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Discovery of N-substituted sulfamoylbenzamide derivatives as novel inhibitors of STAT3 signaling pathway based on Niclosamide

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ABSTRACT

Signal transducer and activator of transcription 3 (STAT3) has been confirmed as an attractive therapeutic target for cancer therapy. Herein, we designed and synthesized a series of N-substituted Sulfamoylbenzamide STAT3 inhibitors based on small-molecule STAT3 inhibitor Niclosamide. Compound B12, the best active compound of this series, was identified as an inhibitor of IL-6/STAT3 signaling with an IC_{50} of 0.61−1.11 μM in MDA-MB-231, HCT-116 and SW480 tumor cell lines with STAT3 overexpression, by inhibiting the phosphorylation of STAT3 of Tyr705 residue and the expression of STAT3 downstream genes, inducing apoptosis and inhibiting the migration of cancer cells. Furthermore, in vivo study revealed that compound B12 suppressed the MDA-MB-231 xenograft tumor growth in nude mice at the dose of 30 mg/kg (i.g.), which has better antitumor activity than the positive control Niclosamide. More importantly, B12 is an orally bioavailable anticancer agent as a promising candidate for further development.

1. Introduction

Signal transducer and activator of transcription 3 (STAT3) is a member of the STAT protein family, which regulates cell growth, differentiation, apoptosis, angiogenesis and immune responses [1−3]. Abnormally activated STAT3 signaling is closely related to the occurrence and development of various malignant tumors (about 70% of human solid tumors and hematological tumors) [4−8]. In terms of STAT3 functional structure, it is mainly composed of six important domains: (1) N-terminal domain; (2) Coil-coil domain (CCD); (3) DNA binding domain (DBD); (4) linker domain; (5) SH2/TAD domain; (6) C-terminal domain [9−11]. STAT3 can be phosphorylated at Tyr705 on the SH2/TAD domain of STAT3 which is mediated by cytoplasmic kinases to form p-STAT3 monomer, which in turn forms a pSTAT3-pSTAT3 dimer with another monomeric p-STAT3. The dimerized STAT3 then translocates into the nucleus and binds to DNA, inducing gene transcription to exert biological activity [12,13]. Therefore, STAT3 is widely recognized as a key target in cancer therapy, and research on anti-tumor drugs targeting STAT3-related signaling pathways has also received much attention in recent years [14,15].

Currently, the most common method to inhibit STAT3 is by blocking the upstream kinase to prevent STAT3 phosphorylation and activation (such as curcumin, resveratrol), or by interfering with the STAT3-SH2 domain to prevent the formation of STAT3 dimers (such as BP-1-102, S3I-201, Niclosamide, LY5, LLL12), thereby blocking this signaling [15,16]. These inhibitors have a certain effect, but they also have shortcomings, including: the lack of membrane permeability and stability, low hydrophobicity, weak binding affinity, or low specificity. So there are currently no STAT3-targeting inhibitor approved by the FDA to date. To discover and develop novel druggable STAT3 inhibitors with high potency remains promising and valuable.

Niclosamide is an FDA-approved antihelminthic drug that has been used for several decades for its high efficiency and low toxicity [17−19]. With the development of drug repurposing initiatives, Niclosamide has become a true hit for screening many diseases, and also shows potential for cancer treatment [20−22]. Studies have shown that: (a) Niclosamide inhibits tumor cell proliferation and induces tumor cell apoptosis by inhibiting abnormal tumor

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Abbreviations used

IL-6	interleukin-6
STAT3	signal transduction and activators of transcription 3
RTK	receptor tyrosine kinase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
DMSO	dimethyl sulfoxide
FBS	fetal bovine serum
PBS	phosphate belanced solution
WB	western blot
PI	propidium iodide
IC_{50}	50% inhibiting concentration
TLC	thin layer chromatography
^{1}HNMR	nuclear Magnetic Resonance-^{1}H
^{13}CNMR	nuclear Magnetic Resonance-^{13}C
CMC-Na	carboxymethylcellulose-Na

signaling pathways such as mTOR, NF-KB, and STAT3; (b) due to the inhibitory effect of Niclosamide on STAT3 and other signaling pathways, it can be combined with traditional chemotherapy drugs to overcome drug resistance [23]; and (c) Niclosamide is used in combination with radiation therapy to overcome the radiation resistance of breast cancer and to act as a radiosensitizer [24]. Furthermore, Niclosamide is a STAT3 inhibitor screened from 1500 listed drugs by dual fluorescein reporter gene assay, which can selectively inhibit the phosphorylation of STAT3 and interfere with the STAT3 signaling pathway, thus playing a role against tumor cells. However, due to its poor hydrophobicity and low oral availability, the possibility of Niclosamide becoming an anti-tumor drug is limited [25]. Therefore, we used Niclosamide as a precursor for structural modification in order to improve its hydrophobicity while maintaining its activity in inhibiting STAT3 signaling pathway.

By analyzing the structure of existing STAT3 inhibitors (Fig. 1), it is not difficult to speculate that $-SO_2NH_2$, which contains both hydrogen bond donor —NH— and hydrogen bond acceptor $-SO_2-$, may be a key interacting group to enhance the ability to bind to STAT3, and that $-SO_2NH_2$ as a hydrophilic group can also improve the hydrophobicity of the compound. So we designed the compound NGT 02 derived from Niclosamide with $-SO_2NH_2$ (see Scheme 1 for related design). Further studies found that although the antitumor activity of compound NGT 02 decreased (see Table 1a), the hydrophobicity of NGT 02 was significantly improved (see Table 2 logP). Therefore, we made further structural modifications of NGT 02. The activity of $-SO_2NH_2$ *ortho*-introduction chlorine was discussed, and the A and B series compounds were designed. According to the preliminary activity screening (see Table 1a), the compounds with good activity were modified by amide linker or further acylated against $-SO_2NH_2$ in order to obtain a more active compound, and thus compounds X, Y and Z series were designed.

2. Results and Discussion

2.1. Chemistry

The synthetic route of the NGT 02 and compounds of A, B, X, Y, Z series were shown in Scheme 2. Intermediate NGT 01 was obtained by amidification of 2-chloro-4-nitroaniline with 2-methoxy-5-sulfamoylbenzoyl chloride obtained by chloration of the starting material 2-methoxy-5-sulfonamidobenzoic acid with dichlorosulfoxide, same as the synthesis method of intermediate NGT 01, compounds of A, B, X series was obtained by amidification of the substituted aromatic amine with 3-sulfonamidobenzoyl chloride or 4-chloro-3-sulfonamidobenzoyl chloride obtained by chloration of the starting material 3-sulfonamidobenzoic acid or 4-chloro-3-sulfonamidobenzoic acid with dichlorosulfoxide. Synthesis of NGT 02 from NGT 01 catalyzed by 5% Pd—C under H_2 conditions. The Y and Z series compounds were based on the reaction of the first three series of compounds with the corresponding substituted linear acid chloride or aromatic acid chloride.

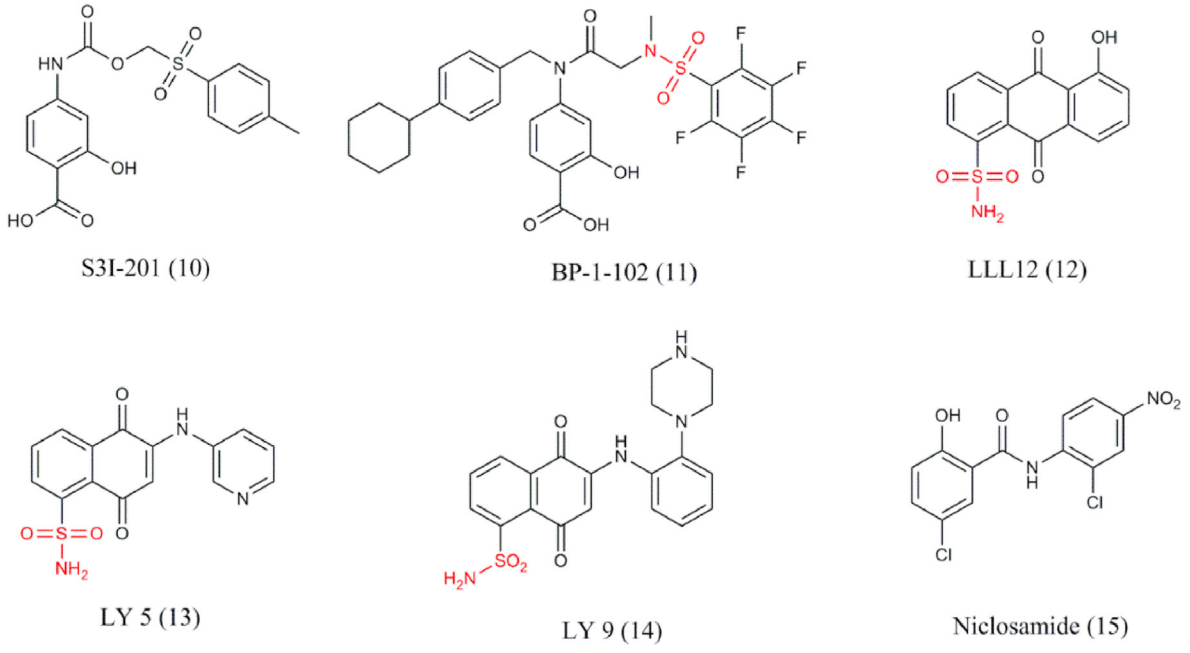
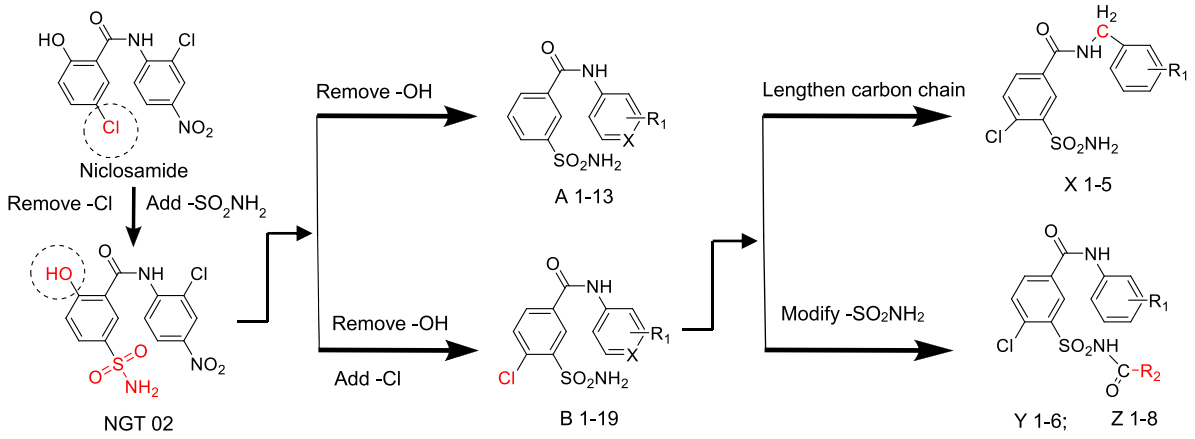
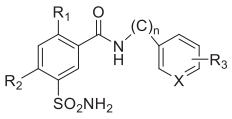


Fig. 1. Representative STAT3 small molecule inhibitors.



Scheme 1. The design based on Niclosamide
Reagents and conditions: (i) $SOCl_2$, 105 °C, 3–5 h; (ii) DIPEA, DCM, DMA, 0 °C, 12 h; (iii) 5% Pd–C, AcOEt, EtOH, H_2, 20 °C, 8 h; (iiii) NaH, THF, N_2, 20 °C, 12 h, 36–59%.

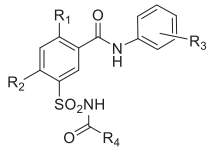
Table 1a
Biological results and Structure-activity Relationship of NGT 02, A, B and X Series.



Comp.	n	X	R_1	R_2	R_3	MDA-MB-231	HCT-116	SW480	L3.6
						(%) Inhibition (10 μM)			
Niclosamide						91.54 ± 3.25	73.77 ± 3.42	76.81 ± 5.24	66.16 ± 5.51
NGT 02	0	CH	-OH	-H	2′-Cl-4′-NO_2	48.23 ± 5.19	63.98 ± 3.15	−8.30 ± 2.77	44.73 ± 2.93
A1	0	CH	-H	-H	2′-OCH_3	27.10 ± 0.93	40.34 ± 1.12	18.16 ± 2.21	9.70 ± 2.33
A2	0	CH	-H	-H	3′-OCH_3	57.62 ± 6.43	51.35 ± 2.32	28.33 ± 1.57	15.21 ± 3.78
A3	0	CH	-H	-H	4′-OCH_3	32.33 ± 4.35	42.73 ± 3.22	38.41 ± 3.41	23.92 ± 2.66
A4	0	CH	-H	-H	3′,5′-diOCH_3	39.56 ± 5.18	47.14 ± 6.06	40.36 ± 4.13	22.19 ± 2.32
A5	0	CH	-H	-H	2′,5′-diOCH_3	37.01 ± 4.13	46.39 ± 6.13	31.93 ± 2.81	20.52 ± 3.18
A6	0	CH	-H	-H	3′,4′,5′-triOCH_3	59.44 ± 6.45	57.74 ± 3.93	26.92 ± 3.22	19.51 ± 1.47
A7	0	CH	-H	-H	2′-CF_3	42.79 ± 4.23	40.33 ± 4.73	24.81 ± 2.64	21.86 ± 3.21
A8	0	CH	-H	-H	3′-CF_3	27.16 ± 4.59	39.72 ± 3.48	34.41 ± 1.76	9.27 ± 1.18
A9	0	CH	-H	-H	4′-CF_3	26.42 ± 4.81	19.13 ± 2.15	38.13 ± 2.71	18.11 ± 1.73
A10	0	CH	-H	-H	2′-Cl-4′-NO_2	43.22 ± 6.23	15.10 ± 1.92	44.14 ± 3.63	21.29 ± 1.85
A11	0	N	-H	-H	H	42.85 ± 6.71	8.11 ± 0.32	28.33 ± 2.51	32.72 ± 2.49
A12	0	CH	-H	-H	4′,5′-diOCH_3-2′-COOH	52.13 ± 2.85	22.14 ± 2.71	22.91 ± 3.22	23.32 ± 3.41
A 13	0	CH	-H	-H	4′-$COOC(CH_3)_3$	91.12 ± 2.13	79.27 ± 2.53	55.35 ± 5.28	31.23 ± 2.77
B1	0	CH	-H	-Cl	2′-OCH_3	19.79 ± 3.27	37.34 ± 3.30	49.34 ± 5.73	22.91 ± 2.32
B2	0	CH	-H	-Cl	3′-OCH_3	42.39 ± 3.64	43.04 ± 3.86	29.32 ± 1.61	22.28 ± 1.66
B3	0	CH	-H	-Cl	4′-OCH_3	30.90 ± 2.10	20.25 ± 1.34	31.92 ± 2.72	22.79 ± 2.13
B4	0	CH	-H	-Cl	3′,5′-diOCH_3	31.80 ± 1.84	42.51 ± 3.03	27.22 ± 1.53	26.33 ± 1.78
B5	0	CH	-H	-Cl	2′,5′-diOCH_3	26.72 ± 3.53	28.22 ± 1.68	23.19 ± 1.47	24.18 ± 1.37
B6	0	CH	-H	-Cl	3′,4′,5′-triOCH_3	93.26 ± 1.45	87.91 ± 2.33	82.19 ± 3.62	42.15 ± 2.90
B7	0	CH	-H	-Cl	3′-Cl-4′-F	41.49 ± 3.54	47.53 ± 3.66	49.14 ± 2.84	30.22 ± 3.41
B8	0	CH	-H	-Cl	4′-Cl-3′-F	43.66 ± 5.64	44.14 ± 2.96	48.15 ± 3.29	26.42 ± 2.22
B9	0	CH	-H	-Cl	2′-CF_3	44.86 ± 4.84	45.38 ± 2.73	30.73 ± 4.12	21.18 ± 1.39
B10	0	CH	-H	-Cl	3′-CF_3	28.99 ± 3.93	41.36 ± 1.51	42.93 ± 3.31	22.29 ± 2.16
B11	0	CH	-H	-Cl	4′-CF_3	27.26 ± 2.45	55.35 ± 2.85	47.39 ± 3.63	29.63 ± 1.33
B12	0	CH	-H	-Cl	4′-$COOC(CH_3)_3$	92.99 ± 2.93	85.28 ± 3.72	80.94 ± 4.72	21.86 ± 1.98
B13	0	CH	-H	-Cl	4′,5′-diOCH_3-2′-COOH	66.66 ± 6.75	47.24 ± 1.55	30.25 ± 1.87	11.81 ± 1.42
B14	0	CH	-H	-Cl	4′-morpholinyl	43.31 ± 2.85	39.32 ± 2.72	21.91 ± 3.11	12.19 ± 2.37
B15	0	CH	-H	-Cl	2′,3′,4′,5′6′-perF	33.10 ± 0.14	34.03 ± 1.71	27.13 ± 2.45	12.56 ± 1.48
B16	0	CH	-H	-Cl	4′-(4′-methylpiperazin-1′-yl)	37.30 ± 2.73	40.48 ± 2.41	38.33 ± 3.50	14.59 ± 0.74
B17	0	CH	-H	-Cl	4′-$SO_2N(NH_2)_2$	46.36 ± 3.35	41.33 ± 5.62	27.32 ± 1.12	11.12 ± 1.33
B18	0	CH	-H	-Cl	3′-SO_2NH_2	52.76 ± 6.93	52.19 ± 6.14	34.29 ± 3.51	12.09 ± 0.37
B19	0	CH	-H	-Cl	2′-Cl-4′-NO_2	56.26 ± 3.48	49.45 ± 4.64	38.24 ± 4.13	12.35 ± 1.81
X1	1	CH	-H	-Cl	2′-OCH3	35.04 ± 4.03	50.05 ± 4.73	42.83 ± 4.16	17.31 ± 0.41
X2	1	CH	-H	-Cl	3′-OCH3	36.30 ± 6.05	64.17 ± 3.18	37.74 ± 2.69	19.42 ± 2.32
X3	1	CH	-H	-Cl	4′-OCH3	54.05 ± 4.44	43.15 ± 3.77	16.21 ± 1.52	29.02 ± 1.34
X4	1	CH	-H	-Cl	2′,3′-diCl	51.55 ± 4.18	70.36 ± 6.15	19.62 ± 2.78	11.61 ± 1.59
X5	1	CH	-H	-Cl	3′,4′,5′-triOCH_3	44.42 ± 2.84	34.32 ± 2.35	27.42 ± 3.16	38.24 ± 2.43

The inhibitory effects of the compounds on the proliferation of the four cell lines were determined by the MTT assay. Results are expressed as means ± SD from three independent experiments.

Table 1b
Biological results and structure-activity relationship of Y and Z Series.



Comp.	R_1	R_2	R_3	R_4	MDA-MB-231	HCT-116	SW480	L3.6
					(%) Inhibition (10 μM)			
Y1	-H	-Cl	3′,4′,5′-triOCH$_3$	-CH=CH$_2$	2.82 ± 0.61	24.20 ± 2.44	28.91 ± 2.91	−12.91 ± 1.72
Y2	-H	-Cl	3′,4′,5′-triOCH$_3$	-C(CH$_3$) = CH$_2$	4.84 ± 0.83	30.02 ± 2.71	22.27 ± 3.12	−11.27 ± 1.95
Y3	-H	-Cl	3′,4′,5′-triOCH$_3$	-CH=C(CH$_3$)$_2$	22.33 ± 2.41	20.14 ± 3.22	22.61 ± 1.66	−7.12 ± 0.51
Y4	-H	-Cl	3′,4′,5′-triOCH$_3$	-Me	19.42 ± 4.82	27.22 ± 1.50	19.52 ± 2.72	−15.17 ± 2.22
Y5	-H	-Cl	3′,4′,5′-triOCH$_3$	-Et	19.61 ± 3.01	27.19 ± 2.12	21.30 ± 1.51	1.47 ± 0.82
Y6	-H	-Cl	3′,4′,5′-triOCH$_3$	(E)-CH=CHCH$_3$	18.42 ± 2.82	29.34 ± 1.91	24.61 ± 2.15	11.71 ± 2.17
Z1	-H	-Cl	3′,4′,5′-triOCH$_3$	-phenyl	2.05 ± 0.32	32.29 ± 3.14	29.53 ± 2.72	−27.84 ± 3.11
Z2	-H	-Cl	3′,4′,5′-triOCH$_3$	2′-methoxyphenyl	13.05 ± 1.50	16.24 ± 1.43	33.92 ± 4.14	−18.31 ± 1.46
Z3	-H	-Cl	3′,4′,5′-triOCH$_3$	3′-methoxyphenyl	23.51 ± 1.81	21.29 ± 1.75	21.97 ± 3.72	−0.51 ± 0.71
Z4	-H	-Cl	3′,4′,5′-triOCH$_3$	4′-methoxyphenyl	13.12 ± 2.76	4.80 ± 0.773	20.13 ± 2.95	−18.30 ± 2.71
Z5	-H	-Cl	3′,4′,5′-triOCH$_3$	3′-nitrophenyl	14.27 ± 2.30	3.74 ± 1.03	12.12 ± 1.72	−6.13 ± 0.85
Z6	-H	-Cl	3′,4′,5′-triOCH$_3$	3′,5′-dimethoxyphenyl	4.11 ± 0.31	−2.37 ± 1.14	16.12 ± 2.37	−13.11 ± 1.41
Z7	-H	-Cl	3′,4′,5′-triOCH$_3$	2′,6′-dimethoxyphenyl	18.13 ± 2.50	10.15 ± 1.62	30.63 ± 3.23	4.06 ± 0.81
Z8	-H	-Cl	3′,4′,5′-triOCH$_3$	6′-chloropyridin-3′-yl	5.01 ± 0.42	−9.17 ± 1.21	8.11 ± 2.38	−15.72 ± 3.32

The inhibitory effects of the compounds on the proliferation of the four cell lines were determined by the MTT assay. Results are expressed as means ± SD from three independent experiments.

Table 2
Anti-proliferative activity of some designed compounds and reference Niclosamide.

Comp.	logP	IC_{50} ± SD (μM)[a]		
		MDA-MB-231	HCT-116	SW480
Niclosamide	3.17 ± 0.15	0.80 ± 0.03	0.45 ± 0.05	
2.16 ± 0.19		NGT 02	1.41 ± 0.08	>20
5.68 ± 0.24	>20			
A2	0.96 ± 0.06	13.58 ± 0.52	11.22 ± 0.63	>20
A6	0.79 ± 0.05	12.72 ± 0.66	10.69 ± 0.51	>20
A12	0.37 ± 0.04	17.88 ± 0.52	>20	>20
A13	1.54 ± 0.08	0.70 ± 0.08	9.82 ± 0.55	
17.12 ± 0.81		B6	1.19 ± 0.07	
1.07 ± 0.28	3.64 ± 0.76	1.75 ± 0.07		
B11	2.76 ± 0.11	7.32 ± 0.51	10.85 ± 0.66	>20
B12	1.83 ± 0.10	0.61 ± 0.03	0.69 ± 0.02	
1.11 ± 0.09		B13	0.73 ± 0.05	
6.84 ± 0.62		12.25 ± 0.31	>20	
B18	1.19 ± 0.07	11.89 ± 0.81	17.15 ± 0.46	>20
B19	2.37 ± 0.13	14.31 ± 0.64	19.06 ± 0.52	>20
X1	1.77 ± 0.09	>20	>20	>20
X2	1.34 ± 0.07	>20	18.63 ± 0.89	>20
X4	2.97 ± 0.15	>20	9.62 ± 0.53	>20

Note: a: SD: standard deviation; all experiments were independently performed at least three times.

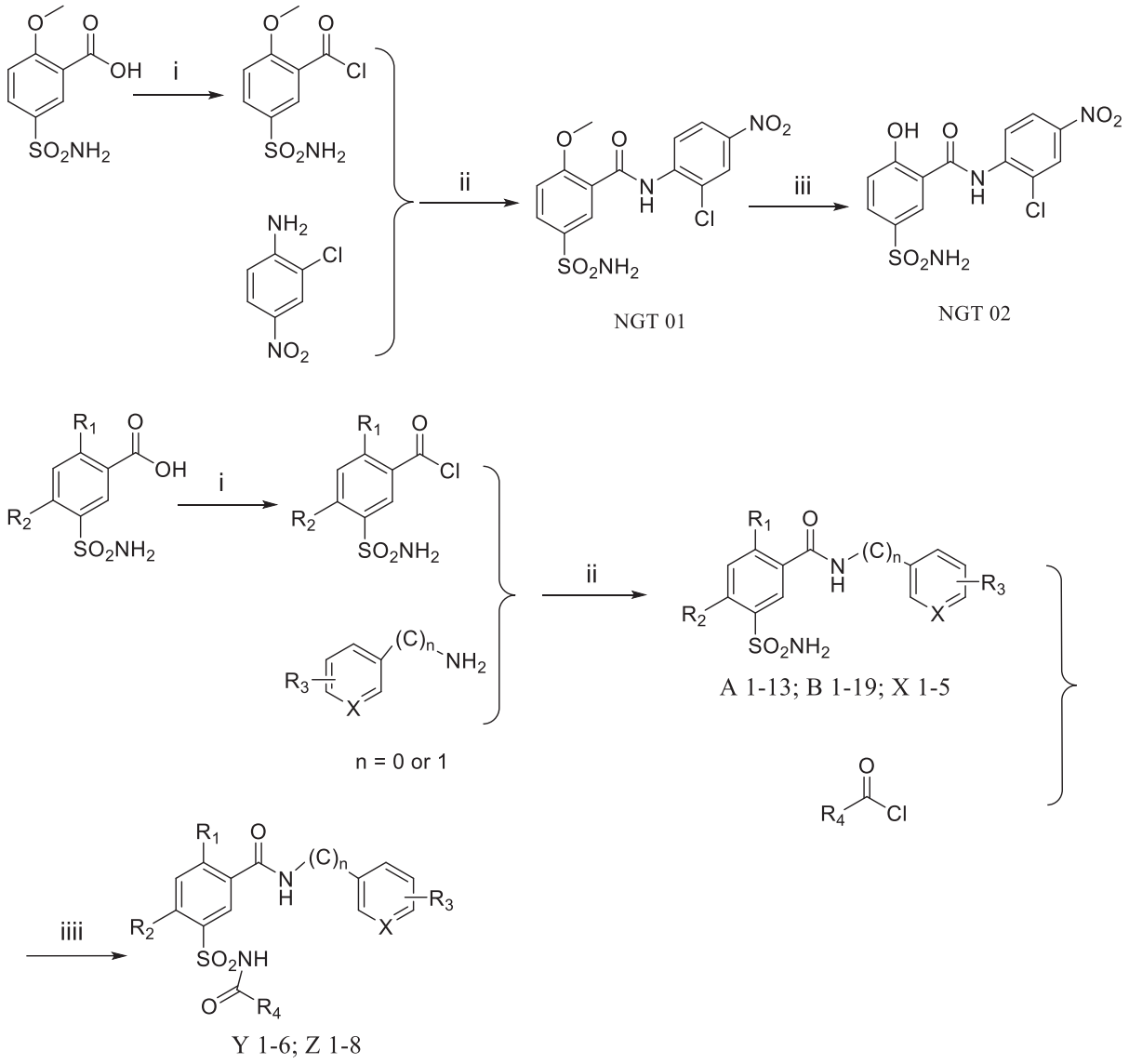
2.2. Biological evaluation

2.2.1. Cell viability assay and SAR analysis

Initially, four cell lines (three STAT3 high-expressing cell lines (MDA-MB-231, HCT-116, SW480) and one non-STAT3 high-expressing cell line (L3.6)) were used to screen the inhibitory rate of all the synthesized compounds (Table 1a, Table 1b). The results showed that the inhibitory effect of the synthesized compounds on STAT3 high expression cell lines was generally better than that of non-STAT3 high expression cell lines, which indicated that the compounds we designed have selectivity for STAT3. Among them, the compounds B6 and B12 performed best. However, the anti-tumor effects of the Y and Z series compounds obtained by further acylation of $-SO_2NH_2$ were generally inferior to those of other series of compounds. This suggest the necessity of $-SO_2NH_2$ for activity. Then, IC_{50} value for the compounds, which the inhibition rate of all three cells was more than 50%, were tested by MTT assays, and Niclosamide was used as a positive control. The most potent compound B12 inhibited the growth of MDA-MB-231, HCT-116, SW480 cells with IC_{50} values of 0.61, 0.79, 1.11 μM showed that it has a good inhibitory effect on these three tumor cells and slightly better than Niclosamide (Table 2).

LogP is a measure of the hydrophobicity of the compound. We tested the logP values of the synthesized compound and the positive control to determine whether the modified compound has improved hydrophobicity compared to the positive control. Table 2 listed the logP values of some active compounds and Niclosamide. It can be seen that the designed compounds are less hydrophobic than the positive control, that is, the hydrophobicity is improved, including the most potent compound B12.

SAR analysis showed that: (1) According to the logP results, replacing the 5-position Cl of Niclosamide with $-SO_2NH_2$ can significantly improve the bioavailability but decrease hydrophobicity of the compound (Table 2). (2) The removal of 2-position-OH not only simplifies the synthesis of the compound but also preserves the anti-proliferative activity of the compound (like NGT02 vs A10 in Table 1a); (3) The structure of N-substituted sulfamoylbenzamide derivatives further acylated of the $-SO_2NH_2$ group can greatly decrease the biological activity (like B6 vs Y, Z series), this result also indicates the importance of the $-SO_2NH_2$ group for the anti-proliferative activity of STAT3 overexpressing tumor cells. (4) The introduction of a chlorine atom at the 4-position of the benzene ring of the compound generally enhances the inhibitory activity of the compound on the above-mentioned cancer cells (like B6 vs A6; A13 vs B12); So, chlorine atoms remain unchanged in the subsequent modification; (5) The carbon chain elongation linked to benzsulfonamide N can sometimes decrease the anti-proliferative activity of the compounds (like X5 vs B6).



Reagents and conditions: (i) $SOCl_2$, 105 °C, 3~5 h; (ii) DIPEA, DCM, DMA, 0 °C, 12 h; (iii) 5% Pd-C, AcOEt, EtOH, H_2, 20 °C, 8 h; (iiii) NaH, THF, N_2, 20 °C, 12 h, 36~59%.

Scheme 2. Synthesis of compound NGT 02, A Series, B Series, X Series, Y Series and Z Series.

Table 3
The IC_{50} of three dominant compounds and Niclosamide in MDA-MB-231 cells.

Compound	IC_{50} ± SD (μM)
Niclosamide	0.21 ± 0.03
A13	0.92 ± 0.07
B6	0.72 ± 0.08
B12	0.23 ± 0.02

2.2.2. Inhibitory effect of STAT3 tyrosine phosphorylation

The ability of three dominant compounds (A13, B6, B12) to inhibit the phosphorylation of STAT3 was determined in MDA-MB-231 cells (Table 3). B12, which exhibits the most potent anti-proliferative activity, inhibits the phosphorylation of STAT3 with an IC50 value of 0.23 μM. And its inhibition is comparable to Niclosamide. Compound A13 which does not introduce a chlorine atom at the 4-position of the compound benzene ring has a weaker inhibitory effect on STAT3 kinase phosphorylation than the compound B6 and B12. This result further confirms that the introduction of chlorine atoms at the 4-position of the benzene ring of the compound increases the selectivity of compounds for STAT3. We also conducted Western blot analysis to confirm the inhibitory activity of B12 on STAT3 phosphorylation. It was showed that B12 markedly suppressed STAT3 phosphorylation at Tyr705 residue in a dose-dependent manner with the expression of total STAT3 not changed, which indicated that the decrease of p-STAT3 (Y705) has no concern with a constitutional decrease of total STAT3 expression (Fig. 2A). STAT3 can be induced by IL-6. We can clearly know that B12 significantly inhibited STAT3 phosphorylation elevated by IL-6. And compound B12 almost completely blocked the stimulation at 5 μM (Fig. 2B). These results was consistent with Table 2.

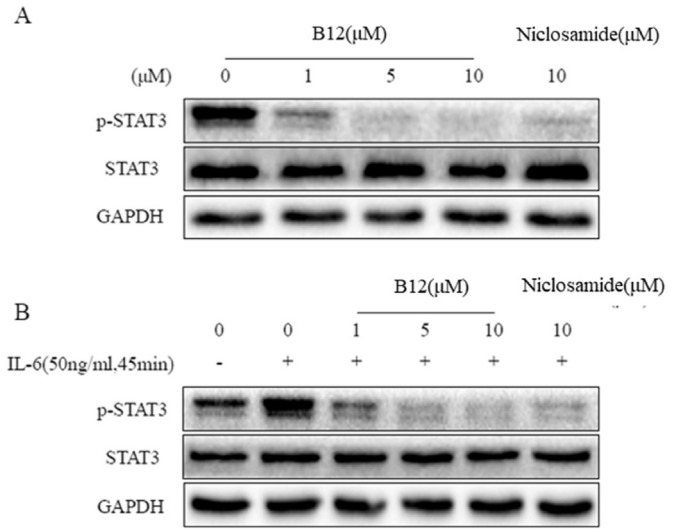


Fig. 2. Compound B12 inhibited STAT3 activation. (A and B) Western blot analysis of p-STAT3 (Y705), STAT3 and IL-6 induced p-STAT3 levels in whole-cell lysates of equal total protein prepared from MDA-MB-231 cells treated with compound B12 for 2 h. The results represent at least 3 independent experiments.

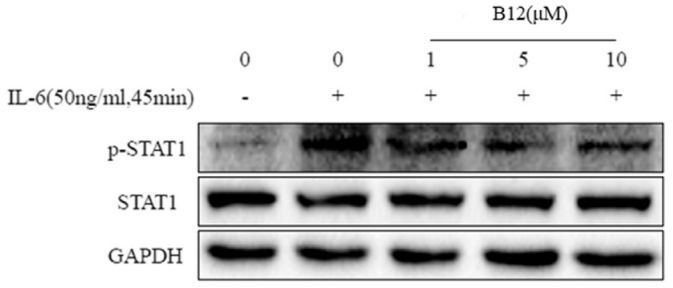


Fig. 3. IL-6 induced STAT1 phosphorylation in MDA-MB-231 cancer cells. The MDA-MB-231 cells were serum-starved overnight, then left untreated or treated with B12 (1–10 μM) for 2 h, followed by stimulation by IL-6 (50 ng/mL). The cells were harvested at 45 min and analyzed by Western blot assays. The results represent at least 3 independent experiments.

Table 4
Kinase inhibition rate after treating with 10 μM B12.

RTK	(%)Inhibition	RTK	(%)Inhibition
EGFR (WT)	4.41 ± 0.25	FLT1	4.33 ± 0.35
EGFR T790 M	2.23 ± 0.03	FLT3	10.20 ± 0.15
EGFR L858R	4.82 ± 0.32	MET	0.13 ± 0.02
JAK1	2.99 ± 0.11	MEK1	1.56 ± 0.25
JAK2	4.77 ± 0.08	ALK	5.19 ± 0.34
JAK3	4.24 ± 0.55	c-Kit	3.33 ± 0.26
TYK2	−1.53 ± 0.11	CDK7	6.45 ± 0.42
SRC	1.42 ± 0.06	CDK1/CyCB1	−2.27 ± 0.26
FGFR1	3.58 ± 0.08	IGF1R	2.93 ± 0.12
FGFR2	5.28 ± 0.43	IKKβ	2.87 ± 0.15
FGFR3	1.25 ± 0.16	JNK1	2.52 ± 0.12
FGFR4	−1.53 ± 0.04	JNK2	4.16 ± 0.27
AKT1	3.48 ± 0.23	PLK1	5.19 ± 0.23
AKT2	3.38 ± 0.21	PAK1	9.32 ± 0.38
Erk1	1.72 ± 0.11	PDGFRβ	−1.19 ± 0.11
Erk2	0.78 ± 0.02	RET	−7.23 ± 0.29
ROS1	−3.38 ± 0.26	WEE1	4.76 ± 0.18

Results are expressed as means ± SD from three independent experiments.

2.2.3. The selectivity of compound B12 to STAT3

STAT1 and STAT3 are structurally homologous, but STAT1 plays a role in inhibiting tumor cell proliferation and promoting apoptosis of tumor cells [26]. Thus, in the design of STAT3 small molecule inhibitors, the selectivity of STAT1 should be detected [27]. So Western blot assays were performed to investigate the effects of B12 on STAT1 phosphorylation. As shown in Fig. 3, B12 had little influence on the extent of STAT1 phosphorylation.

After that, we tested 34 kinases associated with antitumor to further verify the selectivity of B12 with10 μM. Some of these activated kinases could promote the activation of STAT3 directly or indirectly. The results were shown in Table 4. The maximum inhibition rate of B12 on these kinases was only 9.32%. To a certain extent, compound B12 can selectively inhibit STAT3 phosphorylation.

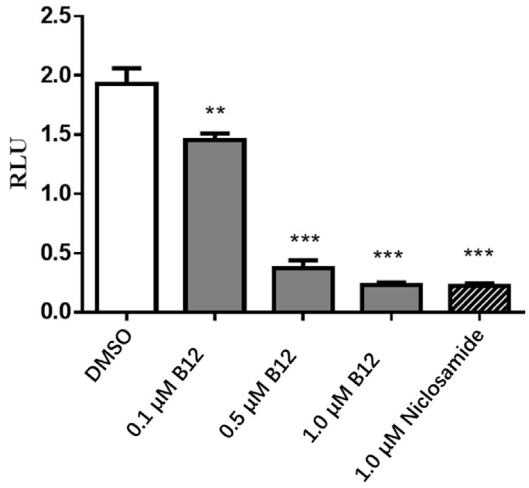


Fig. 4. Compound B12 reduce STAT3 promoter activity by STAT3-mediated luciferase reporter activity in MDA-MB-231 cells. RLU: relative luciferase unit. (**: $p < 0.01$; ***, $p < 0.001$, compared with the DMSO group), the results represent at least 3 independent experiments.

2.2.4. Compound B12 could inhibit promoter activity of STAT3

STAT3 promoter activity was examined in MDA-MB-231 cells transiently transfected with pSTAT3-Luc vector using dual luciferase reporter assay. The STAT3 promoter activity were decreased by 24.7%, 80.6% and 87.9%, in the cells treated with B12 at concentrations of 0.1, 0.5 and 1.0 μM, respectively (Fig. 4). In other words, the inhibition was in a dose-dependent manner. In MDA-MB-231 cells, B12 has an inhibit promoter activity of STAT3 comparable to that of Niclosamide (decreased by 88.2% at concentrations of 1.0 μM).

2.2.5. Compound B12 induced apoptosis and caused changes in related apoptotic proteins

We had demonstrated that compound B12 can effectively inhibit STAT3 activation. To further explore the mechanism of B12 in this pathway, we examined its effect on the related apoptotic proteins. MDA-MB-231 cells were treated with compound B12 and detected by Western blot. As can be seen from Fig. 5, the compound B12 inhibited the expression of the Anti-apoptotic protein, Bcl-xl and induced apoptosis proteins such as Cleaved-PARP, Cleaved-caspase 9, and Cleaved-caspase 3.

We also analyzed the effect of compound B12 on the induction of tumor cell apoptosis. MDA-MB-231 cells were treated with B12 at different concentrations for 24 h. Annexin V-FITC/PI staining was performed and the percentages of apoptotic cells were further determined using flow cytometry. As shown in Fig. 6, B12 significantly induced apoptosis in MDA-MB-231 cells in a dose-

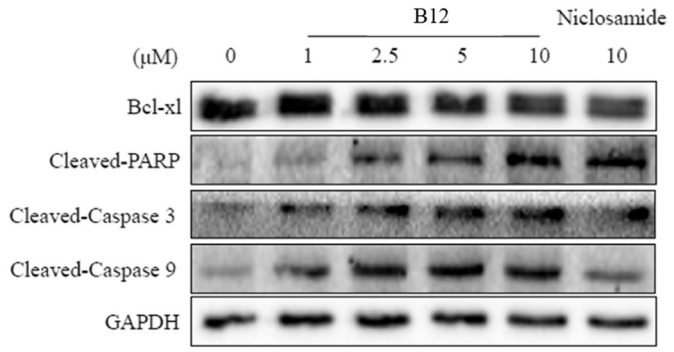


Fig. 5. Effects of compound B12 on the expression of downstream gene, Bcl-xl and the levels of Cleaved-PARP, Cleaved-Caspase 3 and Cleaved-Caspase 9. Western blot analysis of Bcl-xl, Cleaved-PARP, Cleaved-Caspase 3 and Cleaved-Caspase 9 levels in whole-cell lysates of equal total protein prepared from MDA-MB-231 cells treated with compound B12 for 24 h. The results represent at least 3 independent experiments.

dependent manner. In summary, compound B12 induced the apoptosis of tumor cells through activating the caspase-dependent pathway relating to Bcl-xl, which was regulated by the deactivation of STAT3 protein.

2.2.6. Compound B12 reduced tumor cells colony formation in vitro

We used colony formation assays to further assess the in vitro anti-proliferative capacity of B12. After treatment with different concentrations of B12 for 24 h, MDA-MB-231 cells were replaced with fresh medium without B12 for 14 days until the colonies were visible. As shown in Fig. 7, B12 significantly inhibited the formation of colonies at 1 μM, and the inhibitory effect was comparable to that of the positive control.

2.2.7. Compound B12 inhibited migration of cancer cells in vitro

Overexpression of STAT3 is closely linked with the migration of tumor cells. Therefore, the effects of B12 on cell migration of MDA-MB-231 cells were further assessed by transwell assay. MDA-MB-231 cells were plated in the upper chamber containing a membrane, then cells were treated with B12 or Niclosamide for 24 h, and cells on the underside of the membrane was imaged under light microscopy. The results showed that migration of MDA-MB-231 cells was inhibited in a dose-dependent manner when treated with B12 (Fig. 8). Meanwhile, at the same concentration (1000 nM), the inhibition of tumor cell migration in the group of administration B12 has the same effect, compared with Niclosamide.

2.2.8. Compound B12 inhibited tumor growth in a mouse breast cancer model

To further evaluated the inhibitory effect of B12 on breast cancer xenografts in vivo, and tumor volume was measured. After the solid tumor was established, fifteen nude mice were randomly divided into three groups, which were blank control group, B12 administration group and Niclosamide administration group by intragastric gavage. As we could see from the results (Fig. 9), B12 could significantly inhibit tumor growth. and the ability of B12 to inhibit tumor growth was comparable to that of the positive control (Fig. 9B and D). Moreover, perhaps the increase in hydrophilicity improves the bioavailability of B12, resulting B12 being better than the positive control in inhibiting tumor growth, and the results were statistically significant. At the same time, during the administration period, the weight of nude mice did not increase or decrease significantly.

3. Conclusions

Taken together, the query was verified that Niclosamide-modified sulfonamide derivative B12 can improve hydrophobicity and maintain its activity in inhibiting STAT3 signaling pathway. The first reported 52 compounds including A13, B6 and B12 were designed, synthesized and evaluated for their bioactivity. Among all synthesized compounds, compounds A13, B6 and B12 exhibited biological activities equivalent to or even superior to the lead compound Niclosamide; The Western blot assays, kinase activity assays, STAT3 ELISA kit and STAT3 promoter activity studies indicated that B12 could inhibit STAT3 phosphorylation. Additionally,

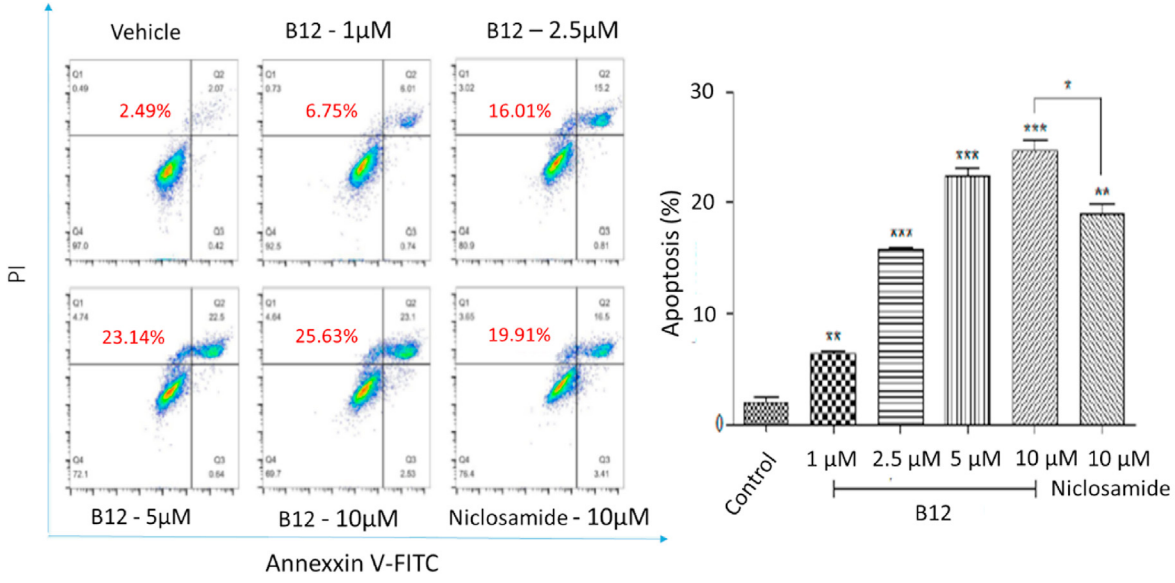


Fig. 6. Compound B12 induced MDA-MB-231 cancer cells apoptosis in Vitro. MDA-MB-231 cells were incubated with B12 at different concentrations (1–10 μM) or Niclosamide (10 μM) for 24 h. Annexin V/PI staining was carried out and the percentages of apoptotic cells were further determined using flow cytometry. *, $p < 0.05$ compared with the blank control group; **, $p < 0.01$; ***, $p < 0.001$; compared with the positive control group, #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$; the results represent at least 3 independent experiments.

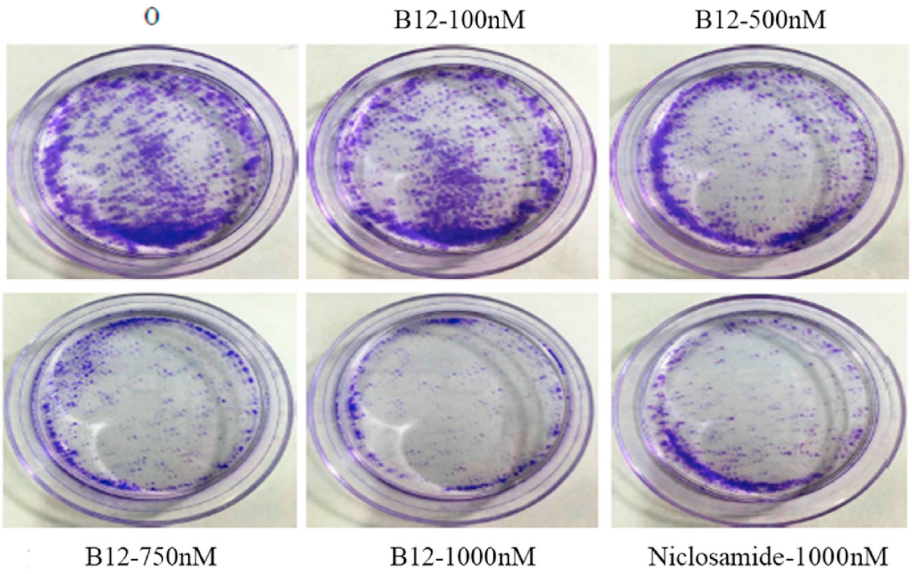


Fig. 7. Compound B12 inhibited the colony formation of MDA-MB-231 cancer cells. MDA-MB-231 cells were treated with compound B12 at 0.1−1 μM or Niclosamide (10 μM) for 24 h and cultured for 14 d until the colonies were visible. Crystal violet solution was used to stain the colonies for 4 h and imaged. The results represent at least 3 independent experiments.

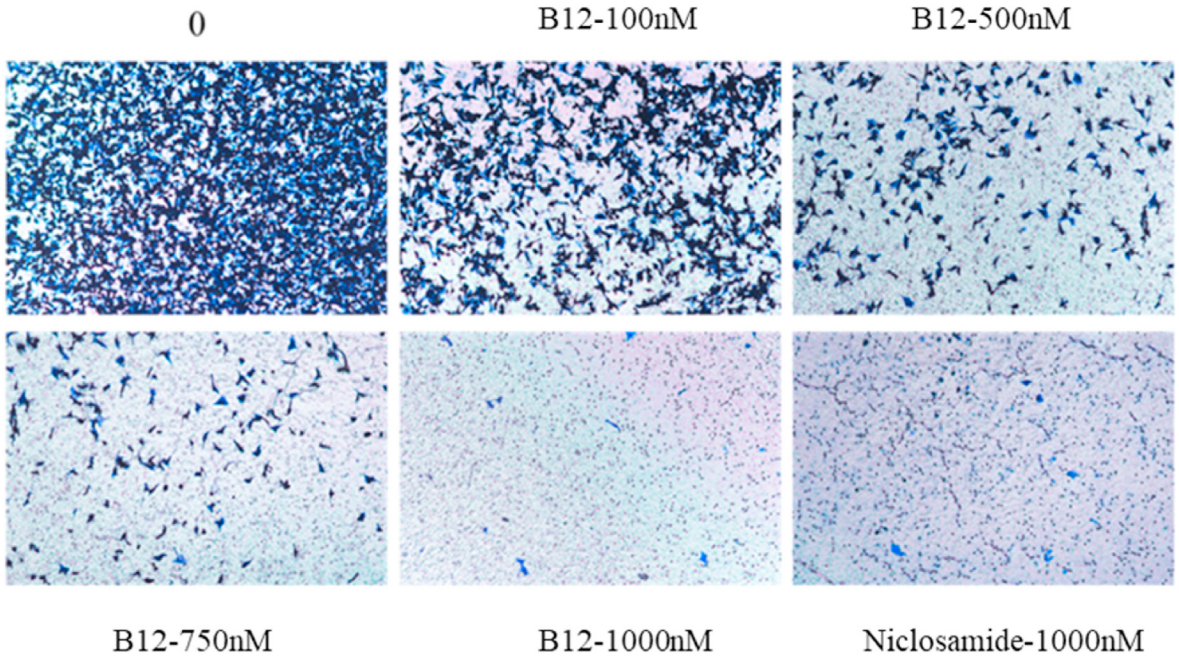


Fig. 8. Compound B12 inhibited Migration of MDA-MB-231 cancer cells in vitro. MDA-MB-231 cells were treated by B12 (0.1−1 μM) or Niclosamide (1 μM) and were allowed to migrate through the porous membrane for 24 h. Then cells in the upper surface of the chamber were completely removed and the lower surfaces of the membranes were stained with crystal violet solution for 20−30 min and imaged. The results represent at least 3 independent experiments.

cell apoptosis assays demonstrated that B12 could promote apoptosis of cancer cells; Transwell assays showed that B12 could be able to inhibit the migration of MDA-MB231 cells; In addition, B12 can inhibit the formation of tumor cell colonies. Importantly, B12 showed the better anti-tumor effects than Niclosamide in vivo though a MDA-MB-231-driven xenograft mouse model. These results have demonstrated that B12 is worthy of further research to develop as novel small-molecule STAT3 inhibitor for human cancer.

4. Materials and methods

4.1. Chemistry

All materials and reagents were purchased from commercial suppliers and used without further purification unless otherwise stated. The progress of the reaction was monitored by analytical thin layer chromatography (TLC) using a silica gel GF254 plate (Qingdao Haiyang Chemical Plant, China), and the spots were observed under UV light of 254 nm or 365 nm. Column

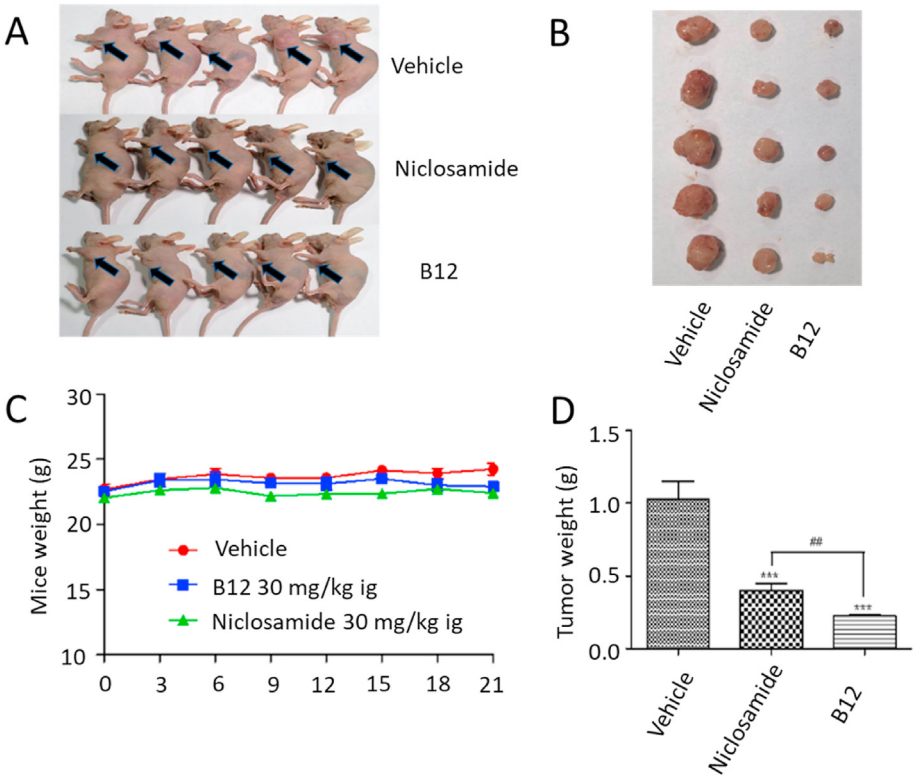


Fig. 9. Effect of compound B12 and positive control Niclosamide on tumor weight and body weight of nude mice in MDA-MB-231 nude mice xenografts; (A) (B) tissue map of nude mice and nude mice xenografts; (C) Body weight of nude mice; (D) Weight of nude mice xenografts; *, $p < 0.05$ compared with the blank control group; **, $p < 0.01$; ***, $p < 0.001$; compared with the positive control group, #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$; the results represent at least 3 independent experiments.

chromatography was carried out on silica gel (90–150 μM; Qingdao Ocean Chemical Co., Ltd.). The melting point measured on the XT-4 micromelting point apparatus without corrected. ^{1}H NMR and ^{13}C NMR spectra were measured on a Bruker 600 MHz Avanve NMR spectrometer or a Bruker 500 MHz Avanve NMR spectrometer using $CDCl_3$ or DMSO-d_6 as a solvent. Mass spectra were obtained on an ACQUITY I-Class UPLC and a XEVO TQD triple quadrupole Mass Spectrometer (Waters Corp., Milford, USA). HPLC (Agilent 1260 , USA) was used to detect the purity of the compounds, and the results showed that all of them exceeded 96%. Elemental analysis for C, H and N were analyzed using elemental analyser (Flash EA1112) and were found within ±0.5% of the theoretical values.

4.1.1. Synthetic routes of N-(2-chloro-4-nitrophenyl)-2-hydroxy-5-sulfamoylbenzamide (NGT 02)

a. 2-methoxy-5-sulfonamidobenzoic acid (1 g, 4.3 mmol) and 30 mL of freshly steamed $SOCl_2$ were refluxed at 105 °C for 3–5 h. The mixture was allowed to stand for cooling, and the thionyl chloride was concentrated under reduced pressure to give 2-methoxy-5sulfonamidobenzoyl chloride;
b. The raw material 2-chloro-4-nitroaniline (460 mg, 2.7 mmol) was weighed and dissolved in 20 mL of dichloromethane, and 350 μL of the base N,N-diisopropylethylamine (DIPEA, 2.7 mmol) was added.), stirring in ice bath for 15 min. Then, 4 mmol of the above-obtained 2-methoxy-5-sulfonamidobenzoyl chloride was added, and the mixture was stirred for 12 h under ice-cooling, and the reaction was monitored by the TLC method. After the reaction is terminated, the product is isolated and purified to obtain the intermediate N-(2-chloro-4-nitrophenyl)-2-methoxy-5-sulfonamidobenzamide (NGT 01);
c. The intermediate NGT 01 (110 mg, 0.28 mmol) and 5% pd/C catalyst (14 mg) were dissolved in 10 mL of ethyl acetate:ethanol (1:1) in H_2 at room temperature. The reaction was carried out for 8 h, and the progress of the reaction was monitored by the TLC method. After the reaction was terminated, the mixture was concentrated under reduced pressure, and then purified by column chromatography to give the product N-(2-chloro-4-nitrophenyl)-2-hydroxy-5-sulfonamidobenzamide (NGT 02).

4.1.2. Synthetic routes of compound a series, B series and X series

a. 3-sulfonamidobenzoic acid or 4-chloro-3-sulfonamidobenzoic acid (3 mmol) and 30 mL of freshly steamed $SOCl_2$, were refluxed at 105 °C for 3–5 h. Cooling and standing, then evaporating the thionyl chloride under reduced pressure to obtain 3-sulfonyl benzoyl chloride or 4-chloro-3-sulfonyl benzoyl chloride;
b. The corresponding substituted aromatic amine (0.5 mmol) was weighed and dissolved in 10 mL of dichloromethane, and 65 μL of base DIPEA (0.5 mmol) was added, and the mixture was stirred for 15 min in an ice bath. Then, 0.75 mmol of the above-obtained 3-sulfonamidobenzoyl chloride or 4-chloro-3-sulfonamidobenzoyl chloride was added, and the mixture was stirred for 12 h under ice-cooling, and the reaction was monitored by TLC. After the reaction is terminated, the product is isolated and purified to obtain three series of compounds: A, B and X.

4.1.3. Synthetic routes of compound Y series and Z series

4-chloro-3-sulfamoyl-N-(3,4,5-trimethoxyphenyl)benzamide

(100 mg, 0.25 mmol), NaH (24 mg, 1 mmol), were dissolved in 5 mL of anhydrous tetrahydrofuran and refluxed at room temperature for 30 min with Nitrogen protection. Then, the corresponding substituted linear acid chloride or aromatic acid chloride dissolved in 5 mL of anhydrous tetrahydrofuran was added, and the mixture was stirred at room temperature for 12 h, and the progress of the reaction was monitored by TLC. After the reaction is terminated, the product is isolated and purified to obtain three series of compounds: Y and Z.

N-(2-chloro-4-nitrophenyl)-2-hydroxy-5-sulfamoylbenzamide (NGT 02). Yield/%: 25.2%; MP: 199.2–201.3 °C; ESI-MS: 372.22 $[M+H]^+$; ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 12.54 (s, 1H, 2-OH), 10.73 (s, 1H), 8.58 (d, 1H, J = 2.0 Hz), 8.45 (d, 1H, J = 2.5 Hz), 8.29 (dd, 1H, J_1 = 7.5 Hz, J_2 = 2.0 Hz), 8.22 (dd, 1H, J_1 = 7.0 Hz, J_2 = 2.0 Hz), 8.01 (d, 1H, J = 7.0 Hz), 7.89 (d, 1H, J = 7.0 Hz), 7.80 (s, 2H); ^{13}C NMR (125 MHz, DMSO-d_6): 162.2, 159.8, 142.8, 140.9, 137.2, 131.8, 129.5, 124.7, 123.8, 122.9, 121.0, 120.4, 113.5. Anal. Calcd for $C_{13}H_{10}ClN_3O_6S$: C, 41.96; H, 2.72; N, 11.30. Found: C, 41.89; H, 2.71; N, 11.26.

N-(2-methoxyphenyl)-3-sulfamoylbenzamide (A1). Yield/%: 83.5%; MP: 183.6–186.1 °C; ESI-MS: 307.11 $[M+H]^+$; ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 9.78 (s, 1H), 8.39 (s, 1H), 8.18 (d, 1H, J = 7.8 Hz), 8.01 (d, 1H, J = 7.8 Hz), 7.73 (t, 1H, J = 7.8 Hz), 7.68 (t, 1H, J = 7.2 Hz), 7.51 (s, 2H), 7.21 (m, 1H), 7.11 (d, 1H, J = 7.8Hz), 6.97 (m, 1H), 3.83 (s, 3H); ^{13}C NMR (151 MHz, DMSO-d_6): 164.0, 151.9, 144.5, 135.3, 130.4, 129.3, 128.4, 126.5, 126.2, 125.1, 124.9, 120.2, 111.6, 55.7. Anal. Calcd for $C_{14}H_{14}N_2O_4S$: C, 54.84; H, 4.57; N, 9.14. Found: C, 54.78; H, 4.58; N, 9.13.

N-(3-methoxyphenyl)-3-sulfamoylbenzamide (A2). Yield/%: 85.7%; MP: 182.8–185.0 °C; ESI-MS: 307.05 $[M+H]^+$; ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 10.48 (s, 1H), 8.38 (s, 1H), 8.17 (d, 1H, J = 7.8 Hz), 8.02 (d, 1H, J = 7.8 Hz), 7.75 (t, 1H, J = 7.8 Hz), 7.51 (s, 2H), 7.46 (t, 1H, J = 1.8 Hz), 7.38 (d, 1H, J = 8.4 Hz), 7.28 (t, 1H, J = 8.4 Hz), 6.72 (dd, 1H, J_1 = 8.4 Hz, J_2 = 1.8 Hz), 3.76 (s, 3H); ^{13}C NMR (151 MHz, DMSO-d_6): 165.0, 160.0, 145.1, 140.6, 136.2, 131.3, 130.1, 129.8, 129.1, 125.7, 113.3, 110.1, 106.8, 55.6. Anal. Calcd for $C_{14}H_{14}N_2O_4S$: C, 54.84; H, 4.57; N, 9.14. Found: C, 54.85; H, 4.56; N, 9.15.

N-(4-methoxyphenyl)-3-sulfamoylbenzamide (A3). Yield/%: 88.9%; MP: 179.3–182.4 °C; ESI-MS: 306.98 $[M+H]^+$; ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 10.38 (s, 1H), 8.38 (s, 1H), 8.17 (d, 1H, J = 7.8 Hz), 8.01 (d, 1H, J = 8.4 Hz), 7.73 (t, 1H, J = 7.8 Hz), 7.67 (d, 2H, J = 9.0 Hz), 7.48 (s, 2H), 6.95 (d, 2H, J = 9.0 Hz), 3.75 (s, 3H); ^{13}C NMR (151 MHz, DMSO-d_6): 164.5, 156.4, 145.0, 136.3, 132.5, 131.2, 129.8, 128.9, 125.6, 122.7, 114.4, 55.8. Anal. Calcd for $C_{14}H_{14}N_2O_4S$: C, 54.84; H, 4.57; N, 9.14. Found: C, 54.76; H, 4.57; N, 9.14.

N-(3, 5-dimethoxyphenyl)-3-sulfamoylbenzamide (A4). Yield/%: 87.6%; MP: 187.1–190.3 °C; ESI-MS: 337.07 $[M+H]^+$; ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 10.43 (s, 1H), 8.37 (s, 1H), 8.16 (d, 1H, J = 7.8 Hz), 8.02 (d, 1H, J = 7.8 Hz), 7.74 (t, 1H, J = 7.8 Hz), 7.51 (s, 2H), 7.08 (d, 2H, J = 2.4 Hz), 6.30 (t, 1H, J = 2.4 Hz), 3.74 (s, 6H); ^{13}C NMR (151 MHz, DMSO-d_6): 165.0, 161.0, 145.1, 141.1, 136.2, 131.3, 129.8, 129.1, 125.7, 99.3, 96.6, 55.8. Anal. Calcd for $C_{15}H_{16}N_2O_5S$: C, 53.51; H, 4.80; N, 8.32. Found: C, 53.50; H, 4.79; N, 8.35.

N-(2, 5-dimethoxyphenyl)-3-sulfamoylbenzamide (A5). Yield/%: 81.9%; MP: 189.3–191.0 °C; ESI-MS: 337.13 $[M+H]^+$; ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 9.70 (s, 1H), 8.38 (s, 1H), 8.16 (d, 1H, J = 7.2 Hz), 8.01 (d, 1H, J = 7.8 Hz), 7.73 (t, 1H, J = 7.8 Hz), 7.49 (s, 2H), 7.43 (d, 1H, J = 3.0 Hz), 7.03 (d, 1H, J = 9.0 Hz), 6.77 (dd, 1H, J_1 = 9.0 Hz, J_2 = 3.0 Hz), 3.79 (s, 3H), 3.72 (s, 3H); ^{13}C NMR (151 MHz, DMSO-d_6): 164.6, 153.4, 146.4, 145.1, 135.8, 131.1, 129.9, 129.1, 127.9, 125.7, 112.9, 111.5, 110.7, 56.8, 56.1. Anal. Calcd for $C_{15}H_{16}N_2O_5S$: C, 53.51; H, 4.80; N, 8.32. Found: C, 53.47; H, 4.80; N, 8.33.

3-sulfamoyl-N-(3, 4, 5-trimethoxyphenyl)benzamide (A6). Yield/%: 84.6%; MP: 243.3–246.1 °C; ESI-MS: 367.09 $[M+H]^+$; ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 10.42 (s, 1H), 8.39 (s, 1H), 8.17 (d, 1H, J = 7.8 Hz), 8.02 (d, 1H, J = 7.8 Hz), 7.75 (t, 1H, J = 7.8 Hz), 7.51 (s, 2H), 7.22 (s, 2H), 3.78 (s, 6H), 3.64 (s, 3H); ^{13}C NMR (151 MHz, DMSO-d_6): 164.8, 153.2, 145.1, 136.2, 135.6, 134.5, 131.2, 129.8, 129.1, 125.6, 98.8, 60.7, 56.4. Anal. Calcd for $C_{16}H_{18}N_2O_6S$: C, 52.40; H, 4.96; N, 7.64. Found: C, 53.46; H, 4.94; N, 7.63.

3-sulfamoyl-N-(2-(trifluoromethyl)phenyl)benzamide (A7). Yield/%: 92.3%; MP: 174.9–177.2 °C; ESI-MS: 345.07 $[M+H]^+$; ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 10.46 (s, 1H), 8.39 (s, 1H), 8.17 (d, 1H, J = 7.8 Hz), 8.04 (d, 1H, J = 8.4 Hz), 7.83 (d, 1H, J = 7.8 Hz), 7.77 (td, 2H, J_1 = 7.2 Hz, J_2 = 2.4 Hz), 7.53 (m, 4H); ^{13}C NMR (151 MHz, DMSO-d_6): 165.3, 144.6, 135.5, 134.6, 133.2, 131.2, 130.5, 129.3, 128.7, 127.7, 126.6, 125.1, 124.7, 122.5. Anal. Calcd for $C_{14}H_{11}F_3N_2O_3S$: C, 48.79; H, 3.23; N, 8.13. Found: C, 48.71; H, 3.22; N, 8.11.

3-sulfamoyl-N-(3-(trifluoromethyl)phenyl)benzamide (A8). Yield/%: 98.6%; MP: 172.3–174.2 °C; ESI-MS: 345.13 $[M+H]^+$; ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 10.81 (s, 1H), 8.41 (s, 1H), 8.23 (s, 1H), 8.21 (d, 1H, J = 7.8 Hz), 8.04 (m, 2H), 7.78 (t, 1H, J = 7.8 Hz), 7.63 (t, 1H, J = 8.4 Hz), 7.53 (s, 2H), 7.49 (d, 1H, J = 7.8 Hz); ^{13}C NMR (151 MHz, DMSO-d_6): 165.4, 145.2, 140.3, 135.7, 131.4, 130.6, 130.1, 130.0, 129.9, 129.4, 125.7, 124.6, 120.9, 117.1. Anal. Calcd for $C_{14}H_{11}F_3N_2O_3S$: C, 48.79; H, 3.23; N, 8.13. Found: C, 48.78; H, 3.24; N, 8.14.

3-sulfamoyl-N-(4-(trifluoromethyl)phenyl)benzamide (A9). Yield/%: 95.6%; MP: 171.3–174.6 °C; ESI-MS: 345.01 $[M+H]^+$; ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 10.81 (br-s, 1H), 8.40 (s, 1H), 8.20 (d, 1H, J = 7.8 Hz), 8.05 (d, 1H, J = 7.8 Hz), 8.02 (d, 2H, J = 8.4 Hz), 7.75 (m, 3H), 7.53 (br-s, 2H); ^{13}C NMR (151 MHz, DMSO-d_6): 165.5, 145.2, 143.1, 135.8, 131.5, 129.9, 129.4, 126.6, 125.8, 124.6, 124.4, 120.9. Anal. Calcd for $C_{14}H_{11}F_3N_2O_3S$: C, 48.79; H, 3.23; N, 8.13. Found: C, 48.77; H, 3.24; N, 8.14.

N-(2-chloro-4-nitrophenyl)-3-sulfamoylbenzamide (A10). Yield/%: 29.6%; MP: 238.3–239.1 °C; ESI-MS: 355.95 $[M+H]^+$; ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 10.68 (s, 1H), 8.43 (m, 2H), 8.29 (dd, 1H, J_1 = 9.0 Hz, J_2 = 2.4 Hz), 8.22 (d, 1H, J = 7.8 Hz), 8.08 (d, 1H, J = 7.2 Hz), 8.01 (d, 1H, J = 7.8 Hz), 7.79 (t, 1H, J = 7.8 Hz), 7.55 (s, 2H); ^{13}C NMR (151 MHz, DMSO-d_6): 165.2, 145.6, 145.3, 141.8, 134.7, 131.6, 130.1, 129.8, 129.4, 128.0, 126.0, 125.6, 123.6. Anal. Calcd for $C_{13}H_{10}ClN_3O_5S$: C, 43.85; H, 2.84; N, 11.81. Found: C, 43.86; H, 2.84; N, 11.83.

N-(pyridin-3-yl*)-3-sulfamoylbenzamide (A11).* Yield/%: 21.9%; MP: 188.2–191.8 °C; ESI-MS: 278.05 $[M+H]^+$; ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 10.71 (s, 1H), 8.93 (d, 1H, J = 1.8 Hz), 8.41 (d, 1H, J = 1.8 Hz), 8.34 (dd, 1H, J_1 = 4.8 Hz, J_2 = 1.2 Hz), 8.19 (m, 2H), 8.05 (d, 1H, J = 7.8 Hz), 7.77 (t, 1H, J = 7.8 Hz), 7.51 (s, 2H), 7.41 (m, 1H); ^{13}C NMR (151 MHz, DMSO-d_6): 164.8, 144.8, 144.6, 142.1, 135.5, 135.0, 130.7, 129.3, 128.7, 127.5, 125.1, 123.5. Anal. Calcd for. $C_{12}H_{11}N_3O_3S$: C, 51.98; H, 4.00; N, 15.15. Found: C, 51.94; H, 4.01; N, 15.13.

4, 5-dimethoxy-2-(3-sulfamoylbenzamido)benzoic acid (A12). Yield/%: 49.6%; MP: 273.3–275.2 °C; ESI-MS: 381.11 $[M+H]^+$; ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 13.67 (br-s, 1H), 12.42 (s, 1H), 8.46 (s, 1H), 8.43 (s, 1H), 8.14 (d, 1H, J = 7.8 Hz), 8.07 (d, 1H, J = 7.8 Hz), 7.81 (t, 1H, J = 7.8 Hz), 7.54 (s, 2H), 7.50 (s, 1H), 3.88 (s, 3H), 3.79 (s, 3H); ^{13}C NMR (151 MHz, DMSO-d_6): 170.5, 163.8, 153.9, 145.6, 144.6, 137.1, 135.8, 130.6, 130.5, 129.6, 125.0, 113.4, 108.7, 104.0, 56.2. Anal. Calcd for $C_{16}H_{16}N_2O_7S$: C, 50.48; H, 4.25; N, 7.36. Found: C, 50.39; H, 4.25; N, 7.38.

4, 5-dimethoxy-2-(3-sulfamoylbenzamido)benzoic acid (A13). Yield/%: 84.3%; MP: 282.3–284.2 °C; ESI-MS: 377.24 $[M+H]^+$; ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 10.79 (s, 1H), 8.41 (s, 1H), 8.21 (d, 1H, J = 7.5 Hz), 8.05 (d, 1H, J = 7.5 Hz), 7.93 (m, 4H), 7.77 (t, 1H, J = 7.5 Hz), 7.50 (s, 2H), 1.56 (s, 9H); ^{13}C NMR (125 MHz, DMSO-d_6): 164.8, 164.6, 144.5, 143.0, 135.3, 130.8, 129.9, 129.2, 128.7, 126.4, 125.2, 119.7, 80.4, 27.8. Anal. Calcd for $C_{18}H_{20}N_2O_5S$: C, 57.38; H,

5.37; N, 7.44. Found: C, 57.27; H, 5.38; N, 7.45.

4-chloro-N-(2-methoxyphenyl)-3-sulfamoylbenzamide (B1). Yield/%: 78.3%; MP: 204.3–206.5 °C; ESI-MS: 341.00 $[M+H]^+$; ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 9.82 (s, 1H), 8.50 (d, 1H, J = 1.8 Hz), 8.14 (dd, 1H, J_1 = 8.4 Hz, J_2 = 1.8 Hz), 7.79 (d, 1H, J = 8.4 Hz), 7.73 (s, 2H), 7.65 (d, 1H, J = 7.2 Hz), 7.19 (m, 1H), 7.09 (d, 1H, J = 7.8 Hz), 6.96 (t, 1H, J = 7.2 Hz), 3.81 (s, 3H); ^{13}C NMR (151 MHz, DMSO-d_6): 163.9, 152.7, 141., 134.1, 134.1, 132.4, 132.3, 129.1, 127.0, 126.9, 125.8, 120.8, 112.2, 56.3. Anal. Calcd for $C_{14}H_{13}ClN_2O_4S$: C, 49.30; H, 3.85; N, 8.22. Found: C, 49.21; H, 3.84; N, 8.19.

4-chloro-N-(3-methoxyphenyl)-3-sulfamoylbenzamide (B2). Yield/%: 30.5%; MP: 206.3–209.8 °C; ESI-MS: 341.06 $[M+H]^+$; ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 10.48 (s, 1H), 8.50 (s, 1H), 8.15 (d, 1H, J = 8.4 Hz), 7.81 (d, 1H, J = 8.4 Hz), 7.73 (s, 2H), 7.42 (s, 1H), 7.34 (d, 1H, J = 7.2 Hz), 7.26 (t, 1H, J = 7.8 Hz), 6.70 (d, 1H, J = 7.8 Hz), 3.74 (s, 3H); ^{13}C NMR (151 MHz, DMSO-d_6): 164.2, 160.0, 141.8, 140.5, 134.4, 134.1, 132.6, 132.3, 130.1, 129.1, 113.3, 110.2, 106.9, 55.7. Anal. Calcd for $C_{14}H_{13}ClN_2O_4S$: C, 49.30; H, 3.85; N, 8.22. Found: C, 49.28; H, 3.85; N, 8.20.

4-chloro-N-(4-methoxyphenyl)-3-sulfamoylbenzamide (B3). Yield/%: 20.4%; MP: 202.2–204.6 °C; ESI-MS: 341.06 $[M+H]^+$; ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 10.57 (s, 1H), 8.57 (m, 1H), 8.20 (m, 1H), 7.77 (dd, 2H, J_1 = 8.4 Hz, J_2 = 1.8 Hz), 7.70 (m, 3H), 6.92 (d, 2H, J = 8.4 Hz), 3.73 (s, 3H); ^{13}C NMR (151 MHz, DMSO-d_6): 163.9, 152.7, 141.8, 134.1, 132.3, 129.1, 127.0, 126.9, 125.8, 120.8, 112.2, 56.3. Anal. Calcd for $C_{14}H_{13}ClN_2O_4S$: C, 49.30; H, 3.85; N, 8.22. Found: C, 49.23; H, 3.85; N, 8.23.

4-chloro-N-(3,5-dimethoxyphenyl)-3-sulfamoylbenzamide (B4). Yield/%: 26.1%; MP: 197.2–199.1 °C; ESI-MS: 371.08 $[M+H]^+$; ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 10.43 (s, 1H), 8.48 (d, 1H, J = 1.2Hz), 8.14 (dd, 1H, J_1 = 7.8 Hz, J_2 = 1.8 Hz), 7.81 (d, 1H, J = 8.4 Hz), 7.74 (s, 2H), 7.03 (d, 2H, J = 1.8 Hz), 6.28 (s, 1H), 3.7 (s, 6H); ^{13}C NMR (151 MHz, DMSO-d_6): 164.2, 161.0, 141.8, 141.0, 134.4, 134.2, 132.5, 132.3, 129.1, 99.3, 96.7, 55.9, 55.8. Anal. Calcd for $C_{15}H_{15}ClN_2O_5S$: C, 48.54; H, 4.09; N, 7.50. Found: C, 48.37; H, 4.10; N, 7.51.

4-chloro-N-(2,5-dimethoxyphenyl)-3-sulfamoylbenzamide (B5). Yield/%: 13.0%; MP: 199.3–201.1 °C; ESI-MS: 371.08 $[M+H]^+$; ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 9.77 (s, 1H), 8.48 (s, 1H), 8.12 (d, 1H, J = 7.8 Hz), 7.79 (d, 1H, J = 7.8 Hz), 7.74 (s, 2H), 7.38 (d, 1H, J = 2.4 Hz), 7.01 (d, 1H, J = 8.4 Hz), 6.76 (dd, 1H, J_1 = 9.0 Hz, J_2 = 3.0 Hz), 3.76 (s, 3H) 3.70 (s, 3H); ^{13}C NMR (151 MHz, DMSO-d_6): 163.9, 153.4, 146.5, 141.8, 134.1, 134.1, 132.4, 132.3, 129.1, 127.7, 112.9, 111.6, 110.9, 56.8, 56.1. Anal. Calcd for $C_{15}H_{15}ClN_2O_5S$: C, 48.54; H, 4.09; N, 7.55. Found: C, 48.45; H, 4.09; N, 7.58.

4-chloro-3-sulfamoyl-N-(3,4,5-trimethoxyphenyl)benzamide (B6). Yield/%: 88.7%; MP: 254.3–256.4 °C; ESI-MS: 401.06 $[M+H]^+$; ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 10.43 (s, 1H), 8.53 (d, 1H, J = 1.8 Hz), 8.17 (dd, 1H, J_1 = 7.8 Hz, J_2 = 1.8 Hz), 7.84 (d, 1H, J = 8.4 Hz), 7.75 (s, 2H), 7.20 (s, 2H), 3.78 (s, 6H), 3.65 (s, 3H); ^{13}C NMR (151 MHz, DMSO-d_6): 163.9, 153.2, 141.8, 135.4, 134.6, 134.4, 134.1, 132.4, 132.3, 129.0, 98.9, 60.7, 56.4. Anal. Calcd for $C_{16}H_{17}ClN_2O_6S$: C, 47.90; H, 4.28; N, 6.99. Found: C, 48.06; H, 4.26; N, 7.02.

4-chloro-N-(3-chloro-4-fluorophenyl)-3-sulfamoylbenzamide (B7). Yield/%: 14.7%; MP: 244.4–246.2 °C; ESI-MS: 363.12 $[M+H]^+$; ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 10.69 (s, 1H), 8.51 (d, 1H, J = 2.4 Hz), 8.15 (dd, 1H, J_1 = 8.4 Hz, J_2 = 2.4 Hz), 8.03 (dd, 1H, J_1 = 6.6 Hz, J_2 = 2.4 Hz), 7.83 (d, 1H, J = 8.4 Hz), 7.75 (s, 2H), 7.68 (m, 1H), 7.42 (t, 1H, J = 9.0 Hz); ^{13}C NMR (151 MHz, DMSO-d_6): 164.2, 155.0, 153.4, 141.9, 136.6, 134.4, 133.9, 132.4, 129.1, 122.6, 121.5, 119.8, 117.6. Anal. Calcd for $C_{13}H_9Cl_2FN_2O_3S$: C, 43.07; H, 2.51; N, 7.73. Found: C, 43.01; H, 2.51; N, 7.75.

4-chloro-N-(4-chloro-3-fluorophenyl)-3-sulfamoylbenzamide (B8). Yield/%: 91.1%; MP: 248.3–250.1 °C; ESI-MS: 362.96 $[M+H]^+$; ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 8.5 (d, 1H, J = 1.8 Hz), 8.16 (dd, 1H, J_1 = 8.4 Hz, J_2 = 2.4 Hz), 7.92 (m, 1H), 7.84 (d, 1H, J = 8.4 Hz), 7.57 (m, 2H), (The chemical shift of CONH and SO2NH2 were not shown); ^{13}C NMR (151 MHz, DMSO-d_6): 164.5, 158.3, 156.6, 142.2, 140.1, 134.5, 133.9, 132.3, 131.1, 129.1, 118.0, 114.3, 109.2. Anal. Calcd for $C_{13}H_9Cl_2FN_2O_3S$: C, 43.07; H, 2.51; N, 7.73. Found: C, 43.01; H, 2.49; N, 7.69.

4-chloro-3-sulfamoyl-N-(2-(trifluoromethyl)phenyl)benzamide (B9). Yield/%: 87.5%; MP: 184.4–185.7 °C; ESI-MS: 378.98 $[M+H]^+$; ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 10.50 (s, 1H), 8.54 (d, 1H, J = 2.4 Hz), 8.15 (dd, 1H, J_1 = 7.8 Hz, J_2 = 2.4 Hz), 7.86 (d, 1H, J = 7.8 Hz), 7.82 (d, 1H, J = 7.8 Hz), 7.75 (m, 3H), 7.54 (m, 2H); ^{13}C NMR (151 MHz, DMSO-d_6): 164.5, 141.9, 140.1, 134.5, 133.9, 132.7, 132.4, 130.1, 129.9, 129.1, 124.6, 123.8, 121.0, 117.2. Anal. Calcd for $C_{14}H_{10}ClF_3N_2O_3S$: C, 44.36; H, 2.67; N, 7.39. Found: C, 44.23; H, 2.66; N, 7.35.

4-chloro-3-sulfamoyl-N-(3-(trifluoromethyl)phenyl)benzamide (B10). Yield/%: 88.4%; MP: 182.1–184.2 °C; ESI-MS: 378.98 $[M+H]^+$; ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 10.82 (s, 1H), 8.56 (d, 1H, J = 2.4 Hz), 8.20 (m, 2H), 8.05 (d, 1H, J = 8.4 Hz), 7.87 (d, 1H, J = 8.4 Hz), 7.77 (m, 2H), 7.63 (t, 1H, J = 8.4 Hz), 7.45 (d, 1H, J = 7.8 Hz); ^{13}C NMR (151 MHz, DMSO-d_6): 164.5, 141.9, 140.1, 134.5, 133.9, 132.7, 132.4, 130.6, 130.1, 129.9, 129.1, 124.6, 121.0, 117.2. Anal. Calcd for $C_{14}H_{10}ClF_3N_2O_3S$: C, 44.36; H, 2.67 N, 7.39. Found: C, 44.13; H, 2.66; N, 7.40.

4-chloro-3-sulfamoyl-N-(4-(trifluoromethyl)phenyl)benzamide (B11). Yield/%: 44.3%; MP: 179.6–181.7 °C; ESI-MS: 378.98 $[M+H]^+$; ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 10.85 (s, 1H), 8.54 (d, 1H, J = 2.4 Hz), 8.20 (dd, 1H, J_1 = 8.4 Hz, J_2 = 2.4 Hz), 8.00 (d, 2H, J = 9.0 Hz), 7.86 (d, 1H, J = 8.4 Hz), 7.75 (m, 4H); ^{13}C NMR (151 MHz, DMSO-d_6): 164.6, 143.0, 141.9, 134.5, 134.0, 132.8, 132.4, 129.2, 126.6, 125.8, 124.7, 124.5, 124.0, 121.0. Anal. Calcd for $C_{14}H_{10}ClF_3N_2O_3S$: C, 44.36; H, 2.67; N, 7.39. Found: C, 44.07; H, 2.67; N, 7.39.

tert-butyl 4-(4-chloro-3-sulfamoylbenzamido)benzoate (B12). Yield/%: 77.2%; MP: 298.2–301.8 °C; ESI-MS: 411.22 $[M+H]^+$; ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 10.81 (s, 1H), 8.53 (d, 1H, J = 2.4 Hz), 8.20 (dd, 1H, J_1 = 8.4 Hz, J_2 = 1.8 Hz), 7.91 (d, 4H, J = 3.0 Hz), 7.85 (d, 1H, J = 8.4 Hz), 7.76 (s, 2H), 1.55 (s, 9H); ^{13}C NMR (151 MHz, DMSO-d_6): 165.2, 164.5, 143.5, 141.8, 134.4, 134.1, 132.7, 132.3, 130.6, 129.2, 127.1, 120.3, 81.0, 28.5. Anal. Calcd for $C_{18}H_{19}ClN_2O_5S$: C, 52.57; H, 4.67; N, 6.81. Found: C, 52.13; H, 4.66; N, 6.84.

2-(4-chloro-3-sulfamoylbenzamido)-4,5-dimethoxybenzoic acid (B13). Yield/%: 55.2%; MP: 284.1–286.1 °C; ESI-MS: 414.99 $[M+H]^+$; ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 13.68 (br-s, 1H), 12.44 (s, 1H), 8.59 (d, 1H, J = 2.4 Hz), 8.43 (s, 1H), 8.12 (dd, 1H, J_1 = 8.4 Hz, J_2 = 2.4 Hz), 7.90 (d, 1H, J = 8.4 Hz), 7.79 (s, 2H), 7.50 (s, 1H), 3.87 (s, 3H), 3.80 (s, 3H); ^{13}C NMR (151 MHz, DMSO-d_6): 170.5, 162.9, 153.8, 144.7, 142.2, 136.9, 134.7, 134.0, 133.0, 131.8, 128.3, 113.5, 109.0, 104.1, 56.2. Anal. Calcd for $C_{16}H_{15}ClN_2O_7S$: C, 46.29; H, 3.65; N, 6.75. Found: C, 46.01; H, 3.66; N, 6.73.

4-chloro-N-(4-morpholinophenyl)-3-sulfamoylbenzamide (B14). Yield/%: 23.6%; MP: 281.8–285.1 °C; ESI-MS: 396.00 $[M+H]^+$; ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 10.56 (s, 1H), 8.61 (s, 1H), 8.23 (d, 1H, J = 8.4 Hz), 7.78 (m, 3H), 7.69 (d, 2H, J = 7.8 Hz), 6.94 (d, 2H, J = 9.0 Hz), 3.73 (t, 4H, J = 4.2 Hz), 3.07 (t, 4H, J = 4.2 Hz); ^{13}C NMR (151 MHz, DMSO-d_6): 164.8, 153.2, 145.1, 136.2, 135.6, 134.5, 131.2, 129.8, 129.1, 125.6, 102.0, 60.7, 56.4. Anal. Calcd for $C_{17}H_{18}ClN_3O_4S$: C, 51.53; H, 4.59; N, 10.61. Found: C, 51.27; H, 4.58; N, 10.57.

4-chloro-N-(perfluorophenyl)-3-sulfamoylbenzamide (B15). Yield/%: 26.8%; MP: 177.2–178.4 °C; ESI-MS: 400.93 $[M+H]^+$; ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 10.92 (s, 1H), 8.59 (d, 1H, J = 1.8 Hz), 8.20 (dd, 1H, J_1 = 7.8 Hz, J_2 = 1.8 Hz), 7.88 (d, 1H, J = 8.4 Hz), 7.78 (s, 2H); ^{13}C NMR (151 MHz, DMSO-d_6): 164.3, 142.0, 135.2, 133.0, 132.8,

131.9, 129.3, 113.3. Anal. Calcd for $C_{13}H_6ClF_5N_2O_3S$: C, 38.93; H, 1.51; N, 6.99. Found: C, 38.73; H, 1.50; N, 6.97.

4-chloro-N-(4-(4-methylpiperazin-1-yl)*phenyl)-3-sulfamoylbenzamide (B16).* Yield/%: 21.6%; MP: 282.3–285.7 °C; ESI-MS: 409.07 $[M+H]^+$; ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 10.46 (s, 1H), 8.56 (d, 1H, J = 1.2 Hz), 8.16 (dd, 1H, J_1 = 8.4 Hz, J_2 = 1.8 Hz), 7.79 (d, 1H, J = 8.4 Hz), 7.67 (m, 2H), 7.62 (d, 2H, J = 9.0 Hz), 6.93 (d, 2H, J = 9.0 Hz), 3.10 (t, 4H, J = 4.8 Hz), 2.45 (t, 4H, J = 4.8 Hz), 2.22 (s, 3H); ^{13}C NMR (151 MHz, DMSO-d_6): 163.5, 141.7, 134.5, 133.8, 132.5, 132.2, 131.3, 129.0, 122.3, 116.1, 52.5, 52.0, 48.6. Anal. Calcd for $C_{18}H_{21}ClN_4O_3S$: C, 52.82; H, 5.19; N, 13.70. Found: C, 52.67; H, 5.18; N, 13.71.

4-chloro-N-(4-(N-(diaminomethylene)sulfamoyl)phenyl)-3-sulfamoylbenzamide (B17). Yield/%: 79.1%; MP: 243.8–246.4 °C; ESI-MS: 431.99 $[M+H]^+$; ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 10.78 (s, 1H), 8.53 (d, 1H, J = 2.4 Hz), 8.19 (dd, 1H, J_1 = 8.4 Hz, J_2 = 1.8 Hz), 7.84 (m, 3H), 7.75 (m, 4H), 6.69 (br-s, 4H); ^{13}C NMR (151 MHz, DMSO-d_6): 164.5, 158.7, 141.8, 141.8, 140.2, 134.4, 134.1, 132.7, 132.4, 129.2, 127.1, 120.5. Anal. Calcd for $C_{14}H_{14}ClN_5O_5S_2$: C, 38.94; H, 3.27; N, 16.22. Found: C, 38.90; H, 3.26; N, 16.16.

4-chloro-3-sulfamoyl-N-(3-sulfamoylphenyl)benzamide (B18). Yield/%: 79.5%; MP: 171.5–173.3 °C; ESI-MS: 389.92 $[M+H]^+$; ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 10.83 (s, 1H), 8.56 (d, 1H, J = 2.4 Hz), 8.32 (d, 1H, J = 1.2 Hz), 8.21 (dd, 1H, J_1 = 8.4 Hz, J_2 = 2.4 Hz), 7.97 (m, 1H), 7.86 (d, 1H, J = 8.4 Hz), 7.77 (s, 2H), 7.57 (m, 2H), 7.41 (s, 2H); ^{13}C NMR (151 MHz, DMSO-d_6): 164.4, 145.2, 141.8, 139.7, 134.4, 134.0, 132.7, 132.4, 130.1, 129.2, 124.0, 121.8, 118.1. Anal. Calcd for $C_{13}H_{12}ClN_3O_5S_2$: C, 40.02; H, 3.11; N, 10.77. Found: C, 39.95; H, 3.10; N, 10.74.

4-chloro-N-(2-chloro-4-nitrophenyl)-3-sulfamoylbenzamide (B19). Yield/%: 28.6%; MP: 246.6–248.1 °C; ESI-MS: 390.01 $[M+H]^+$; ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 10.72 (s, 1H), 8.57 (d, 1H, J = 1.8 Hz), 8.43 (d, 1H, J = 2.4 Hz), 8.28 (dd, 1H, J_1 = 9.0 Hz, J_2 = 2.4 Hz), 8.20 (dd, 1H, J_1 = 7.8 Hz, J_2 = 1.8 Hz), 8.00 (d, 1H, J = 9.0 Hz), 7.88 (d, 1H, J = 7.8 Hz), 7.79 (s, 2H); ^{13}C NMR (151 MHz, DMSO-d_6): 164.4, 145.6, 141.9, 141.6, 134.9, 133.0, 132.9, 132.6, 129.4, 129.3, 128.1, 125.6, 123.6. Anal. Calcd for $C_{13}H_9Cl_2N_3O_5S$: C, 39.98; H, 2.33; N, 10.76. Found: C, 39.76; H, 2.33; N, 10.76.

4-chloro-N-(2-methoxybenzyl)-3-sulfamoylbenzamide (X1). Yield/%: 29.7%; MP: 188.2–191.5 °C; ESI-MS: 355.09 $[M+H]^+$; ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 9.16 (t, 1H, J = 5.4 Hz), 8.50 (d, 1H, J = 2.4 Hz), 8.10 (dd, 1H, J_1 = 8.4 Hz, J_2 = 1.8 Hz), 7.77 (d, 1H, J = 8.4 Hz), 7.71 (s, 2H), 7.23 (m, 1H), 7.17 (d, 1H, J = 7.2 Hz), 7.00 (d, 1H, J = 7.8 Hz), 6.91 (t, 1H, J = 7.2 Hz), 4.46 (d, 2H, J = 6.0 Hz), 3.82 (s, 3H); ^{13}C NMR (151 MHz, DMSO-d_6): 165.0, 157.2, 141.7, 133.9, 133.8, 132.3, 132.2, 128.8, 128.7, 128.0, 127.0, 120.7, 111.1, 56.0, 38.5. Anal. Calcd for $C_{15}H_{15}ClN_2O_4S$: C, 50.73; H, 4.27; N, 7.89. Found: C, 50.51; H, 4.28; N, 7.88.

4-chloro-N-(3-methoxybenzyl)-3-sulfamoylbenzamide (X2). Yield/%: 22.8%; MP: 189.1–191.0 °C; ESI-MS: 355.16 $[M+H]^+$; ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 9.32 (t, 1H, J = 6.0 Hz), 8.49 (d, 1H, J = 1.8 Hz), 8.08 (dd, 1H, J_1 = 7.8 Hz, J_2 = 1.8 Hz), 7.77 (d, 1H, J = 8.4 Hz), 7.71 (s, 2H), 6.81 (m, 4H), 4.46 (d, 2H, J = 6.0 Hz), 3.73 (s, 3H); ^{13}C NMR (151 MHz, DMSO-d_6): 164.9, 161.7, 159.9, 141.7, 141.4, 133.9, 132.3, 132.1, 130.0, 128.8, 120.0, 113.7, 112.8, 55.6, 43.4. Anal. Calcd for $C_{15}H_{15}ClN_2O_4S$: C, 50.73; H, 4.27; N, 7.89. Found: C, 50.35; H, 4.26; N, 7.89.

4-chloro-N-(4-methoxybenzyl)-3-sulfamoylbenzamide (X3). Yield/%: 82.2%; MP: 184.9–186.2 °C; ESI-MS: 355.09 $[M+H]^+$; ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 9.27 (t, 1H, J = 5.4 Hz), 8.48 (d, 1H, J = 1.8 Hz), 8.06 (dd, 1H, J_1 = 7.8 Hz, J_2 = 1.8 Hz), 7.76 (d, 1H, J = 8.4 Hz), 7.70 (s, 2H), 7.25 (d, 2H, J = 8.4 Hz), 6.89 (d, 2H, J = 8.4 Hz), 4.41 (d, 2H, J = 5.4 Hz), 3.72 (s, 3H); ^{13}C NMR (151 MHz, DMSO-d_6): 164.7, 161.5, 158.9, 141.7, 134.0, 133.8, 132.3, 132.1, 131.8, 131.5, 129.3, 128.8, 114.3. Anal. Calcd for $C_{15}H_{15}ClN_2O_4S$: C, 50.73; H, 4.27; N, 7.89. Found: C, 50.43; H, 4.26; N, 7.91.

4-chloro-N-(2,3-dichlorobenzyl)-3-sulfamoylbenzamide (X4). Yield/%: 85.3%; MP: 174.2–177.6 °C; ESI-MS: 392.92 $[M+H]^+$; ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 9.41 (t, 1H, J = 6.0 Hz), 8.51 (d, 1H, J = 2.4 Hz), 8.11 (dd, 1H, J_1 = 8.4 Hz, J_2 = 2.4 Hz), 7.79 (d, 1H, J = 8.4 Hz), 7.57 (dd, 1H, J_1 = 6.6 Hz, J_2 = 2.4 Hz), 7.55 (d, 1H, J = 7.8 Hz), 7.34 (m, 3H), 4.58 (d, 2H, J = 5.4 Hz); ^{13}C NMR (151 MHz, DMSO-d_6): 165.1, 141.8, 139.3, 134.1, 133.5, 132.3, 132.3, 130.6, 129.7, 128.8, 128.7, 128.0, 42.2. Anal. Calcd for $C_{14}H_{11}Cl_3N_2O_3S$: C, 42.68; H, 2.82; N, 7.11. Found: C, 42.54; H, 2.81; N, 7.14.

4-chloro-N-(2-methoxybenzyl)-3-sulfamoylbenzamide (X5). Yield/%: 87.9%; MP: 199.2–202.0 °C; ESI-MS: 414.96 $[M+H]^+$; ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 9.28 (t, 1H, J = 5.5 Hz), 8.50 (d, 1H, J = 1.5 Hz), 8.09 (dd, 1H, J_1 = 8.5 Hz, J_2 = 2.0 Hz), 7.77 (d, 1H, J = 8.0 Hz), 7.68 (s, 2H), 6.66 (s, 2H), 4.44 (d, 1H, J = 5.5 Hz), 3.76 (s, 6H), 3.64 (s, 3H); ^{13}C NMR (125 MHz, DMSO-d_6): 164.3, 152.8, 141.2, 136.6, 134.8, 133.4, 133.2, 131.6, 131.5, 128., 105.0, 60.0, 55.9, 43.1. Anal. Calcd for $C_{17}H_{19}ClN_2O_6S$: C, 49.17; H, 4.63; N, 6.75. Found: C, 49.03; H, 4.61; N, 6.77.

3-(N-acryloylsulfamoyl)-4-chloro-N-(3, 4, 5-trimethoxyphenyl) benzamide (Y1). Yield/%: 55.9%; MP: 174.9–177.0 °C; ESI-MS: 455.20 $[M+H]^+$; ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 10.56 (s, 1H), 8.58 (d, 1H, J = 1.8 Hz), 8.22 (d, 1H, J = 6.6 Hz), 7.71 (d, 1H, J = 6.6 Hz), 7.25 (s, 2H), 6.05 (m, 2H), 5.62 (s, 1H), 3.77 (s, 6H), 3.64 (s, 3H), (The chemical shift of SO2NHCO was not shown); ^{13}C NMR (125 MHz, DMSO-d_6): 163.6, 162.9, 152.6, 137.0, 134.6, 134.2, 133.9, 133.6, 133.4, 131.9, 131.4, 98.6, 60.1, 55.8. Anal. Calcd for $C_{19}H_{19}ClN_2O_7S$: C, 50.12; H, 4.22; N, 6.16. Found: C, 49.97; H, 4.22; N, 6.14.

4-chloro-3-(N-methacryloylsulfamoyl)-N-(3,4,5-trimethoxyphenyl)benzamide (Y2). Yield/%: 44.3%; MP: 184.8–187.8 °C; ESI-MS: 469.23 $[M+H]^+$; ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 12.69 (br-s, 1H), 10.58 (s, 1H), 8.59 (s, 1H), 8.23 (m, 1H), 7.74 (m, 1H), 7.25 (s, 2H), 5.83 (m, 1H), 5.49 (m, 1H), 3.77 (s, 6H), 3.64 (s, 3H), 1.75 (s, 3H); ^{13}C NMR (125 MHz, DMSO-d_6): 163.6, 162.9, 153.2, 152.6, 137.0, 134.6, 134.0, 133.6, 133.5, 131.9, 131.4, 115.6, 106.8, 98.6, 60.1, 56.2, 20.0. Anal. Calcd for $C_{20}H_{21}ClN_2O_7S$: C, 51.18; H, 4.52; N, 5.97. Found: C, 51, 04; H, 4.52; N, 5.94.

4-chloro-3-(N-(3-methylbut-2-enoyl)sulfamoyl)-N-(3,4,5-trimethoxyphenyl)benzamide (Y3). Yield/%: 41.6%; MP: 194.9–196.2 °C; ESI-MS: 483.19 $[M+H]^+$; ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 12.4 (br-s, 1H), 10.51 (s, 1H), 8.64 (d, 1H, J = 2.4 Hz), 8.25 (dd, 1H, J_1 = 8.4 Hz, J_2 = 2.4 Hz), 7.87 (d, 1H, J = 8.4 Hz), 7.19 (s, 2H), 5.80 (s, 1H), 3.78 (s, 6H), 3.65 (s, 3H), 1.97 (s, 3H), 1.84 (s, 3H); ^{13}C NMR (125 MHz, DMSO-d_6): 163.6, 162.9, 153.2, 152.6, 137.0, 134.6, 134.2, 133.9, 133.6, 133.4, 131.9, 131.5, 131.4, 98.6, 60.1, 55.8, 27.3, 20.0. Anal. Calcd for $C_{21}H_{23}ClN_2O_7S$: C, 52.18; H, 4.81; N, 5.80. Found: C, 52.02; H, 4.80; N, 5.82.

3-(N-acetylsulfamoyl)-4-chloro-N-(3, 4, 5-trimethoxyphenyl)benzamide (Y4). Yield/%: 49.1%; MP: 210.0–211.5 °C; ESI-MS: 443.21 $[M+H]^+$; ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 12.66 (br-s, 1H), 10.50 (s, 1H), 8.61 (d, 1H, J = 2.4 Hz), 8.23 (dd, 1H, J_1 = 8.4 Hz, J_2 = 1.8 Hz), 7.88 (d, 1H, J = 8.4 Hz), 7.19 (s, 2H), 3.78 (s, 6H), 3.65 (s, 3H), 1.98 (s, 3H); ^{13}C NMR (125 MHz, DMSO-d_6): 169.1, 162.9, 152.6, 134.6, 134.2, 133.9, 133.7, 133.5, 131.9, 131.3, 98.6, 60.1, 55.8.23.1. Anal. Calcd for $C_{18}H_{19}ClN_2O_7S$: C, 48.77; H, 4.33; N, 6.32. Found: C, 48.67; H, 4.32; N, 6.33.

4-chloro-3-(N-propionylsulfamoyl)-N-(3,4,5-trimethoxyphenyl) benzamide (Y5). Yield/%: 33.9%; MP: 217.9–219.2 °C; ESI-MS: 457.24 $[M+H]^+$; ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 12.61 (br-s, 1H), 10.50 (s, 1H), 8.62 (d, 1H, J = 1.8 Hz), 8.24 (dd, 1H, J_1 = 8.4 Hz, J_2 = 1.8 Hz), 7.86 (d, 1H, J = 8.4 Hz), 7.19 (s, 2H), 3.78 (s, 6H), 3.65 (s, 3H), 2.27 (q, 2H, J = 7.8 Hz), 0.90 (t, 3H, J = 7.8 Hz); ^{13}C NMR (125 MHz, DMSO-d_6): 172.8, 162.9, 152.6, 134.7, 134.2, 133.8, 133.6, 133.3, 131.8, 131.3, 98.6, 60.1, 55.8, 28.9, 8.4. Anal. Calcd for $C_{19}H_{21}ClN_2O_7S$: C, 49.90; H, 4.64; N, 6.13. Found: C, 49.87; H, 4.64;

N, 6.16.

(E)-3-(N-(but-2-enoyl)sulfamoyl)-4-chloro-N-(3,4,5-trimethoxyphenyl)benzamide (Y6). Yield/%: 39.9%; MP: 198.0−199.7 °C; ESI-MS: 469.16 $[M+H]^+$; ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 12.66 (br-s, 1H), 10.50 (s, 1H), 8.64 (d, 1H, J = 1.8 Hz), 8.25 (dd, 1H, J_1 = 8.4 Hz, J_2 = 1.8 Hz), 7.86 (d, 1H, J = 8.4 Hz), 7.19 (s, 2H), 6.77 (m, 1H), 6.01 (d, 1H, J = 15.6 Hz), 3.78 (s, 6H), 3.65 (s, 3H), 1.82 (d, 3H, J = 5.4 Hz); ^{13}C NMR (125 MHz, DMSO-d_6): 162.9, 152.6, 134.6, 134.208, 133.9, 133.6, 131.9, 131.4, 98.6, 60.1, 55.8, 17.7. Anal. Calcd for $C_{20}H_{21}ClN_2O_7S$: C, 51.18; H, 4.52; N, 5.97. Found: C, 51.09; H, 4.51; N, 5.97.

3-(N-benzoylsulfamoyl)-4-chloro-N-(3, 4, 5-trimethoxyphenyl) benzamide (Z1). Yield/%: 22.9%; MP: 201.0−203.5 °C; ESI-MS: 505.13 $[M+H]^+$; ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 10.42 (s, 1H), 8.59 (d, 1H, J = 1.8 Hz), 8.01 (d, 1H, J = 7.2 Hz), 7.89 (m, 2H), 7.57 (d, 1H, J = 7.8 Hz), 7.39 (m, 2H), 7.33 (t, 1H, J = 14.4 Hz), 7.24 (s, 2H), 3.78 (s, 6H), 3.65 (s, 3H), (The chemical shift of SO2NHCO was not shown); ^{13}C NMR (125 MHz, DMSO-d_6): 163.2, 163.0, 152.6, 134.7, 134.2, 133.9, 133.5, 131.8, 131.4, 130.7, 129.3, 113.9, 113.3, 98.6, 60.1, 55.8. Anal. Calcd for $C_{23}H_{21}ClN_2O_7S$: C, 54.66; H, 4.20; N, 5.55. Found: C, 54.57; H, 4.21; N, 5.57.

4-chloro-3-(N-(2-methoxybenzoyl)sulfamoyl)-N-(3,4,5-trimethoxyphenyl)benzamide (Z2). Yield/%: 29.8%; MP: 211.6−213.2 °C; ESI-MS: 535.14 $[M+H]^+$; ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 12.48 (br-s, 1H), 10.52 (s, 1H), 8.70 (d, 1H, J = 1.8 Hz), 8.29 (d, 1H, J = 7.8 Hz), 7.91 (d, 1H, J = 8.4 Hz), 7.20 (s, 2H), 6.99 (m, 4H), 3.84 (s, 3H), 3.789 (s, 6H), 3.66 (s, 3H); ^{13}C NMR (125 MHz, DMSO-d_6): 162.9, 157.0, 152.6, 134.7, 134.2, 133.9, 133.6, 133.4, 132.9, 132.4, 132.0, 131.5, 130.7, 130.5, 129.4, 120.4, 120.0, 112.4, 112.2, 112.0, 98.6, 60.1, 55.9, 55.8. Anal. Calcd for $C_{24}H_{23}ClN_2O_8S$: C, 53.84; H, 4.34; N, 5.23. Found: C, 53.81; H, 4.35; N, 5.22.

4-chloro-3-(N-(3-methoxybenzoyl)sulfamoyl)-N-(3,4,5-trimethoxyphenyl)benzamide (Z3). Yield/%: 33.9%; MP: 214.8−215.1 °C; ESI-MS: 535.16 $[M+H]^+$; ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 10.46 (s, 1H), 8.60 (s, 1H), 8.42 (d, 1H, J = 2.4 Hz), 7.85 (d, 1H, J = 7.8 Hz), 7.32 (m, 3H), 7.19 (s, 2H), 7.08 (d, 1H, J = 7.8 Hz), 3.77 (s, 3H), 3.68 (s, 6H), 3.62 (s, 3H), (The chemical shift of SO2NHCO was not shown); ^{13}C NMR (125 MHz, DMSO-d_6): 172.4, 159.0, 158.8, 153.1, 135.9, 135.0, 129.7, 121.2, 120.8, 118.2, 114.0, 113.1, 106.4, 98.5, 60.0, 56.2, 55.8. Anal. Calcd for $C_{24}H_{23}ClN_2O_8S$: C, 53.84; H, 4.34; N, 5.23. Found: C, 53.82; H, 4.33; N, 5.23.

4-chloro-3-(N-(4-methoxybenzoyl)sulfamoyl)-N-(3,4,5-trimethoxyphenyl)benzamide (Z4). Yield/%: 36.8%; MP: 207.7−209.4 °C; ESI-MS: 535.23 $[M+H]^+$; ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 12.91 (br-s, 1H), 10.52 (s, 1H), 8.70 (d, 1H, J = 2.4 Hz), 8.25 (d, 1H, J = 7.2 Hz), 7.92 (d, 2H, J = 9.0 Hz), 7.83 (m, 1H), 7.20 (s, 2H), 7.02 (d, 2H, J = 9.0 Hz), 3.82 (s, 3H), 3.79 (s, 6H), 3.65 (s, 3H); ^{13}C NMR (125 MHz, DMSO-d_6): 163.2, 163.0, 152.6, 134.7, 134.2, 133.9, 133.6, 131.8, 131.4, 130.7, 129.3, 113.9, 113.3, 98.6, 60.1, 55.8, 55.5, 55.3. Anal. Calcd for $C_{24}H_{23}ClN_2O_8S$: C, 53.84; H, 4.34; N, 5.23. Found: C, 52.79; H, 4.33; N, 5.21.

4-chloro-3-(N-(3-nitrobenzoyl)sulfamoyl)-N-(3,4,5-trimethoxyphenyl)benzamide (Z5). Yield/%: 37.5%; MP: 217.8−218.2 °C; ESI-MS: 550.15 $[M+H]^+$; ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 10.42 (s, 1H), 8.68 (t, 1H, J = 1.8 Hz), 8.59 (d, 1H, J = 2.4 Hz), 8.23 (m, 3H), 8.01 (dd, 1H, J_1 = 8.4 Hz, J_2 = 2.4 Hz), 7.57 (d, 1H, J = 7.8 Hz), 7.24 (s, 2H), 3.78 (s, 6H), 3.65 (s, 3H), (The chemical shift of SO2NHCO was not shown); ^{13}C NMR (125 MHz, DMSO-d_6): 164.1, 159.0, 158.8, 152.6, 147.5, 135.0, 134.5, 130.6, 130.2, 129.3, 122.7, 118.2, 114.0, 113.2, 106.4, 98.5, 60.1, 55.8. Anal. Calcd for $C_{23}H_{20}ClN_3O_9S$: C, 50.19; H, 3.67; N, 7.64. Found: C, 50.15; H, 3.67; N, 7.65.

4-chloro-3-(N-(3,5-dimethoxybenzoyl)sulfamoyl)-N-(3,4,5-trimethoxyphenyl)benzamide (Z6). Yield/%: 21.3%; MP: 199.0−202.2 °C; ESI-MS: 565.12 $[M+H]^+$; ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 10.43 (s, 1H), 8.57 (d, 1H, J = 2.4 Hz), 8.01 (d, 1H, J = 8.4 Hz), 7.55 (d, 1H, J = 8.4 Hz), 7.25 (s, 2H), 7.07 (s, 2H), 6.50 (s, 1H), 3.77 (s, 6H), 3.72 (s, 6H), 3.64 (s, 3H), (The chemical shift of SO2NHCO was not shown); ^{13}C NMR (125 MHz, DMSO-d_6): 167.1, 163.9, 159.0, 153.1, 147.5, 134.9, 131.6, 129.7, 121.2, 120.8, 118.2, 113.9, 113.1, 106.3, 102.0, 98.5, 60.0, 56.2, 55.8, 55.3, 55.3, 55.0. Anal. Calcd for $C_{23}H_{20}ClN_3O_9S$: C, 48.85; H, 3.58; N, 7.43. Found: C, 48.81; H, 3.57; N, 7.45.

N-((2-chloro-5-((3,4,5-trimethoxyphenyl)carbamoyl)phenyl)sulfonyl)-2,6-dimethoxybenzamide (Z7). Yield/%: 19.6%; MP: 194.8−196.4 °C; ESI-MS: 565.12 $[M+H]^+$; ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 10.43 (s, 1H), 8.57 (d, 1H, J = 2.4 Hz), 8.01 (d, 1H, J = 8.4 Hz), 7.55 (d, 1H, J = 8.4 Hz), 7.25 (s, 2H), 7.07 (d, 2H, J = 2.4 Hz), 6.50 (t, 1H, J = 2.4 Hz), 3.77 (s, 6H), 3.72 (s, 6H), 3.64 (s, 3H), (The chemical shift of SO2NHCO was not shown); ^{13}C NMR (125 MHz, DMSO-d_6): 169.4, 164.1, 159.7, 152.6, 144.1, 141.4, 135.0, 133.9, 133.6, 132.8, 130.2, 129.7, 106.1, 102.4, 98.5, 60.1, 55.8, 55.1. Anal. Calcd for $C_{23}H_{20}ClN_3O_9S$: C, 48.85; H, 3.58; N, 7.43. Found: C, 48.88; H, 3.57; N, 7.42.

6-chloro-N-((2-chloro-5-((3,4,5-trimethoxyphenyl)carbamoyl)phenyl)sulfonyl)nicotinamide (Z8). Yield/%: 24.5%; MP: 191.2−192.8 °C; ESI-MS: 540.07 $[M+H]^+$; ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 10.38 (s, 1H), 8.80 (d, 1H, J = 1.8 Hz), 8.58 (d, 1H, J = 1.8 Hz), 8.19 (dd, 1H, J_1 = 7.8 Hz, J_2 = 1.8 Hz), 7.97 (dd, 1H, J_1 = 7.8 Hz, J_2 = 1.8 Hz), 7.57 (d, 1H, J = 7.8 Hz), 7.48 (d, 1H, J = 7.8 Hz), 7.22 (s, 2H), 3.78 (s, 6H), 3.65 (s, 3H), (The chemical shift of SO2NHCO was not shown); ^{13}C NMR (125 MHz, DMSO-d_6): 166.9, 164.1, 152.6, 151.4, 150.0, 139.3, 135.0, 133.6, 133.5, 133.0, 130.6, 130.1, 130.0, 123.4, 98.5, 60.1, 55.8. Anal. Calcd for $C_{22}H_{19}Cl_2N_3O_7S$: C, 48.90; H, 3.54; N, 7.78. Found: C, 48.82; H, 3.54; N, 7.77.

4.2. Oil-water partition coefficient (logP) measurement experiment

At room temperature, take two larger Erlenmeyer flasks, add n-octanol and enough water, water and enough n-octanol, respectively, then place them in a constant temperature shaker at 150•r/min and shake for 24 h, then transfer into the normal pressure separating funnel, and then obtain water-saturated n-octanol and n-octanol-saturated water, for use. Weigh 2 mg of the target compound accurately, place it in a 2 ml brown volumetric flask, add anhydrous methanol to ultrasonically dissolve, dilute to the mark and shake well to obtain a 1 mg/ml reference substance stock solution. Dilute and prepare 1 μg/ml, 1.5 μg/ml, 2 μg/ml, 2.5 μg/ml, 5 μg/ml, 10 μg/ml series of reference standard solutions, and draw the standard curve by UV−visible spectrophotometer. Excessively dissolve the analyte in water-saturated n-octanol, shake at 150•r/min for 24 h in a constant temperature shaker to make a saturated solution. After standing still, centrifuge, and take 1 ml of the supernatant in a 4 ml centrifuge tube. Add 1 ml of n-octanol saturated water, place it in a constant temperature shaker at 150•r/min and shake for 24 h, let it stand for 8 h and then centrifuge, pipette an appropriate amount of n-octanol phase before and after equilibration, dilute with methanol appropriately, and then pass the previously drawn standard. The curve obtains the n-octanol phase concentration before and after equilibrium, C_0 and C_1, and the n-octanol saturated aqueous phase concentration is $C_w = C_0 - C_1$, that is, $\log P_{ow} = \log_{10} C_0/C_w$.

4.3. Cell lines and reagents

Human colon cell lines (HCT-116 and SW480), human breast cancer cell line (MDA-MB-231) and human pancreatic cancer cell line (L3.6) were purchased from Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences

(Shanghai, China). Human colon cancer cells HCT-116 and SW480 were cultured in RPMI 1640 medium supplemented with 10% (vol/vol) FBS, 50 μg/ml penicillin and 50 μg/ml streptomycin; Human pancreatic cancer cell line L3.6 was cultured in Dulbecco modified Eagle medium (DMEM) medium supplemented with 10% (vol/vol) FBS, 50 μg/ml penicillin and 50 μg/ml streptomycin; Human breast cancer cell MDA-MB-231 was cultured in L15 medium supplemented with 10% (vol/vol) FBS, 50 μg/ml penicillin and 50 μg/ml streptomycin; All cells were cultured at 37 °C under a 5% CO_2 and 90% humidified atmosphere.

4.4. Cell viability assays

In this experiment, MTT assay was used to detect cell viability in breast cancer cells MDA-MB-231, colon cancer cells HCT-116, SW480 and pancreatic cancer cells L3.6. Each tumor cell (MDA-MB-231, HCT-116, SW480 and L3.6) in the logarithmic growth phase was cultured in a 96-well plate at a concentration of 6×10^3 cells per well, incubated for 24 h at 37 °C. Then, 1 μL of the positive compound Niclosamide and 57 target compound solutions dissolved in DMSO at a final concentration of 10 μM or different concentration gradients (0.1−2 μM) were administered; After 48 h of administration, 20 μL of MTT solution dissolved in PBS to 5 mg/mL was added to each well, and continued to culture for 4 h. Discard the solution in each well, add 150 μL of DMSO to each well to dissolve the formazan crystals and shake it on a shaker for 10 min. Finally, the absorbance of each well at the ultraviolet absorption wavelength of 490 nm was detected by a microplate reader, and the corresponding cell survival rate, inhibition rate or IC_{50} value were calculated. This experiment requires at least three repeated experiments to reduce the experimental error.

4.5. STAT3 kit

The PathScan® Phospho-STAT3 (Tyr 705) Sandwich ELISA kit (Cell Signaling Technology, #7300) was used under the guidance of manufacturer's instructions to measure the level of STAT3 phosphorylation with the treatment of tested compounds. The measurements were repeated at least three times.

4.6. Western blot analysis

Breast cancer cells MDA-MB-231 were plated in 6-well plates and cultured overnight; then different concentrations (0 μM, 1 μM, 5 μM, 10 μM) of the active compound B12 and 10 μM Niclosamide were administered for 2 h. The corresponding cells were collected, washed with PBS, and lysed with RIPA buffer (1% Triton X-100, 1% deoxycholate, 0.1% SDS) to extract the total proteins. The extracted protein was loaded, subjected to SDS-PAGE electrophoresis, and then the protein was transferred to a PDVF membrane and incubated in the corresponding Primary antibody overnight (STAT3, p-STAT3, p-STAT1, STAT1, Cleaved Caspase-3, Cleaved Caspase-9, Cleaved PARP, Bcl-xl and GAPDH); the next day, the Primary antibody was recovered and labeled and the corresponding Secondary antibody was incubated. Finally, bound immune-complexes were detected using ChemiDOC™ XRS t system (Bio-Rad Laboratories, Hercules, CA).

4.7. IL-6 induction of STAT3 phosphorylation

MDA-MB-231 cells were seeded in 6-well plates and allowed to adhere overnight. The following day, the cells were serum-starved. The cells were then left untreated or were treated with B12 (0.5−10 mM) or Niclosamide (10 mM). After 2 h, the untreated and treated cells were stimulated by IL-6 (50 ng/mL). The cells were harvested after 45 min and analyzed by Western blot assay.

4.8. Kinase inhibition assay

The procedure was as follows: Prepare 2.5 × enzyme solution; add kinase in 1 kinase base buffer. Prepare 2.5 × peptide solution, add FAM-labeled (GL Biochem) peptide and ATP (Sigma) in a 1 × kinase base buffer. The assay plate already contains 5 μL of compound in 10% DMSO (Sigma). Transfer 2.5 × enzyme solution to the assay plate, add 10 μL of 2.5 × enzyme solution to each well of the 384-well (Corning) assay plate. Incubate at room temperature for 10 min. Transfer 2.5 × peptide solution to the assay plate. Add 10 μL of 2.5 × peptide solution to each well of the 384-well assay plate. Incubate at 28 °C for specified period of time. Add 25 μL of buffer to quench reaction. The data was collected using Caliper. Convert conversion values to inhibition values, percent inhibition = (max-conversion)/(max-min) × 100, that "max" stands for DMSO control; "min" stands for low control. The same method was used to perform the kinase selectivity test of 34 kinases. FLT1and FLT3 were purchased from Invitrogen. AKT1 and AKT2 wrer purchased from BPS. The other 30 kinases were purchased from Carna.

4.9. Transient transfection and dual luciferase reporter assays

In 24-well plates MDA-MB-231 cells were seeded at a density of 1.5×10^4 cells per well in DMEM supplemented with 50 mg/ml penicillin, 50 mg/ml streptomycin and 10% (v/v) FBS. Utilizing the method reported, transient transfections were conducted 4 h after plating [30−32]. The amount of transfected DNA including internal control vector renilla and pSTAT3-Luc was 0.5 μg per well. After 6 h of transfection, the cells were treated with 0.1, 0.5, 1.0 μM B12, or 1.0 μM Niclosamide for 24 h, and then reporter activity was determined using dual luciferase reporter assay kit (Thermo Scientific™ Varioskan™ LUX Microplate Luminometer). Each experiment was independently conducted three times, and the results were averaged. The relative luciferase units were expressed as the ratio of absolute activity of firefly luciferase to absolute activity of renilla luciferase.

4.10. Flow cytometry analysis of apoptotic cells

MDA-MB-231 cells at a density of 5×10^5 per well were cultured in regular growth medium in 6-well plates for 24 h and treated with different concentrations of B12 or Niclosamide for 24 h. The cells were harvested, washed and stained with 3 μL Annexin V-FITC and 3 μL propidium iodide at room temperature for 15 min and 1 × binding buffer was used for dilution to 500 μL. The cells were then stained with PI, Annexin-V alone as positive control. The samples were measured with a BD Accuri™ C6 flow cytometer (Becton Dickinson) and the data were processed using FlowJo 7.6.1.

4.11. Colony survival assay

MDA-MB-231 cells were cultured 1000 per well in 6-well plate with regular growth medium. Cells were treated by B12 (0.1−1 μM) or Niclosamid (1 μM) on the following day for 24 h. Cells were allowed to grow for 10−14 d until the colonies were visible. Crystal violet solution (Sigma, St. Louis, MO, USA) was used to stain the colonies for 4 h and imaged.

4.12. Transwell assay

2×10^5 MDA-MB-231 cells were seeded into the upper chamber of one well of a transwell system. Cells were treated by B12 (0.1−1 μM) or Niclosamide (1 μM) and were allowed to migrate

through the porous membrane for 24 h at 37 °C in 5% CO_2. Then cells in the upper surface of the chamber were completely removed and the lower surfaces of the membranes were stained with crystal violet solution for 20–30 min and imaged.

4.13. In vivo studies

In vivo studies, the animal experiments were approved by the Animal Policy and Ethics Committee of Wenzhou Medical University (ID Number: wxdw2018-1206). Six-week old BALB/c (NU/NU) female mice were injected subcutaneously with 0.2 ml of MDA-MB-231 tumor cells, and the number of cells was about 5×10^6 cells. After the tumor developed, the mice were randomly divided into groups. The mice in the blank control group (solvent group), the compound B12 (30 mg/kg) group and the positive control group Niclosamide (30 mg/kg) were intraperitoneally injected or intragastric administered every two days until the mice were sacrificed. The body weight of nude mice was recorded every two days, and the tumor weight was recorded on the day of death of nude mice.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

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