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Design and synthesis of thiourea derivatives containing a benzo[5,6]cyclohepta[1,2-*b*]pyridine moiety as potential antitumor and anti-inflammatory agents

Wukun Liu [a,b], Jinpei Zhou [a,*], Tong Zhang [a], Haiyang Zhu [a], Hai Qian [a], Huibin Zhang [a], Wenlong Huang [a], Ronald Gust [b,c,*]

[a] Department of Medicinal Chemistry, China Pharmaceutical University, Tongjia Xiang 24, 210009 Nanjing, PR China
[b] Institute of Pharmacy, Freie Universität Berlin, Königin-Luise-Str, 2+4, 14195 Berlin, Germany
[c] Institute of Pharmacy, University of Innsbruck, Innrain 80/82, A-6020 Innsbruck, Austria

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ABSTRACT

Thiourea derivatives (**6a–e**) were developed and screened for antitumor and anti-inflammatory activity. Most of the compounds exhibited growth inhibitory effects comparable to 5-fluorouracil in vitro against mammary (MCF-7 and MDA-MB 231) as well as colon (HT-29) carcinoma cells. They also showed stronger anti-inflammatory activity than ibuprofen in vivo in the xylene-induced ear swelling assay in mice.

Cancer is a group of malignant diseases responsible for tremendous health costs associated with high level of mortality and morbidity.[1] Apart from the use of surgical treatment and irradiation, chemotherapy still remains an important option for its treatment. However, the use of available chemotherapeutics is often restricted mainly due to undesirable side effects and a limited choice of available anticancer drugs. Still, the successful treatment of cancer remains a challenge in the 21st century, and this clearly underlies the urgent need of developing novel and safe chemotherapeutic agents with more potent antitumor activities.

The thiourea derivatives represent one of the most promising classes of anticancer agents with a wide range of activities against various leukemia and solid tumors.[2–10] They play an important role as anticancer agents because of their good inhibitory activity against protein tyrosine kinases (PTKs),[3–6] human sirtuin type proteins 1 and 2 (SIRT1 and SIRT2),[7] topoisomerase II[8] and DNA repair synthesis.[9] For example, Liu and Jiang's group[3,4] reported a series of N-substituted-thiourea derivatives as epidermal growth factor receptor (EGFR) (one of important PTKs) inhibitors. The antitumor activity studies focused on optimizing activity against EGFR as this kinase plays an important role in tumor angiogenesis. In addition, thiourea derivatives also exhibit other various biological properties such as antiviral,[11] antimalaria,[12] antibacterial[13,14] and anti-inflammatory[14] activities and have therefore attracted considerable pharmaceutical interest.[11–16] For these reasons, the synthesis of thiourea and their functionalized derivatives is a primary objective.

The benzo[5,6]cyclohepta[1,2-*b*]pyridine is a highly efficient pharmacophore and widely used in drug molecular design. Derivatives containing this group such as loratadine (**4**), desloratadine (**5**), rupatadine and lonafarnib (Sch-66336) could exhibit antihistamine as well as antitumor and anti-inflammatory activities.[17–26] For instance, it has been demonstrated that loratadine, a second-generation H_1 histamine antagonist used to treat allergies, induced a cell cycle arrest in G2/M by interfering with the activity of these regulatory proteins.[19] Further investigations indicated that this drug had potential as a chemotherapeutic agent and as a modifier of radiation responsiveness in the treatment of cancer and may warrant further clinical evaluation.[20] In addition, desloratadine, a third-generation H_1 antihistamine, has been shown to have direct effects on inflammatory mediators in vitro.[21]

The above mentioned results induced us to investigate whether there would be some new beneficial properties if the benzo[5,6]cyclohepta[1,2-*b*]pyridine moiety was introduced in thiourea derivatives. Here we described the synthesis and the preliminary in vitro cytotoxic activity as well as the in vivo anti-inflammatory effects.

* Corresponding authors. Tel.: +86 25 83471532; fax: +86 25 83271480 (J.Z.); tel.: +43 512 507 58200; fax: +43 512 507 58299 (R.G.).
E-mail addresses: jpzhou668@163.com (J. Zhou), ronald.gust@uibk.ac.at (R. Gust).

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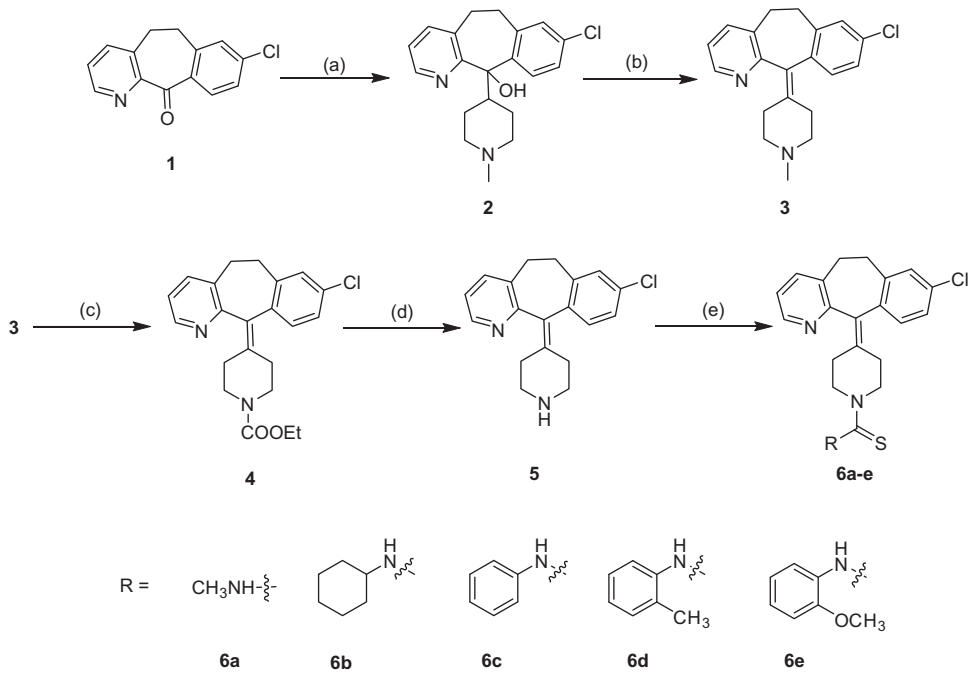

Scheme 1. Synthetic routes of thiourea derivatives **6a–e**. Reagents and conditions: (a) (1-methylpiperidin-4-yl)magnesium chloride (Grignard species provided in situ from 4-chloro-1-methylpiperidine and Mg 1:1), absolute THF, 3 h, reflux, 95%; (b) H_2SO_4 (85%), 4.5 h, rt, 65%; (c) $ClCO_2CH_2CH_3$, absolute toluene, Et_3N, 3 h, reflux, 72%; (d) KOH, EtOH (80%), 6 h, reflux, 93%; (e) RNCS, absolute EtOH, over 1 h, rt, 69–76%.

The synthetic routes to the thiourea derivatives **6a–e** are outlined in Scheme 1. Compounds **4** (loratadine) and **5** (desloratadine) were synthesized according to previously published methods.[24–26] The commercially available ketone **1** was treated with a Grignard reagent to give the corresponding tertiary carbinol **2** which was dehydrated with 85% H_2SO_4 affording the 8-chloro-11-piperidinylidene derivative **3**. Then **3** was converted to the corresponding carbamate **4** by employing ethyl chloroformate in toluene. The carbamate **4** was further cleaved under alkaline or acidic conditions to release the amine **5**. The yield of this reaction depended on the hydrolytic agent and solvent. In this study, several experimental conditions were investigated (Table 1). Among them using KOH as hydrolytic agent and EtOH (80%) as solvent led to the best yield (93%). Finally, **5** was treated with isothiocyanate derivatives in ethanol at room temperature (rt) to give the target compounds **6a–e** in high yield (69–76%).[27]

In vitro cytotoxicity assays were performed with **6a–e** according to established procedures[28–31] to get an insight into the antitumor activity. In addition, loratadine (**4**), desloratadine (**5**), the isothiocyanate derivatives and the antitumor drug 5-FU were screened against hormone-dependent MCF-7, hormone-independent MDA-MB 231 breast cancer and HT-29 colon cancer cell lines. In this assay, a known number of cells were exposed to increasing concentrations of compounds on a 96-well tissue culture plate and incubated for a given period of time. IC_{50} values for these compounds were calculated (OriginPro 8) and are presented in Table 2.

Interestingly, as mentioned in Table 2, our target compounds **6a–e** displayed IC_{50} values in the range of 4.7–10.4 μM in the tested cell lines. Therefore, these thiourea derivatives have a comparable activity as 5-FU which is widely employed in the treatment of cancer. Besides the compounds without benzo[5,6]cyclohepta[1,2-*b*]pyridine moiety (isothiocyanate derivatives, IC_{50} >40 μM, data not shown) were inactive against the tumor cells.

In addition, loratadine (**4**) caused comparable effects to compounds **6a–e** with IC_{50} values between 6.2 and 8.4 μM, while desloratadine (**5**) was less active with IC_{50} values of about 10 μM. These results indicated that the high growth inhibitory effects of **6a–e** might be due to the combination of benzo[5,6]cyclohepta[1,2-*b*]pyridine group with the thiourea structure.

Moreover, **6a–e** showed promising antiproliferative activities at MCF-7 cells (IC_{50} values of 5.1–9.4 μM; 5-FU: IC_{50} 4.7 μM). While **6a** (IC_{50} = 9.4 μM) and **6e** (IC_{50} = 7.6 μM) were less active than

Table 1
Experimental conditions to prepare **5** and its yields

Entry	Solvent	Hydrolytic agent	Reagent (**4**) (g)	Solvent volume (mL)	Reaction time (h)	Yield (%)
1	H_2O	KOH	2.0	10	24	85.4
2	EtOH (80%)	KOH	2.0	17	6	93.0
3	EtOH	KOH	2.0	17	10	92.3
4	EtOH	NaOH	2.0	84	56	75.2
5	EtOH (80%)	NaOH	2.0	17	24	73.8
6	H_2O	HCl	2.0	8	24	61.6
7	EtOH	HCl	2.0	17	8	55.4

Table 2
Antiproliferative effects against MCF-7, MDA-MB 231 and HT-29 cells

Compound	Cytotoxicity IC_{50}, (μM)[a]		
	MCF-7	MDA-MB 231	HT-29
6a	9.4 ± 0.7	7.1 ± 1.9	9.6 ± 2.6
6b	5.1 ± 0.2	8.1 ± 0.5	6.2 ± 0.4
6c	5.6 ± 0.5	6.4 ± 1.1	6.7 ± 0.5
6d	5.4 ± 1.9	4.7 ± 0.6	6.6 ± 0.6
6e	7.6 ± 0.1	10.4 ± 0.1	9.2 ± 1.4
Loratadine (**4**)	7.5 ± 0.7	8.4 ± 1.3	6.2 ± 2.4
Desloratadine (**5**)	10.5 ± 0.6	12.1 ± 1.1	11.2 ± 0.7
5-FU	4.7 ± 0.4	9.6 ± 0.3	7.3 ± 1.0

[a] The IC_{50} values represent the concentration which results in a 50% decrease in cell growth after 72 h incubation.

5-FU (IC_{50} = 4.7 μM) the IC_{50} values of about 5 μM determined for **6b–d** were comparable to this reference.

In contrast, at the MDA-MB 231 cell line only **6e** showed an IC_{50} = 10.6 μM higher than 5-FU (IC_{50} = 9.6 μM). Especially **6d** (IC_{50} = 4.7 μM) possessed high cytotoxicity. At HT-29 cells, compounds **6b–d** (IC_{50} = 6.2–6.7 μM) were slightly more active than 5-FU (IC_{50} = 7.3 μM), whereas the growth inhibitory effects of **6a** and **6e** were significantly lower (IC_{50} values of 9.6 and 9.2 μM, respectively).

Next, the anti-inflammatory effects of the thiourea derivatives were studied in vivo using the well-known xylene-induced ear swelling assay in mice.[28,32] Overnight fasted mice (12 h) of either sex weighing approximately 20 g were divided into groups of eight animals each. One group of mice, which served as control was only given vehicle (0.5% CMC in water in a volume of 20 mL/kg). Test compounds (4 mg/kg b.w.) and ibuprofen (4 and 30 mg/kg b.w.) suspended in vehicle (20 mL/kg) were administered intraperitoneally (ip) to respective groups for 5 days. 30 min after the last administration, 0.1 mL of xylene was applied to the anterior and posterior surfaces of the right ear. The left ear was considered as control. One hour after xylene application, mice were killed and both ears were removed. Circular sections were taken, using a cork borer with a diameter of 9 mm, and measured. The degree of ear swelling was calculated based on the weight of left ear without xylene.

As shown in Table 3, **6a–e** exhibited good anti-inflammatory activity at a dose of 4 mg/kg b.w. Especially, **6a** and **6b** showed the highest anti-inflammatory inhibition rate with 26.08% and 30.40%, respectively. In addition, the target compounds **6a–e** (13.04 to 30.40% inhibition) were more active than ibuprofen (8.69% inhibition) at the same dose (4 mg/kg b.w.). To achieve the same activity as our compounds ibuprofen had to be used in a dose of 30 mg/kg b.w. (39.13% inhibition).

Finally, in order to study if the inhibition of COX-1/2 enzymes contributed to the anti-inflammation in vivo, we evaluated the COX inhibitory properties of the most active compound **6b** by using an established assay with isolated ovine COX-1 and human recombinant COX-2.[28,29,33] In these experiments the reference substance aspirin showed only a weak inhibition of COX-1 (29% at: 10 μM) and was inactive at COX-2 while the second reference indomethacin was much more active (COX-1 inhibition: 56%; COX-2 inhibition: 100%; concentration: 10 μM). Unfortunately, at a concentration of 10 μM, **6b** caused only weak COX inhibition (COX-1 (11.6%) and COX-2 (11.1%)). On the basis of these observations it could be assumed that the new compounds might inhibit other enzymes, which were responsible for modifications of arachidonic acid to produce inflammatory molecules such as prostanoids and leukotrenes.[28,29]

In conclusion, a series of thiourea derivatives was synthesized and tested for tumor cell growth inhibitory effects as well as for anti-inflammatory activity. Preliminary in vitro bioassay data documented that all compounds represent potential antitumor drugs, while in vivo anti-inflammatory tests indicated stronger inhibition than ibuprofen in the xylene-induced ear swelling assay. Further investigations on structural optimization and biological activities with these derivatives are still underway in our laboratory.

Table 3
Effect of the compounds on xylene-induced ear swelling in mice (n = 8, $\bar{x} \pm S$)

	Dose (mg/kg b.w.)	Swollen extent; weight (g)	Inhibition (%)
Control		0.023 ± 0.003	
6a	4	0.017 ± 0.005*	26.08
6b	4	0.016 ± 0.006*	30.40
6c	4	0.018 ± 0.004*	21.74
6d	4	0.020 ± 0.004	13.04
6e	4	0.018 ± 0.008	21.74
Ibuprofen	4	0.021 ± 0.002*	8.69
Ibuprofen	30	0.014 ± 0.004*	39.13

* P <0.05, data were subjected to one-way ANOVA.

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27. General procedure for the synthesis of **6a–e**. The flask was charged with 10 mL of absolute ethanol, 1.0 g (3.4 mmol) of desloratadine and 6.8 mmol of RNCS. The resulting solution was stirred at rt for over 1 h. Then the white suspension was filtered under suction and the solid was washed with a small amount of cold ethanol to obtain the white product.
4-(8-Chloro-5,6-dihydro-11*H*-benzo[5,6]cyclohepta[1,2-*b*]pyridin-11-ylidene) -*N*-methyl-1-piperidinecarbothioamide (**6a**).Yield: 70%; A white solid; mp: 234∼236 °C; ESI-MS *m*/*z*: 384.1 $[M+H]^+$, 406.0 $[M+Na]^+$, 422.1 $[M+K]^+$; IR (cm^{-1}): 3468, 3420, 3283, 2909, 2837, 1634, 1588, 1535, 1466, 1431, 138, 1347, 1257, 1205, 1055, 997, 803; ^1H NMR ($CDCl_3$): δ 2.41∼2.45 (m, 2H, $=CCH_2$); 2.51∼2.61 (m, 2H, $=CCH_2$); 2.81∼2.85 (m, 2H, NCH_2); 3.18 (s, 3H, SCH_3); 3.36∼3.41 (m, 2H, NCH_2); 3.66∼3.68 (m, 2H, CH_2); 4.14 (m, 2H, CH_2); 5.64 (s, 1H, NH); 7.19∼7.24 (m, 4H, Ar-H, pyridin-H); 7.58 (dd, 1H, J = 4.5 Hz, pyridin-H); 8.46 (dd, 1H, J = 2.9 Hz, pyridin-H); Anal. Calcd for $C_{21}H_{22}ClN_3S$: C, 65.69; H, 5.78; N, 10.94. Found C, 65.40; H, 5.82; N, 10.92.

4-(8-Chloro-5,6-dihydro-11*H*-benzo[5,6]cyclohepta[1,2-*b*]pyridin-11-ylidene) -*N*-cyclohexyl-1-piperidinecarbothioamide (**6b**). Yield: 76%; A white solid; mp: 184∼186 °C; ESI-MS *m*/*z*: 452.1 $[M+H]^+$, 474.1 $[M+Na]^+$, 490.1 $[M+K]^+$; IR (cm^{-1}): 3448, 3303, 2927, 2851, 1636, 1532, 1477, 1436, 1408, 1365, 1333, 1177, 979, 828, 646; ^1H NMR ($CDCl_3$): δ 1.13∼1.20 (m, 3H, CH_2); 1.40∼1.44 (m, 2H, CH_2); 1.76∼1.72 (m, 3H, CH_2); 2.08∼2.11 (m, 2H, CH_2); 2.38∼2.42 (m, 2H, $=CCH_2$); 2.82∼2.90 (m, 4H, $=CCH_2$, NCH_2); 3.32∼3.37 (m, 2H, NCH_2); 3.62∼3.67 (m, 2H, CH_2); 4.07∼4.11 (m, 2H, CH_2); 4.34 (m, 1H, –CH–); 5.23 (s, 1H, NH); 7.14∼7.23 (m, 4H, Ar-H, pyridine-H); 7.56 (dd, 1H, *J* = 7.1 Hz, pyridine-H); 8.44 (dd, 1H, *J* = 1.1 Hz, pyridine-H); Anal. Calcd for $C_{26}H_{30}ClN_3S$: C, 69.08; H, 6.69; N, 9.30. Found C, 69.11; H, 6.58; N, 9.25.

4-(8-Chloro-5,6-dihydro-11*H*-benzo[5,6]cyclohepta[1,2-*b*]pyridin-11-ylidene) -*N*-phenyl-1-piperidinecarbothioamide (**6c**). Yield: 72%; A white solid; mp: 187∼188 °C; ESI-MS *m*/*z*: 446.1 $[M+H]^+$, 468.1 $[M+Na]^+$, 484.0 $[M+K]^+$; IR (cm^{-1}): 3309, 2928, 2899, 2855, 1591, 1524, 1476, 1436, 1321, 1304, 1200, 1174, 1071, 991, 828, 761, 698, 409; ^1H NMR ($CDCl_3$): δ 2.41∼2.46 (m, 2H, $=CCH_2$); 2.66 (m, 2H, $=CCH_2$); 2.82∼2.93 (m, 2H, NCH_2); 3.38∼3.44 (m, 2H, NCH_2); 3.67 (m, 2H, CH_2); 4.14∼4.21 (m, 2H, CH_2); 7.15∼7.22 (m, 5H, Ar-H); 7.28∼7.35 (m, 3H, Ar-H); 7.65 (m, 2H, pyridine-H); 8.45 (dd, 1H, *J* = 2.5 Hz, pyridine-H); Anal. Calcd for $C_{26}H_{24}ClN_3S$: C, 70.02; H, 5.42; N, 9.42. Found C, 70.11; H, 5.31; N, 9.53.

4-(8-Chloro-5,6-dihydro-11*H*-benzo[5,6]cyclohepta[1,2-*b*]pyridin-11-ylidene) -*N*-(2-methylphenyl)-1-piperidinecarbothioamide (**6d**). Yield: 69%; A white solid; mp: 204∼205 °C; ESI-MS *m*/*z*: 460.1 $[M+H]^+$, 482.1 $[M+Na]^+$, 498.0 $[M+K]^+$; IR (cm^{-1}): 3470, 3224, 2902, 1512, 1497, 1401, 1329, 1264, 1220, 1205, 1102, 994, 831, 719, 673; ^1H NMR ($CDCl_3$): δ 2.25 (s, 3H, CH_3); 2.36∼2.43 (m, 2H, $=CCH_2$); 2.51∼2.59 (m, 2H, $=CCH_2$); 2.75∼2.84 (m, 2H, NCH_2); 3.32∼3.37 (m, 2H, NCH_2); 3.54∼3.57 (m, 2H, CH_2); 4.05∼4.09 (m, 2H, CH_2); 6.88 (s, 1H, Ar-H); 7.10∼7.24 (m, 7H, Ar-H, pyridine-H); 7.44∼7.47 (d, 1H, *J* = 7.4 Hz, pyridine-H); 8.38∼8.40 (dd, 1H, *J* = 4.7 Hz, pyridine-H); Anal. Calcd for $C_{27}H_{26}ClN_3S$: C, 70.49; H, 5.70; N, 9.13. Found C, 70.45; H, 5.77; N, 9.21.

4-(8-Chloro-5,6-dihydro-11*H*-benzo[5,6]cyclohepta[1,2-*b*]pyridin-11-ylidene) -*N*-(2-methoxyphenyl)-1-piperidinecarbothioamide (**6e**). Yield: 71%; A white solid; mp: 140∼141 °C; ESI-MS *m*/*z*: 476.1 $[M+H]^+$, 498.1 $[M+Na]^+$, 514.0 $[M+K]^+$; IR (cm^{-1}): 3450, 3285, 1634, 1524, 1506, 1438, 1401, 1320, 1188, 1022, 828, 752; ^1H NMR ($CDCl_3$) δ: 2.43∼2.48 (m, 2H, $=CCH_2$); 2.51∼2.60 (m, 2H, $=CCH_2$); 2.79∼2.88 (m, 2H, NCH_2); 3.34∼3.38 (m, 2H, NCH_2); 3.68∼3.71 (m, 2H, CH_2); 3.84 (s, 3H, OCH_3); 4.13∼4.16 (m, 2H, CH_2); 6.86 (m, 1H, Ar-H); 6.88∼6.97(m, 1H, Ar-H); 7.03∼7.09 (m, 1H, Ar-H); 7.16∼7.18 (m, 4H, Ar-H, pyridine-H); 7.41 (m, 1H, pyridine-H); 7.81∼7.84 (dd, 1H, *J* = 7.9 Hz, Ar-H); 8.41∼8.42 (dd, 1H, *J* = 3.5 Hz, pyridine-H); Anal. Calcd for $C_{27}H_{26}ClN_3OS$: C, 68.12; H, 5.51; N, 8.83. Found C, 68.21; H, 5.43; N, 8.71.

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31. Cell Culture: The human MCF-7, MDA-MB 231 breast s and HT-29 colon cancer cell lines were obtained from the American Type Culture Collection. All cell lines were maintained as a monolayer culture in L-glutamine containing Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose (PAA Laboratories, Austria), supplemented with 5% fetal bovine serum (FBS; Biochrom, Germany) in a humidified atmosphere (5% CO_2) at 37 °C.

Cytotoxicity: In 96 well plates 100 μL of a cell suspension in culture medium at 7500 cells/mL (MCF-7 and MDA-MB 231) or 3000 cells/mL (HT-29) were plated into each well and were incubated for three days under culture conditions. After addition of various concentrations of the test compounds, cells were incubated for up to 144 h. Then the medium was removed, the cells were fixed with glutardialdehyde solution (1%) and stored under phosphate buffered saline (PBS) at 4 °C. Cell biomass was determined by a crystal violet staining, followed by extracting of the bound dye with ethanol and a photometric measurement at 590 nm. Mean values were calculated and the effects of the compounds were expressed as % Treated/Control$_{corr}$ values according to the following equations: $T/C_{corr}[\%] = \frac{T-C_0}{C-C_0} \cdot 100$ C_0 control cells at the time of compound addition; C control cells at the time of test end; T probes/samples at the time of test end).

The IC_{50} value was determined as the concentration causing 50% inhibition of cell proliferation and calculated as mean of at least three independent experiments (OriginPro 8).
32. Xylene-induced ear edema. Animals: The experiments applied with animals were approved by Research Ethic Committee of Jiang-Shu province, China. Kunming male mice of approximately 20 g were delivered from experimental animal centre of China Pharmaceutical University, and fed with food and water ad libitum. All animals were fasted for 12 h before the experiments. The temperature (25 °C) and humidity (60%) in the animal room were well controlled.

Method: Mice were allotted to groups of 8 animals each. One group of mice, which served as control was only given vehicle (0.5% CMC in water in a volume of 20 mL/kg). Test compounds (4 mg/kg b.w.) and ibuprofen (4 mg/kg b.w. and 30 mg/kg b.w.) suspended in vehicle (20 mL/kg) were administered ip to respective groups for 5 days. 30 min after the last administration, 0.1 mL of xylene was applied to the anterior and posterior surfaces of the right ear. The left ear was considered as control. One hour after xylene application, mice were killed and both ears were removed. Circular sections were taken, using a cork borer with a diameter of 9 mm, weighed and measured. The degree of ear swelling was calculated based on the weight of left ear without xylene.

Statistical analysis: The measurement data were expressed as the mean ± SD. Data were subjected to one-way analysis of variance (ANOVA), followed by multiple comparison with least significant differences (LSD) test or Dunnett's test as appropriate. Statistical significance was considered with P <0.05. The analysis of data was performed by software SPSS 13.0.
33. The inhibition of isolated ovine COX-1 and human recombinant COX-2 was determined with 10 μM of the respective compounds by ELISA ('COX inhibitor screening assay', Cayman Chemicals). Experiments were performed according to the manufacturer's instructions. Absorption was measured at 415 nm (Victor2, Perkin Elmer). Results were calculated as the means of duplicate determinations.