_6) δ: 8.14 (s, 1H), 8.01 (s, 1H), 7.91 (d, J = 8.7 Hz, 1H), 7.85 (dt, J = 28.0, 8.7 Hz, 4H), 7.49 (d, J = 16.5 Hz, 1H, —CH=CH—), 7.42 (d, J = 16.5 Hz, 1H, —CH=CH—), 7.33 (dd, J = 13.6, 7.2 Hz, 1H), 7.24 (t, J = 8.3 Hz, 2H), 5.80 + 5.25 (2brs, 4H, —C(=NH)NHNH$_2$). HR-ESI-MS: m/z $[M+H]^+$ calcd for $C_{19}H_{16}FN_3$: 306.1401; found: 306.1403.

5e: 6-[2-(4-Methoxy-phenyl)-vinyl]-naphthalene-2-amidrazone, light yellow solid, yield, 71%; mp 228–229 °C; ^{1}H NMR (600 MHz, DMSO-d_6) δ: 8.12 (s, 1H), 7.93–7.86 (m, 2H), 7.81 (dt, J = 20.6, 8.6 Hz, 3H), 7.58 (d, J = 8.7 Hz, 2H), 7.33 (d, J = 16.4 Hz, 1H, —CH=CH—), 7.24 (d, J = 16.4 Hz, 1H, —CH=CH—), 6.96 (t, J = 6.9 Hz, 2H), 5.84 + 5.26 (2brs, 4H, —C(=NH)NHNH$_2$), 3.77 (s, 3H, —OCH$_3$). HR-ESI-MS: m/z $[M+H]^+$ calcd for $C_{20}H_{19}N_3O$: 318.1601; found: 318.1601.

5f: 6-[2-(3-Fluoro-phenyl)-vinyl]-naphthalene-2-amidrazone, light yellow solid, yield, 65%; mp 261–262 °C; ^{1}H NMR (600 MHz, DMSO-d_6) δ: 8.14 (s, 1H), 7.97 (d, J = 7.4 Hz, 1H), 7.93–7.89 (m, 1H), 7.83 (ddd, J = 13.4, 12.6, 6.9 Hz, 3H), 7.51 (d, J = 7.9 Hz, 1H), 7.48–7.45 (m, 2H), 7.44–7.36 (m, 2H), 7.10 (t, J = 8.4 Hz, 1H), 5.80 + 5.25 (2brs, 4H, —C(=NH)NHNH$_2$). HR-ESI-MS: m/z $[M+H]^+$ calcd for $C_{19}H_{16}FN_3$: 306.1401; found: 306.1401.

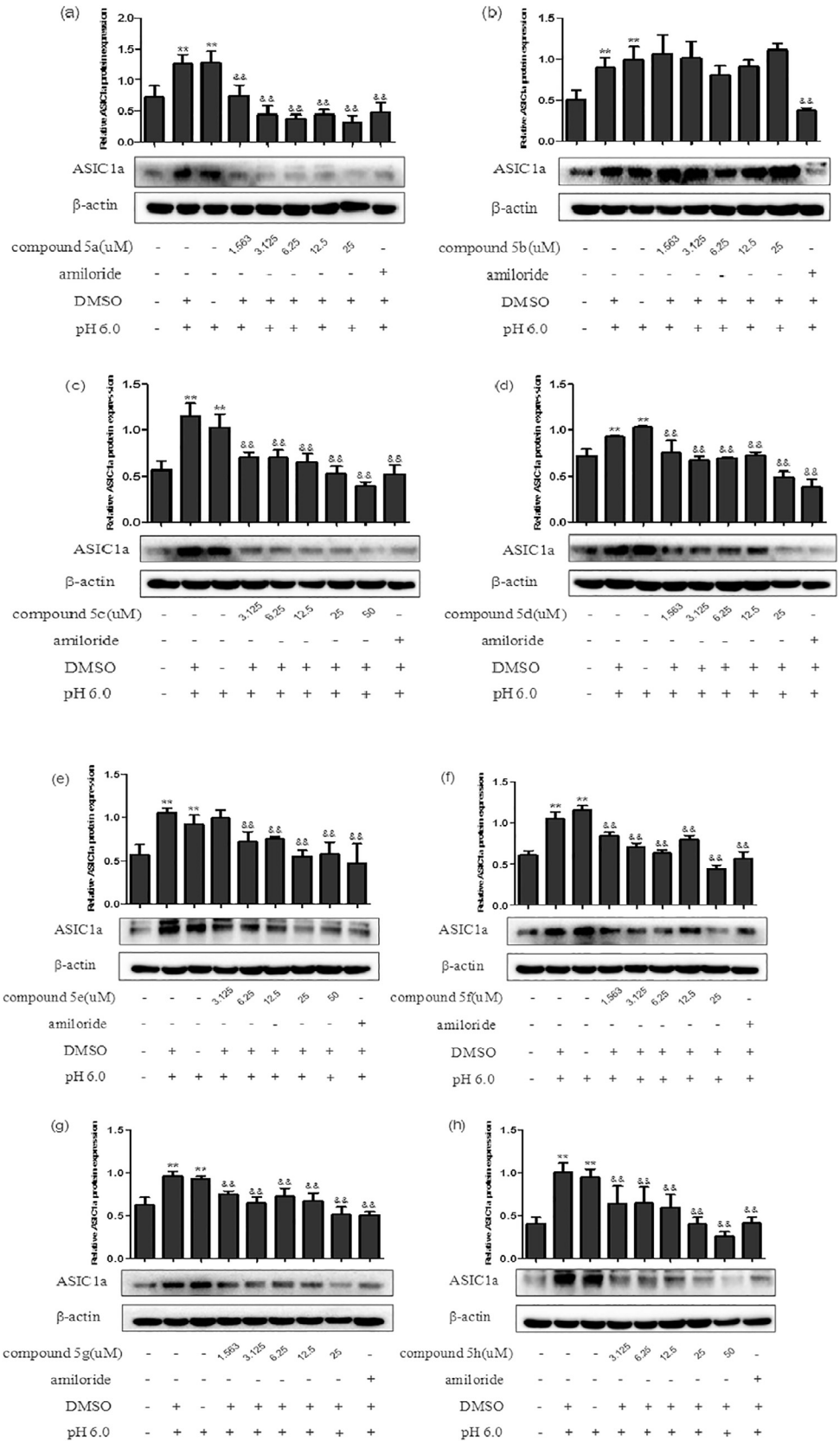


Fig. 4. Target compound **5** and amiloride (100 μM) inhibited acid medium (pH 6.0) modulation of ASIC1a protein expression. Cultured chondrocytes were pretreated with or without target compound for 24 h followed by stimulation with acid medium (pH 6.0) for 3 h (Fig. 4a–h). The level of ASIC1a protein was assessed by western blotting. β-Actin was chosen as an internal control. Data were presented as mean ± SD obtained in three independent experiments. $^{**}p < 0.01$ versus control group; $^{\&\&}p < 0.01$ versus pH 6.0 + DMSO group.

5g: 6-[2-(3-Chloro-phenyl)-vinyl]-naphthalene-2-amidrazone, light yellow solid, yield, 37%; mp 213–214 °C; ^{1}H NMR (600 MHz, DMSO-d_6) δ: 8.16 (s, 1H), 8.00 (s, 1H), 7.97–7.81 (m, 4H), 7.75 (s, 1H), 7.62 (t, J = 8.2 Hz, 1H), 7.53 (d, J = 16.5 Hz, 1H), 7.42 (dd, J = 19.6, 12.0 Hz, 2H), 7.34 (d, J = 7.9 Hz, 1H), 5.94 + 5.39 (2brs, 4H, —C(=NH)NHNH$_2$). HR-ESI-MS: m/z [M+H]$^+$ calcd for $C_{19}H_{16}ClN_3$: 322.1106; found: 322.1106.

5h: 6-(2-p-Tolyl-vinyl)-naphthalene-2-amidrazone, light yellow solid, yield, 25%; mp 243–244 °C; ^{1}H NMR (600 MHz, DMSO-d_6) δ: 8.13 (s, 1H), 7.95–7.77 (m, 5H), 7.54 (t, J = 8.5 Hz, 2H), 7.40–7.33 (m, 2H), 7.20 (d, J = 7.7 Hz, 2H), 5.92 (s, 2H), 5.35 (s, 2H), 2.30 (s, 3H, —CH$_3$). HR-ESI-MS: m/z [M+H]$^+$ calcd for $C_{20}H_{19}N_3$: 302.1652; found:302.1650.

4.2. Isolation of articular chondrocytes and treatment

Rat articular chondrocytes were isolated using the method described previously.[22] Cartilage tissue from knee joint of three or four rats was merged for each isolation. Cartilage from the knee joint was cutted into small pieces (about 1 × 1 mm) and firstly digested with Trypsin-EDTA Solution (Beyotime, Hangzhou, China) and then digested with 0.2% type II collagenase (Sigma Chemical

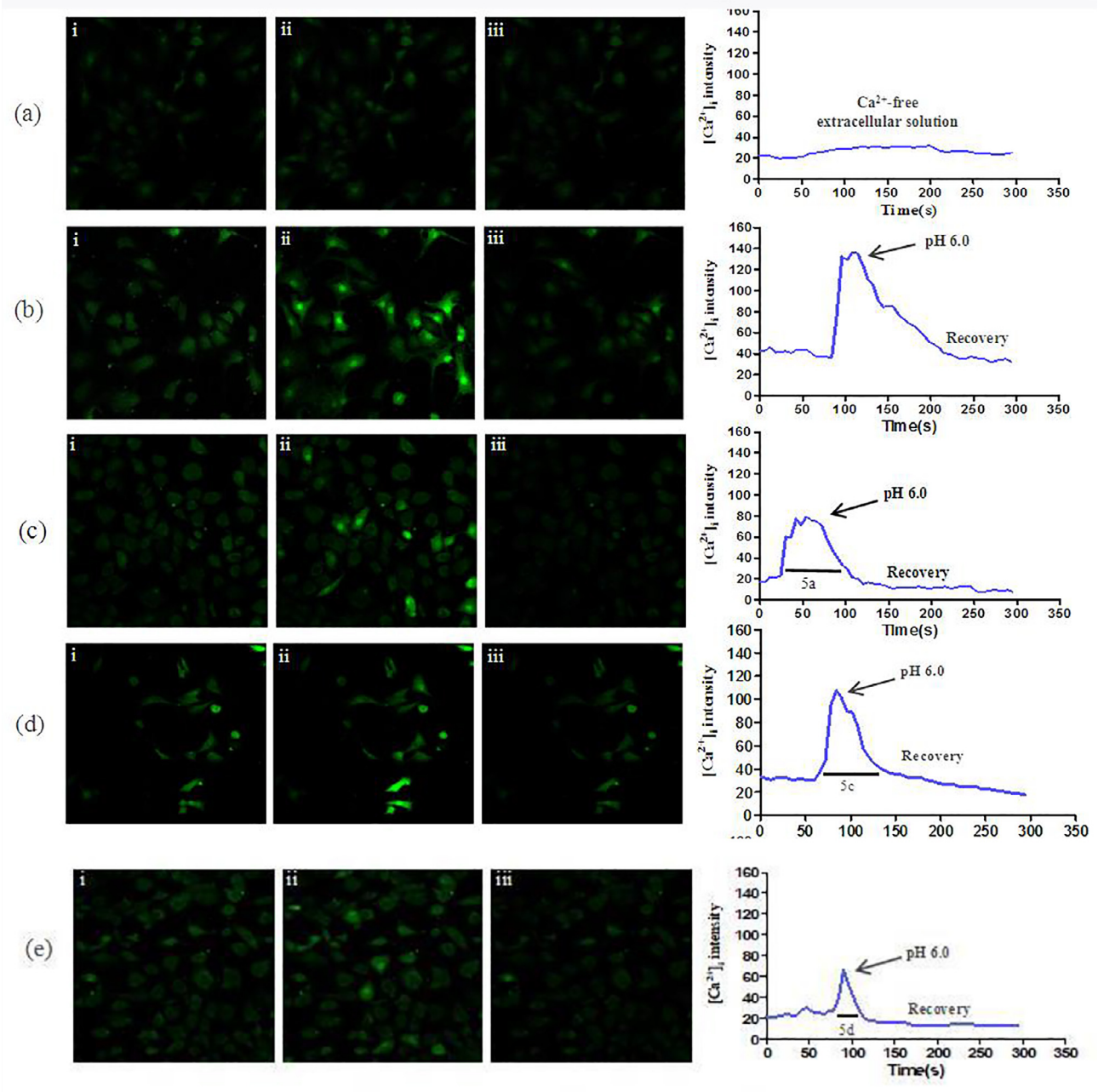


Fig. 5. Blockade of ASIC1a with compounds **5** and amiloride reduced acid-induced elevation of $[Ca^{2+}]_i$ level in articular chondrocytes. Cellular confocal micrographs of the same scale showed the changes in the $[Ca^{2+}]_i$ intensity, as visualized by Fluo-3-AM, in articular chondrocytes. (**a**) Acid-induced elevation of $[Ca^{2+}]_i$ in Ca^{2+}-free extracellular solution; (**b**) Acid-induced elevation of $[Ca^{2+}]_i$ in extracellular Ca^{2+} solution; (**c**) Acid-induced elevation of $[Ca^{2+}]_i$ in chondrocytes treated with compound **5a** (25 μM); (**d**) Acid-induced elevation of $[Ca^{2+}]_i$ in chondrocytes treated with compound **5c** (50 μM); (**e**) Acid-induced elevation of $[Ca^{2+}]_i$ in chondrocytes treated with compound **5d** (25 μM); (**f**) Acid-induced elevation of $[Ca^{2+}]_i$ in chondrocytes treated with compound **5e** (25 μM); (**g**) Acid-induced elevation of $[Ca^{2+}]_i$ in chondrocytes treated with compound **5g** (25 μM); (**h**) Acid-induced elevation of $[Ca^{2+}]_i$ in chondrocytes treated with compound **5h** (50 μM); (**i**) Acid-induced elevation of $[Ca^{2+}]_i$ in chondrocytes treated with amiloride (100 μM). The amplitude of $[Ca^{2+}]_i$ intensity in chondrocytes induced by acid treatment was quantified as the maximal rise of $[Ca^{2+}]_i$ above basal levels. **i**: Before exposure to acid solution. **ii**: Increased Ca^{2+} intensity when pH was decreased to 6.0. **iii**: A few minutes after exposure to the acid solution (n = 3–4).

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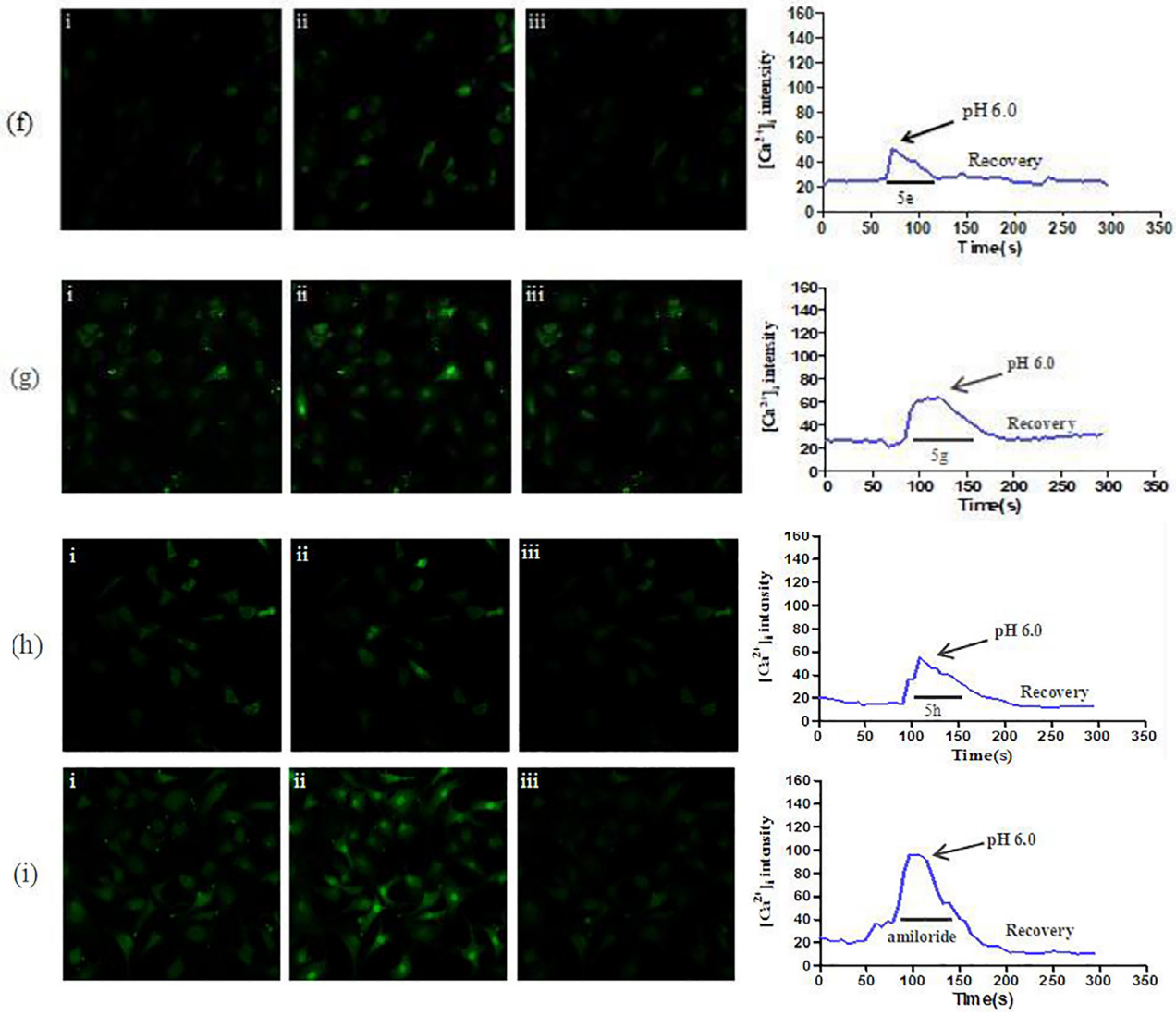

Fig. 5 (*continued*)

Co., St. Louis, MO, USA) in PBS. After digestion, isolated cells were centrifuged and washed three times with PBS. The freshly isolated chondrocytes were plated at a density of 2×10^4 cells/well, which had been immersed previously in plastic dishes filled with Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Sigma, St. Louis, MO, USA), 100 IU/ml penicillin, and 100 μg/mL streptomycin. These cultures were maintained under sterile conditions at 37 °C in a humidified 5% CO_2 incubator. Primary rat articular chondrocytes were subjected to different treatments with increased concentrations of compound **5a**–**h** (1.563–50 μM) in DMEM supplemented with 10% FBS for 24 h. After exposure to a normal extracellular solution (pH 7.4) with compound **5a**–**h**, chondrocytes were cultured in a low pH solution (pH 6.0) for 3 h. Chondrocytes cultured in the pH 7.4 solution were used as control group. After treatment, the cells were washed three times with PBS and cell lysates were subjected to Western blot.

4.3. Cell viability assay

Cells were planted into 96-well plates (5×10^3 cells/well) and incubated for 24 h. After treatment, 20 μL of MTT (Sigma, St. Louis, MO, USA) reagent solution (5 mg/mL) was added to each well, and the cells were incubated in complete DMEM solution at 37 °C for 4 h. At the end of incubation, MTT solutions were removed, 150 μL dimethyl sulfoxide (DMSO) was added to each well and stirred to dissolve the water-insoluble formazan salt. Then, the optical density (OD) of each well was measured at 492 nm using an enzyme-linked immunosorbent assay (ELISA) microplate reader (Biotek, Vermont, USA), and the well without cells served as blank control. All experiments were performed in triplicate and should be repeated at least three times.

4.4. Laser scanning confocal microscopy

The intracellular calcium level of isolated rat articular chondrocytes was monitored using fluorescence imaging.[23] 1×10^5 cells were planted into small plate that was only used for laser scanning confocal microscopy and after 24 h, cells were washed three times with D-Hanks' solution and incubated with 10 μM Fluo-3-AM and 0.02% Pluronic F-127 (Biotium, Hayward, California, USA) for 30 min at 37 °C. After incubation, the cells were washed three times with Hank's solution to remove the extracellular Fluo3-AM. To eliminate the effects of voltage-gated Ca^{2+} channels from intracellular stores, nimodipine (5 μM) was added to the extracellular fluid. Then cells were perfused initially with D-Hank's solution

(pH 6.0), or pretreated with Amiloride (100 μM) or compounds **5a–h** (compounds **5a**, **5b**, **5d**, **5e**, **5f**, **5g** 25 μM; compounds **5c**, **5h** 50 μM) and then perfused with Hank's solution (pH 6.0). The fluorescence of intracellular Fluo-3 was quantitated by confocal laser scanning fluorescence microscopy (Zeiss 710, Germany) with excitation at 488 nm and emission at 525 nm. Gray scale images with 0–50 steps were collected at different time points before and up to 5 min after fluorescence microscopy and archived as TIFF image files for later analysis. Digitized images were acquired, stored and analyzed on a computer controlled by Leica-sp5 LAS AF software. Images (340/380 ratio) were analyzed by averaging pixel ratio values in circumscribed regions of cells in the field of view. The values were exported from Axon Imaging Workbench software to SigmaPlot for additional analysis and plotting.

Author contributions

Xin-Hua Liu and Fei-Hu Chen designed the research; Zhi Sen Wang and Hua Zhou conducted the studies and prepared the manuscript; all authors read and approved the manuscript.

Additional informations

Supplemental Informations include synthesis of all intermediates, ^{1}H NMR and HR-ESI-MS of title compounds. Supplementary information accompanies this paper at http://www.nature.com/scientificreports.

Competing financial interests

The authors declare no competing financial interests.

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bmc.2018.04.042.

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