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Synthesis and biological activity of fused furo[2,3-d]pyrimidinone derivatives as analgesic and antitumor agents

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Abstract Tumor growth is usually associated with persistent pain, especially during mid and terminal stages of cancer development. Nonetheless, a medicinal compound that possesses both anticancer and analgesic properties has not been identified. The 2-alkylthio-benzofuro[3,2-d]pyrimidin-4(3H)-ones (Code **5a–d**) and 1-aryl-2-alkylthio-benzofuro[3,2-d]-1,2,4-triazolo[1,5-a]pyrimidin-5(1H)-ones (Code **10a–g**) were synthesized by using the bioisostere concept, which were obtained via the *aza*-Wittig reaction of functionalized iminophosphoranes reacted with carbon disulfide and further reaction of the product with alkyl halides or halogenated aliphatic esters. The analgesic properties of **5a–d** and **10a–g** were studied using rat chronic constriction injury model and the antitumor properties of these chemicals were assessed using MTS cell proliferation assay. Results showed that **5a–d** and **10a–g** were found to attenuate thermal and mechanical allodynia induced by neuropathy and inhibited the proliferation of three human cancer cell lines

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(A459, HepG2, and HeLa). Among these compounds, **10g** showed highly positive effects in both assessments, and would be selected for future work.

Keywords Fused furo[2,3-d]pyrimidinone derivatives · Synthesis · Analgesic and antitumor

Introduction

At terminal stages of cancer development, patients can experience severe cancer-associated pain. In normal practice, both chemotherapeutic and analgesic agents are prescribed simultaneously. However, none of these drugs possess dual activities in a single remedy. Even if there were drugs that exerted strong action in one area with some effect on the other, the power would be usually weak. During the course of our drug discovery works aiming to synthesize new nitrogen heterocyclic compounds, we revealed that furo[2,3-d]pyrimidines and fused benzofuro[2,3-d]pyrimidinones have potent antitumor properties [1, 2]. It was reported that 2,3-disubstituted quinazolin-4-(3H)-ones may exhibit good analgesic effects [3–5]. Bioisosterism is a useful strategy during the lead optimization process and molecular modification in rational drug design [6, 7]. On the basis of the bioisostere concept, we postulated that replacement of the condensed quinazolinone ring system with furopyrimidinone bioisostere group could generate compounds with improved biological activities (Fig. 1).

In such respect, as a continuation of our ongoing efforts towards the design and synthesis of nitrogen-containing heterocycle compounds via aza-Wittig reaction [8–12], we now report a number of tricyclic and tetracyclic fused benzofuro[2,3-d]pyrimidinone derivatives **5** and **10** based on the development and identification of lead molecules by the bioisostere concept. In addition, these fused furo[2,3-d]pyrimidinone derivatives were screened for their analgesic and antitumor activities.

Results and discussion

Chemistry

As described in detail previously [12], the synthesis of compounds **5** and **10** started from commercially available salicylic nitrile **1**, and the reaction of **2**, **3**, **4**, and **5** had taken place smoothly at room temperature with good yields (Scheme 1).

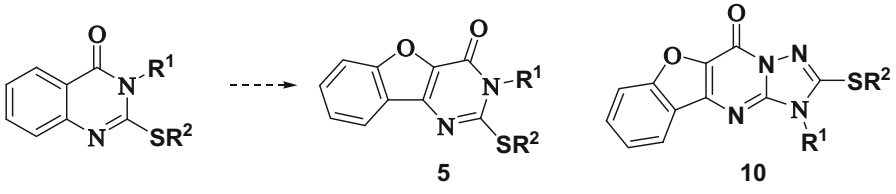
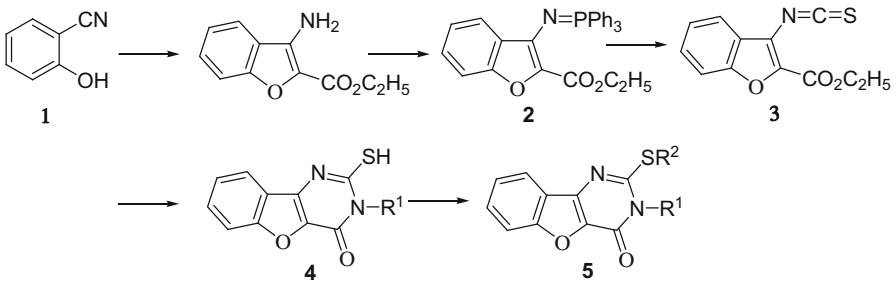
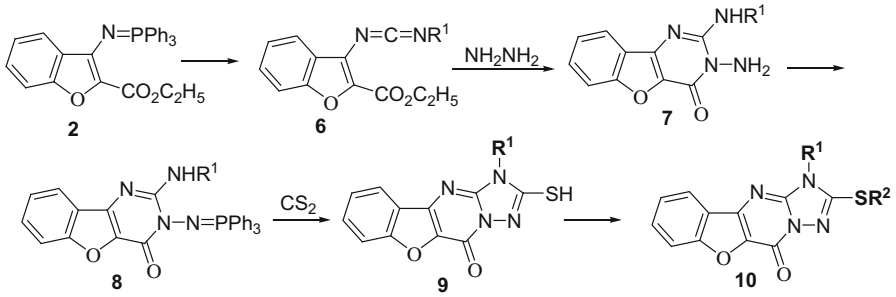


Fig. 1 Bioisostere-based drug structure design



Scheme 1 Preparation of 3-alkyl-2-alkylthio-benzofuro[3,2-d]pyrimidin-4(3H)-one **5**



Scheme 2 Preparation of 1-aryl-2-alkylthio-benzofuro[3,2-d]-1,2,4-triazolo[1,5-a]pyrimidin-5(1H)-one **10**

The synthetic strategy for **6–10** is outlined in Scheme 2. The syntheses of the key intermediates **6–8** have been described earlier [9, 11]. The functionalized iminophosphoranes **8** reacted with excess carbon disulfide to give 1-aryl-2-thioxo-benzofuro[3,2-d]-1,2,4-triazolo[1,5-a]pyrimidin-5(1H)-one **9**. S-Alkylation of **9** with alkyl halides or halogenated aliphatic esters in the presence of potassium carbonate produced 1-aryl-2-alkylthio- benzofuro[3,2-d]-1,2,4-triazolo[1,5-a]pyrimidin-5(1H)-one **10** (Scheme 2) in good yield. The results are listed in Table 1. The structures of the compounds **9** and **10** were deduced based on their ^{1}H NMR, MS, IR, and elementary analyses. Furthermore, the structure of **10f** was confirmed by X-ray crystallographic analysis [11].

Biological evaluation

The synthesized compounds **5a–5d** and **10a–10g** were evaluated for analgesic and antitumor activities. Because of poor water solubility, all the synthesized compounds were administered in the form of a suspension in 5 % DMSO. Briefly, accurate weighing the synthesized compounds were dissolved in 0.5 ml 100 % DMSO, respectively, and then diluted to 10 ml with normal saline as suspension in 10 % v/v Tween-80. Final concentrations of DMSO in assay solutions never exceed 1 %. The animals used in this experiment were provided by the

Table 1 Preparation of fused furo[2,3-d]pyrimidinone derivatives (**5**[b] and **10**) and the intermediates **9**

Compound	R^1	R^2	Condition	Yield[a] (%)
5a	$CH_2CH_2CH_3$	$CH_2CH_2CH_3$	50 °C/4 h	86
5b	$CH_2CH_2CH_3$	$CH_2CH_2CH_2CH_3$	50 °C/4 h	82
5c	$CH_2CH_2CH_3$	$PhCH_2$	50 °C/4 h	85
5d	$CH_2CH_2CH_3$	$CH_2CO_2C_2H_5$	50 °C/4 h	86
9a	Ph	H	40–50 °C/24 h	86
9b[b]	4-F-Ph	H	40–50 °C/24 h	80
10a	Ph	$CH_2CH_2CH_2CH_3$	50–60 °C/6 h	75
10b	Ph	$C(CH_3)_2CO_2C_2H_5$	50–60 °C/6 h	72
10c	Ph	$CH_2CH_2CO_2C_2H_5$	50–60 °C/6 h	78
10d	Ph	$CH_2CO_2C_2H_5$	50–60 °C/6 h	83
10e	Ph	$HC\equiv CCH_2$	50–60 °C/6 h	80
10f[#]	4-F-Ph	$CH_3CH_2CH_2CH_2CH_2CH_2$	50–60 °C/6 h	72
10g	4-F-Ph	$CH_2CO_2C_2H_5$	50–60 °C/6 h	81

[a] Isolated yields were estimated based on those of **4**, **8**, and **9**

[b] Known compounds were identified [11, 12]

Experimental Animal Center of Hubei University of Medicine. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Hubei University of Medicine. The protocol was approved by the Committee on the Ethics of Animal Experiments of Hubei University of Medicine. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering. Human A549 (lung carcinoma), HepG2 (hepatocellular carcinoma), and HeLa (cervical adenocarcinoma) cells (kindly provided by Institute of Basic Medical Sciences, Hubei Medical University, Shiyan, China) were cultured in a proper medium supplemented with 10 % fetal bovine serum under humidified atmosphere of 5 % CO_2 at 37 °C.

Analgesic activity

The analgesic activity of the synthesized compounds **5a–5d** and **10a–10g** was evaluated using the rat paw withdrawal technique. Namely, test for analgesic activity was performed by using the Plantar and the electronic von Frey Test Analgesia Meter for detection of thermal paw hyperalgesia (IITC Life Science, USA). For the establishment of chronic pain, a constriction injury was applied to the sciatic nerve, which induced neuropathic pain characterized by hyperalgesia and allodynia. All injections were given intraperitoneally with the compounds 500 mg/kg of body weight. Results showed that the replacement of 2,3-disubstituted quinazolinones by its polysubstitution fused furo[2,3-d]pyrimidinone derivatives promoted potential analgesic activities (Table 2). The test compounds (**10a**, **10g**) showed good analgesic activity.

Table 2 Behavioral studies of paw withdrawal latency ($n = 5$, $\bar{x} \pm s$)

Compound	Concentration (mg/ml)	Paw withdrawal latency (PWL) (TS(s), MS(g))			
		Before drug treatment		After drug treatment	
				30 min	60 min
5a	50	TS	8.90 ± 0.72	11.65 ± 0.61	10.81 ± 0.70
		MS	35.00 ± 2.00	35.98 ± 3.50	57.85 ± 4.50*
5b	50	TS	7.09 ± 0.68	8.40 ± 0.87	11.66 ± 0.92
		MS	36.72 ± 3.50	44.98 ± 1.07*	40.14 ± 1.07
5c	50	TS	6.45 ± 1.82	7.67 ± 1.26	13.07 ± 3.16*
		MS	36.38 ± 2.80	36.56 ± 1.80	44.52 ± 2.80
5d	50	TS	9.51 ± 2.80	12.80 ± 2.23	6.96 ± 1.67
		MS	36.74 ± 2.67	45.42 ± 1.61	47.92 ± 1.31*
10a	50	TS	11.44 ± 1.36	32.43 ± 5.67*	27.99 ± 4.17*
		MS	35.56 ± 1.27	57.22 ± 3.24*	61.12 ± 5.82*
10b	50	TS	10.99 ± 2.37	16.66 ± 3.09	16.12 ± 3.79
		MS	39.02 ± 3.84	44.42 ± 1.00	61.70 ± 4.00*
10c	50	TS	12.25 ± 2.78	18.51 ± 2.55*	18.81 ± 6.85*
		MS	32.50 ± 3.00	59.54 ± 2.41*	47.88 ± 2.48
10d	50	TS	13.80 ± 2.92	25.23 ± 3.51*	15.66 ± 3.77
		MS	38.80 ± 2.46	62.40 ± 2.01*	44.12 ± 4.30
10e	50	TS	6.07 ± 1.21	15.66 ± 3.37*	7.89 ± 1.50
		MS	38.4 ± 4.31	55.14 ± 2.60*	48.06 ± 5.70*
10f	50	TS	8.65 ± 0.84	12.18 ± 2.15	11.54 ± 1.48
		MS	35.70 ± 4.14	49.30 ± 1.60*	44.80 ± 4.8*
10 g	50	TS	9.63 ± 1.46	17.97 ± 0.94*	28.01 ± 2.70*
		MS	32.09 ± 2.68	52.10 ± 4.0*	69.80 ± 2.80*
Control	Normal saline	TS	11.40 ± 2.89	8.51 ± 0.82	11.58 ± 2.92
		MS	29.50 ± 1.64	31.20 ± 1.07	27.73 ± 1.26

TS thermal hyperalgesia, *MS* mechanical hyperalgesia

* Different from normal saline group ($p < 0.05$)

Antitumor activity

For evaluation of antitumor activities, the MTS cell proliferation assay was performed, which was dependent on the amount of MTS being reduced to an aqueous, soluble formazan by dehydrogenase enzymes found in metabolically active cells. The antiproliferative activities of **5a–5d** and **10a–10g** were investigated, and the results showed that these compounds exhibited cytotoxicity against human cancer cell lines: A459 (lung cancer), HepG2 (hepatocellular carcinoma) and HeLa (cervical cancer) with cisplatin as the reference drug. The results are listed in Table 3 and expressed as IC_{50} values. Among these compounds, **10g** is the most active, which showed potent anticancer efficacy against the human lung cancer A549 cell line with an IC_{50} value of 14.8 μM.

Table 3 Cytotoxicity of compounds **5** and **10** in representative human cancer cell lines (IC_{50}, μM)[a]

Compound	A549[b]	HepG2[c]	HeLa[d]
5a	115.2 ± 0.6	259.3 ± 1.5	138.1 ± 1.8
5b	124.5 ± 0.3	166.6 ± 0.8	128.6 ± 2.3
5c	156.5 ± 0.5	262.9 ± 0.3	129.3 ± 1.7
5d	131.4 ± 0.5	183.2 ± 1.0	174.6 ± 2.6
10a	91.6 ± 0.8	168.1 ± 1.3	121.1 ± 0.6
10b	97.6 ± 1.1	163.1 ± 3.4	115.6 ± 1.8
10c	134.5 ± 1.0	210.2 ± 2.0	99.4 ± 0.9
10d	122.7 ± 1.2	142.8 ± 1.7	223.6 ± 2.0
10e	156.2 ± 0.4	185.3 ± 2.0	206.2 ± 2.2
10f	49.3 ± 1.2	156.4 ± 1.4	89.4 ± 1.1
10g	**14.8 ± 0.6**	78.8 ± 2.4	53.5 ± 0.8
Cisplatin	**32.7 ± 1.3**	–	–

[a] Half maximal inhibitory concentration (IC_{50}); values are the mean of three independent experiments performed in triplicate determined after 24 h of treatment

[b] Lung cancer

[c] Liver hepatocellular carcinoma

[d] Cervical cancer

Conclusions

In the present study, making use of the bioisostere concept for replacement of 2, 3-disubstituted quinazolinones by fused furo[2,3-d]pyrimidinone derivatives, we have developed an efficient synthesis for fused furo[2,3-d]pyrimidinone derivatives via the functionalized iminophosphoranes. Bioassay of the compounds indicated that 2-alkylthiobenzo-furo[3,2-d]pyrimidin-4(3H)-ones and 1-aryl-2-alkylthio-benzofuro[3,2-d]-1,2,4-triazolo[1,5-a]pyrimidin-(1H)-ones showed potential analgesic and antitumor activities, for which these compounds can be established as lead molecules for developing novel analgesic and antitumor drugs. Upon vigorous screening, compound **10g** was found to provide promising dual analgesic and antitumor activities as a single compound, which could be further developed into an effective chemotherapeutic agent in treating patients with cancer pain. Further bioassay, optimization, and structure–activity relationships of the title compounds are underway.

Experimental

General

Melting points were rectified. MS were measured by using a Finnigan Trace MS spectrometer. IR were recorded by a PE-983 infrared spectrometer as KBr pellets with absorption in cm^{-1}. ^1H NMR were recorded in $CDCl_3$ or DMSO-d_6 by the Varian Mercury 400 spectrometer with resonances relative to TMS. Elementary analyses were performed by a Vario EL III elementary analysis instrument. X-ray diffraction data were collected by a Bruker SMART AXS CCD diffractometer. Male albino rats weighing 120–150 g were used throughout this assay. They were kept in the animal house under standard conditions of light and temperature with free access to food and water. The animals were randomly divided into groups of

five rats each. Test for analgesic activity was performed by using the Plantar Test Analgesia Meter for detection of thermal paw hyperalgesia (IITC Life Science, USA).

Synthesis of 1-aryl-2-thioxo-benzofuro[3,2-d]-1,2,4-triazolo[1,5-a] pyrimidin-5(1H)-ones 9a–9b

Excess carbon disulfide (15 ml) was added to a solution of iminophosphorane **8** (20 mmol) in anhydrous methylene dichloride and acetonitrile (20 ml, v/v = 1:1). After the reaction mixture was refluxed for 24–30 h at 40–50 °C, the solvent was condensed under reduced pressure, with addition of ether (10 ml) to precipitate triphenylphosphine sulfide. The precipitate was then removed by filtration, while the filtrate was evaporated to give 1-aryl-2-thioxo-benzofuro[3,2-d]-1,2,4-triazolo[1,5-a] pyrimidin-5(1H)-ones **9a–9b.**

*1-phenyl-2-thioxo-benzofuro[3,2-d]-1,2,4-triazolo[1,5-a]pyrimidin-5(1H)-ones (**9a**)*

White crystals (86 % yield), M.p. > 300 °C. ^{1}H NMR (400 MHz, DMSO-d_6)δ: 7.51–7.27 (m, 9H, Ar–H). MS (70 eV) *m/z* (%): 334/333 (M^+, 55/100), 300 (23), 275 (42), 261 (38), 235 (16), 185 (46), 160 (42), 130 (55), 114 (61), 102 (99), 77 (81). Anal. Calcd for $C_{17}H_{10}N_4O_2S$ (334.4): C, 61.07; H, 3.01; N, 16.76. Found: C, 61.13; H, 3.18; N, 16.70.

*1-(4-flurophenyl)-2-thioxo-benzofuro[3,2-d]-1,2,4-triazolo[1,5-a]pyrimidin-5(1H)-ones **(9b)***

As described in detail previously [11].

Synthesis of 1-aryl-2-alkylthio-benzofuro[3,2-d]-1,2,4-triazolo[1,5-a] pyrimidin-5(1H)-ones 10a–10g

Alkyl halide (3.5 mmol) and solid potassium carbonate (4 mmol) were added to a solution of 1-aryl-2-thioxo- benzofuro[3,2-d]-1,2,4-triazolo[1,5-a]pyrimidin-5(1H)-ones **9** (3 m mol) in dry DMF (10 ml). The mixture was stirred for 5–6 h at 50–60 °C. The resulting solution was cooled and diluted with ice water (30 ml). The solid product obtained was filtered and recrystallized from methylene dichloride/ petroleum ether (*v/v*) to give 1-aryl-2-alkylthio-benzofuro[3,2-d]-1,2,4-triazolo[1,5-a] pyrimidin-5(1H)-ones **10a–10g.**

*1-phenyl-2-n-butylthio-benzofuro[3,2-d]-1,2,4-triazolo[1,5-a] pyrimidin-5(1H)-ones (**10a**)*

White crystals; Mp: 210–212 °C. ^{1}HNMR (400 MHz, $CDCl_3$): δ = 0.94 (t, *J* = 7.2, 3H, CH_3), 1.46–1.52 (m, 2H, CH_2), 1.68–1.81 (m, 2H, CH_2), 3.45–3.48 (s, 2H, CH_2), 7.34–7.99 (m, 9H, Ar–H). IR (KBr) nu = 1700 (C=O), 1513, 1227 cm^{-1}; MS (70 eV) *m/z* (%): 390 (69, M^+), 342 (34), 332 (100), 260 (19),

184 (17), 159 (17), 129 (38), 113 (74), 100 (52), 76 (36). Anal. Calcd for $C_{21}H_{18}N_4O_2S$ (390.5): C, 64.60; H, 4.65; N, 14.35. Found: C, 64.53; H, 4.77; N, 14.19.

*1-phenyl-2-(1,1-dimethyl-1-ethoxycarbonyl-methylthio)-benzofuro[3,2-d]-1,2,4-triazolo[1,5-a]-pyrimidin- 5(1H)-ones (**10b**)*

White crystals; Mp: 204–206 °C; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 1.29$ (t, $J = 7.2$, 3H, CH_3), 1.78 (s, 6H, $2 \times CH_3$), 4.22 (q, $J = 7.2$, 2H, OCH_2), 7.35–7.98 (m, 9H, Ar–H). ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 13.9$, 26.5, 55.2, 62.4, 112.7, 121.8, 122.4, 123.3, 127.4, 129.6, 129.8, 130.2, 131.2, 134.0, 144.2, 147.1, 147.4, 150.8, 157.4, 172.2. MS (70 eV) *m/z* (%): 448 (35, M^+), 333 (100), 276 (33), 260 (40), 185 (17), 129 (31), 113 (35), 101 (24), 76 (18). Analytical calculation for $C_{23}H_{20}N_4O_4S$ (448.5): C, 61.59; H, 4.49; N, 12.49. Found: C, 61.53; H, 4.58; N, 12.35.

*1-phenyl-2-(2-ethoxycarbonyl-ethylthio)-benzofuro[3,2-d]-1,2,4-triazolo[1,5-a]-pyrimidin-5(1H)-ones (**10c**)*

White crystals; Mp:223–225 °C. ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 1.22$ (t, $J = 7.2$, 3H, CH_3), 2.96–2.98 (m, 2H, CH_2), 3.65–23.71 (m, 2H, CH_2), 4.14 (q, $J = 7.2$, 2H, OCH_2), 7.34–7.98 (m, 9H, Ar–H).MS (70 eV) *m/z* (%): 434 (M^+, 54), 389 (7), 333 (100), 261 (7), 185 (3), 130 (7), 114 (9), 77 (7). Analytical calculation for $C_{22}H_{18}N_4O_4S$ (434.5): C, 60.82; H, 4.18; N, 12.90. Found: C, 60.76; H, 4.25; N, 12.82.

*1-phenyl-2-(1-ethoxycarbonyl-methylthio)-benzofuro[3,2-d]-1,2,4-triazolo[1,5-a]-pyrimidin-5(1H)- ones (**10d**)*

White crystals; Mp: 252–254 °C; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 1.31$ (t, $J = 7.2$, 3H, CH_3), 4.24–4.30 (m, 4H, $2 \times CH_2$), 7.35–7.98 (m, 9H, Ar–H). IR (KBr) nu = 1713 (C=O), 1540, 1345, 1105 cm^{-1}. MS (70 eV) *m/z* (%): 420 (M^+, 100), 333 (79), 260 (7), 185 (3), 129 (3), 113 (3). Analytical calculation for $C_{21}H_{16}N_4O_4S$ (420.4): C, 59.99; H, 3.84; N, 13.33. Found: C, 59.93; H, 3.80; N, 13.20.

*1-phenyl-2-propargylthio-benzofuro[3,2-d]-1,2,4-triazolo[1,5-a]-pyrimidin-5(1H)-ones (**10e**)*

White crystals; Mp: 275–277 °C; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 2.33$ (s, H, CH), 4.22 (s, 2H, CH2), 7.34–7.99 (m, 9H, Ar–H). MS (70 eV) *m/z* (%): 372 (M^+, 89), 333 (73), 275 (95), 260 (100), 185 (29), 169 (60), 129 (82), 113 (97), 102 (76), 76 (58). Analytical calculation for $C_{20}H_{12}N_4O_2S$ (372.4): C, 64.50; H, 3.25; N, 15.04. Found: C, 64.39; H, 3.19; N, 14.96.

*1-(4-flurophenyl)-2-(1-ethoxycarbonyl-methylthio)-benzofuro[3,2-d]-1,2,4-triazolo[1,5-a]-pyrimidin-5(1H)- ones (**10g**)*

White crystals; Mp: 198–200 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.32 (t, J = 7.2, 3H, CH_3), 4.25–4.28 (m, 4H, 2 × CH_2), 7.34–7.95 (m, 8H, Ar–H). IR (KBr) nu = 1724 (C = O), 1580, 1513, 1307, 1183 cm^{-1}. MS (70 eV) *m/z* (%): 438 (M^+, 7), 436 (100), 353 (44), 350 (84), 279 (27), 240 (4), 114 (14), 102 (9), 95 (6). Analytical calculation for $C_{21}H_{15}FN_4O_4S$ (438.4): C, 57.53; H, 3.45; N, 12.78; Found: C, 57.43; H, 3.53; N, 12.61.

Pharmacological studies

Anticancer activity

Cancer cells (9 × 10^4 cells/well) were seeded into each well of a 96-well microplate, supplemented with 10 % fetal bovine serum (PBS) in each well of 96-well microculture plates, and incubated for 24 h at 37 °C in a CO_2 incubator. Compounds, diluted to the desired concentrations in culture medium, were added to the wells with respective vehicle control. After 24 h of incubation, MT stetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-5- (3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium) (MTS) (10 µl, 5 mg/ml) was added to each well, and the plates were further incubated for 4 h. Then the supernatant from each well was carefully removed, formazan crystals were dissolved in DMSO (100 µl), and absorbance at 570 nm wavelength was recorded. Three independent experiments were performed in triplicate. An equal amount of DMSO was added to the cells as negative control. All experiments were performed in triplicate.

Analgesic activity

CCI surgery A neuropathic pain model of sciatic nerve chronic constriction injury (CCI) was employed to induce sciatic nerve injury [13]. Briefly, rats were anesthetized with pentobarbital sodium (60 mg/kg, intraperitoneal); following this, the left sciatic nerve was exposed at mid-thigh level. The distance between two adjacent ligatures was 1 mm. The wound was irrigated with normal saline (0.9 %) and closed in two layers with 4–0 silk sutures and surgical skin staples. To confirm the consequence of nerve injury, a sham operation was performed with exposure of the left sciatic nerve without ligation. The CCI-treated rats were tested 6 days after the operation.

Von Frey filament test To evaluate the mechanical sensitivity, a Von Frey filament method was used. For the test, each rat was placed on a mesh floor inside a transparent plastic cage. The rats were kept in a silent environment at room temperature for 20 min, and then force (in grams) was applied to the rats through the plantar hind paw up-down method. The minimum paw withdrawal threshold was defined as the minimum gram strength producing two sequential responses at 3-min

intervals (withdrawal from pressure). The upper pressure limit was 100 g, and the point at which each rat pulled its paw back was recorded.

Plantar test To evaluate the magnitude of thermal hyperalgesia, plantar test was used. For this method, each rat was placed in an acrylic compartment on a clear glass surface. A moveable heat source was then put under the plantar surface of the hind paw and activated with a light beam. The intensity of the light beam was adjusted such that the withdrawal latencies in sham-operated rats were around 8–10 s. The built-in digital timer automatically recorded the response latency for paw withdrawal to the nearest 0.1 s. A cutoff time of 20 s was used to prevent tissue damage when a rat failed to respond after 20 s.

Statistical analysis

Statistical analysis of the biological activities caused by the synthesized compounds in animals was evaluated using one-way analysis of variance (ANOVA). In all cases, post hoc comparisons of the means of individual groups were performed using t test. A significance level of $p < 0.05$ denoted significance. All values are expressed as mean ± standard deviations (SD).

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