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From COX-2 Inhibitor Nimesulide to Potent Anti-Cancer Agent: Synthesis, In Vitro, In Vivo and Pharmacokinetic Evaluation

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From COX-2 inhibitor nimesulide to potent anti-cancer agent: Synthesis, *in vitro*, *in vivo* and pharmacokinetic evaluation

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Introduction

Numerous studies have demonstrated the overexpression of cyclooxygenase 2 (COX 2) in solid malignancies [1–9]. Epidemiological, clinical, and preclinical investigations also provide compelling evidence that COX 2 inhibitors could act as chemopreventive agents [8,10–12]. The anti cancer effects of COX 2 inhibitors are based on the assumption that prostaglandins generated by COX 2 promote tumor growth in an autocrine and/or paracrine manner [13,14].

Theoretically, COX 2 inhibitors exhibit all the anti cancer or cancer preventive activity by blocking COX 2, thereby decrease the concentration of prostaglandins inside the tumor. However, these small molecules may also target other growth pathway, which may lead to cell growth inhibition, apoptosis or necrosis [8,15,16]. Many COX 2 inhibitors can suppress the growth of non COX 2 expressing tumor cells, while supplementation with exogenous prostaglandin cannot rescue the cells from growth inhibition caused by COX 2 inhibitors [17–22]. Therefore, it is speculated that COX 2 independent effects may contribute to or even be fully responsible for the anti cancer properties of some COX 2 inhibitors. Furthermore, the relative potency of COX 2 inhibitors to inhibit COX 2 enzyme does not match their potency to inhibit cancer cell growth [19]. In addition to the lack of correlation between COX 2 inhibition and anti cancer activities, the required concentrations of these COX 2 inhibitors to inhibit tumor cell growth significantly exceed the concentrations required to inhibit COX 2. This phenomenon suggests that the COX 2 inhibitors mainly target other pathways which need much higher concentration for COX 2 inhibitors to block [19,23–26]. The strongest evidence for a COX independent mechanism is that some non COX 2 inhibitory derivatives of certain COX 2 inhibitors still exhibit significant anti cancer activity [27,28].

The COX 2 selective inhibitor nimesulide, *N* (2 phenoxy 4 nitrophenyl)methanesulfonamide, is a promising lead compound for anti cancer drug discovery. In several *in vivo* experiments, nimesulide exhibits chemopreventive activity against 2 amino 1 methyl 6 phenylimidazo [4,5 *b*] pyridine induced mammary carcinogenesis in rats and against the post initiation development of squamous cell carcinomas in 4 nitroquinoline 1 oxide induced rat tongue carcinogenesis [29–31]. In addition, nimesulide is

shown to protect against *N* nitrosobis(2 oxopropyl)amine induced pancreatic tumors in hamster [32]. In some *in vitro* experiments, nimesulide is able to inhibit the proliferation and to increase the apoptosis rate of various types of cancer cells [18,25,33–38]. However, the nimesulide concentrations used in these studies are ranged from 200 to 500 μM, which greatly exceed the concentra tion necessary to inhibit COX 2 activity. These facts suggest that nimesulide inhibits cancer cell growth and induces cancer cell apoptosis independent of COX 2.

JCC76 is a non COX 2 active nimesulide analog (Fig. 1) [39–41], and it inhibits SKBR 3 breast cancer cell growth with an IC_{50} of 1.38 μM, which is about 100 fold more active than nimesulide (Table 1). The N Methylation of JCC76 blocks the ionization of the sulfonamide group, which abolishes the potential COX 2 activity [42–44]. In addition, the aromatic nitro group is converted to an amide moiety on the structure of JCC76, which could diminish the potential hepatotoxicity, since nimesulide shows hepatotoxicity due to the multistep nitroreductive bioactivation that produces the hazardous nitroanion radical and nitroso intermediate [45]. Based on the structure of JCC76, more analogs were designed and synthesized in the current studies. Some new analogs such as CSUOH0901 (NSC751382) inhibited SKBR 3 breast cancer cell growth with IC_{50}s around 0.1 μM–0.2 μM, which is about 10 fold more active than JCC76 and almost 1000 fold more active than nimesulide. In addition, CSUOH0901 inhibited the growth of a broad range of cancer cell lines with IC_{50}s of 0.2 μM to 0.5 μM. It also inhibited the growth of HT29 colorectal xenograft in nude mice as well. All the studies suggest that the newly developed JCC76 derivatives are promising anti cancer drug candidates.

Results and discussion

Compound design and parallel synthesis of JCC76 derivatives

In previous studies, systematic modification was performed on the structure of nimesulide to improve the anti cancer activity and erase the COX 2 inhibitory activity [41,46]. SAR result suggests that the dimethyl benzyl and methylsulfonamide moieties are critical for the nimesulide analogs to inhibit cancer cell growth (Fig. 1). Further, the conversion of the nitro group to a bulky amide moiety generated novel anti cancer agent JCC76 [39–41,46]. In the current study, dimethyl benzyl and methylsulfonamide groups which are important for the anti cancer activity of the derivatives were maintained, and we focused on the modification of the amide moiety of JCC76. Various chemical structures including alkyl amide groups, electron donating or withdrawing group substituted ben zamides, bulky or small group substituted benzamides, and heterocyclic amides were introduced at this moiety.

The synthesis is described in Scheme 1. The starting material 2 amino 5 nitrophenol was refluxed with K_2CO_3 and 2, 5 dimethyl benzyl chloride to obtain compound **a**. Sodium hydride and methanesulfonyl chloride were added to compound **a** in dry dimethylformamide (DMF) at room temperature and the reaction mixture was stirred at room temperature overnight to obtain the N,N bimethanesulfonamido **b**. Compound **b** was hydrolyzed with 10% NaOH solution to generate **c** as a monomethanesulfonamido compound. Compound **c** was treated with sodium hydride and methyl iodine in DMF at room temperature to obtain compound **d**. Then the nitro group was reduced to an amine group to obtain compound **e**. Compound **e** was treated with different substituted acetyl chloride and K_2CO_3 to generate the substituted benzamides **1**–**39**, respectively. Structures of all the synthesized compounds were determined by ^1H NMR, MS, and their purity was confirmed by HPLC with two mobile phases.

Biological evaluation of the new analogs with breast cancer cell line SKBR 3

The compounds were tested for the inhibition of SKBR 3 breast cancer cell growth (Table 1). Subsequently, a detailed structure activity analysis was performed based on the structure and the anti cancer activity. The new derivatives have same core structure as JCC76, the different activity is correlated with the different amide moieties.

For the substituted benzamide moiety in the structure, *para* position bulky halogen substituted benzamide such as **9** (bromine) with an IC_{50} of 0.22 μM and **29** (iodine) with an IC_{50} of 0.13 μM show better potency than JCC76, whereas small halogen chlorine at *para* position (**28**) slightly decreases the activity with an IC_{50} of 2.15 μM. It is also possible that the activity is related to the elec tronegativity or electron density of the halogens. Iodine has the lowest electronegativity and highest electron density, which may contribute to the potent anti cancer activity. **2**, **8**, **25**, **20**, and **27**, which have single *meta* substitution groups, display weaker inhibitory activity with IC_{50}s ranging from 1.97 μM to 7.34 μM. It seems that single *meta* substitution is predominated by steric effect. The activity is decreased no matter the substitution is electron donating or withdrawing group. Dual chlorine substitu tion (**4** and **14**) slightly increases the activity compared to JCC76, with IC_{50} of 0.91 μM and 0.80 μM respectively. **1**, **2**, **3**, **7**, **8**, **15**, **21** and **34**, which have electron withdrawing groups as substitution, exhibit IC_{50}s among 1.0 μM–3.0 μM. The results suggest that electron withdrawing groups such as nitro, cynao, multi fluoro, and trifluoro methyl substituted benzamide do not increase the activity. **26** shows very weaker activity with an IC_{50} of 55.35 μM, which possibly is due to the very strong electron withdrawing effect and the *meta* position steric effect of the di trifluoro methyl groups. Most of the strong electron donating groups such as methoxyl and multi methoxyl substituted benzamide (**10**, **11**, **16**, **18** and **19**) increase the activity with IC_{50}s among 0.19 μM–0.68 μM. However, *para* methyl benzamide (**33**) and *para* ethyl benzamide (**36**) show lesser potency with IC_{50}s of 2.48 μM and 2.82 μM respectively, although both methyl and ethyl groups are electron donating substitution. It suggests that *para* position steric effect reduces the activity, but strong electron donating group at *para* position overall increase the activity because the electron donating

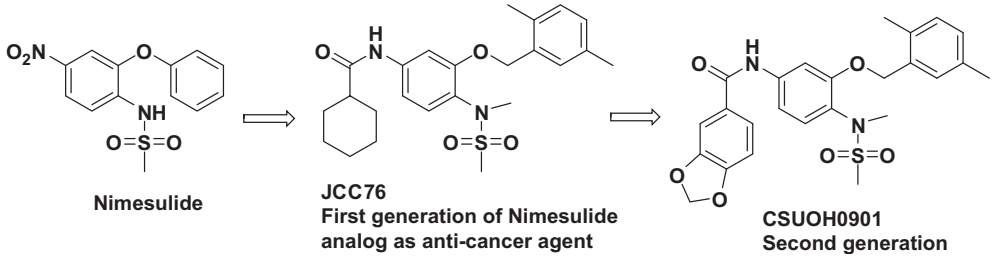


Fig. 1. Anti-cancer drug development based on COX-2 inhibitor nimesulide.

Table 1
Inhibition of cancer cell growth of the JCC76 anlogs.

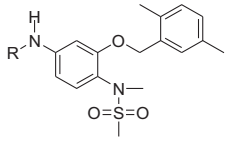
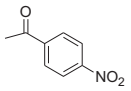
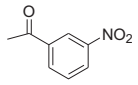
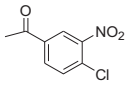
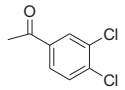
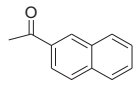
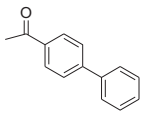
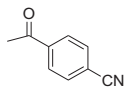
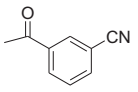
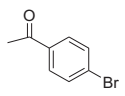
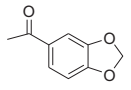
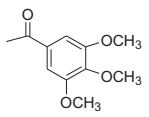
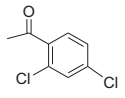
Entry		IC_{50} against SKBR-3 breast cancer cell growth
JCC76	R= 	1.38 ± 0.10 μM
1	R = 	1.13 ± 0.10 μM
2	R = 	1.97 ± 0.21 μM
3	R = 	3.35 ± 0.40 μM
4	R = 	0.91 ± 0.05 μM
5	R = 	0.21 ± 0.01 μM
6	R = 	2.28 ± 0.09 μM
7	R = 	1.46 ± 0.06 μM
8	R = 	3.01 ± 0.12 μM
9	R = 	0.22 ± 0.01 μM
10 (CSUOH0901; NSC751382)	R = 	0.20 ± 0.01 μM
11	R = 	0.30 ± 0.02 μM
12	R = 	43.27 ± 7.38 μM
13	R = 	11.05 ± 4.76 μ3M
14	R = 	0.80 ± 0.01 μM

Table 1 (*continued*)

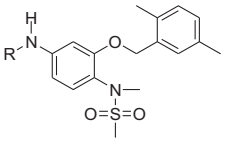
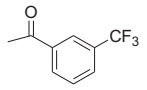
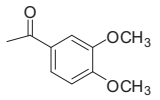
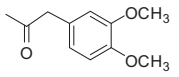
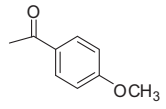
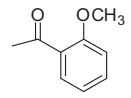
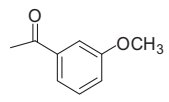
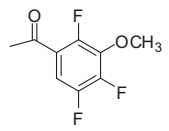
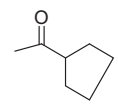
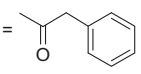
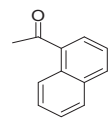
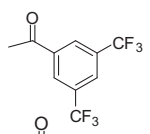
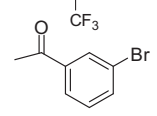
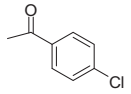
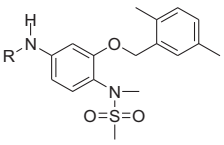
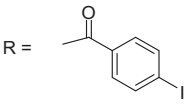
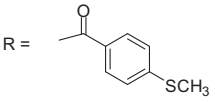
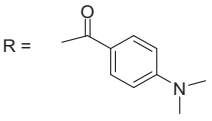
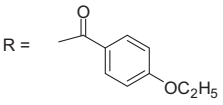
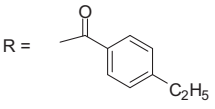
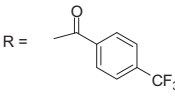
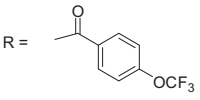
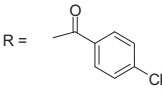
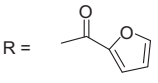
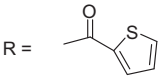
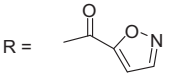
Entry		IC_{50} against SKBR-3 breast cancer cell growth
15	R = 	2.78 ± 0.29 μM
16	R = 	0.19 ± 0.14 μM
17	R = 	34.02 ± 2.01 μM
18	R = 	0.15 ± 0.05 μM
19	R = 	0.68 ± 0.32 μM
20	R = 	6.88 ± 3.18 μM
21	R = 	2.16 ± 1.08 μM
22	R = 	51.24 ± 4.49 μM
23	R = 	11.21 ± 4.47 μM
24	R = 	>100 μM
25	R = 	3.95 ± 2.09 μM
26	R = 	55.35 ± 4.11 μM
27	R = 	7.34 ± 3.99 μM
28	R = 	2.15 ± 0.94 μM

Table 1 (*continued*)

Entry		IC_{50} against SKBR-3 breast cancer cell growth
29		0.13 ± 0.07 μM
30		0.66 ± 0.32 μM
31		1.01 ± 0.52 μM
32		0.41 ± 0.03 μM
33		2.48 ± 1.44 μM
34		1.20 ± 0.59 μM
35		0.58 ± 0.29 μM
36		2.82 ± 1.51 μM
37		16.65 ± 2.26 μM
38		20.12 ± 5.28 μM
39		21.88 ± 5.08 μM

Cells were treated with indicated compounds at various concentrations by six replicates for 48 h and cell viability was measured by MTT assay as described in the experimental section.

effect overcomes the steric effect. 2 Naphthyl amide (**5**) increases the activity with an IC_{50} of 0.21 μM, but 1 Naphthyl amide (**25**) decreases the activity with an IC_{50} of 3.95 μM. Because 2 Naphthyl group is more like a dual *para* and *meta* position substituted benzamide, and the substitution has very strong electron donating effect at *para* position due to the super conjugation effect. 1 Naphthyl group is more like a dual *meta* and *ortho* position substituted benzamide, which decreases the activity due to steric effect of *meta* substitution. **6** (*para* phenyl benzamide) shows weaker activity with an IC_{50} of 2.28 μM. Because the phenyl group cannot form super conjugation with the benzamide due to the steric effect of the two phenyl rings. It makes the phenyl substitution predominated mainly by steric effect. Substituted Benzylamide, namely **17** and **24**, display much weaker activity with IC_{50}s above 30 μM. Heterocyclic amides (**37**, **38**, **39**) decrease the activity, with IC_{50}s of 16.65 μM, 20.12 μM, and 21.88 μM respectively. The results suggest that benzamide moiety is better for the activity compared to the heterocyclic amides.

For the alkyl substituted amide, lead compound JCC76 is still the most potent one with an IC_{50} of 1.38 μM. Small alkyl groups such as ethyl (**12**), 3 pentyl (**22**), and cyclopentyl (**23**) greatly decrease the activity, and the corresponding IC_{50} are 43.27 μM, 51.24 μM, and 11.21 μM respectively. Very bulky alkyl group such as hexadacanyl (**13**) decreases the activity as well, with an IC_{50} of 11.05 μM. It appears that the best alkyl substitution is a middle size and closed ring.

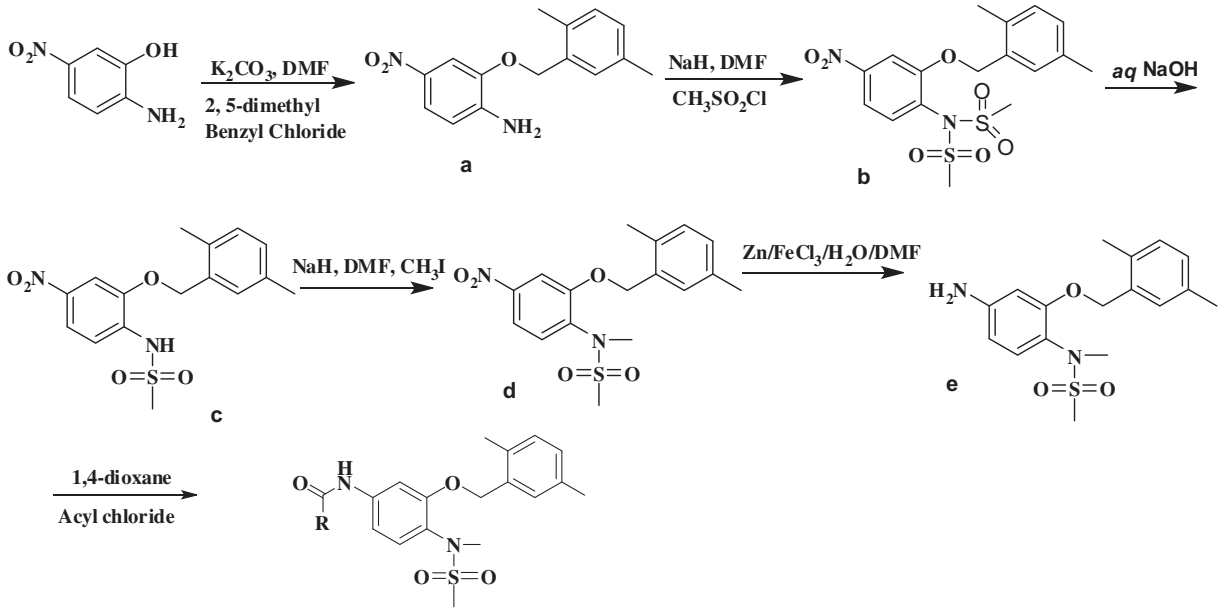
Overall, *para* position of the benzamide is very critical for the anti cancer activity of the JCC76 analogs. Strong electron donating groups at *para* position significantly increase the activity. Single *meta* substituted benzamide greatly decreases the activity, no matter it is electron donating or withdrawing group as substitution. Several very potent analogs (**5**, **9**, **10**, **16**, **18**, **29**), which are about 5–10 fold more active than JCC76, share some structure similarity: *para* position substituted benzamide, or *para* and *meta* both electron donating group substituted benzamide. **10**, discovered earlier in the study, was named CSUOH0901 and submitted to the Developmental Therapeutic Program at the National Cancer Institute for screening against 60 human tumor cell lines. It was also investigated for the anti cancer mechanism and *in vivo* activity in the study.

CSUOH0901 caused cell population concentrated at sub G1 and G2 phase

Compound CSUOH0901 significantly inhibited SKBR 3 breast cancer cell growth. It is important to elucidate the anti cancer mechanisms of the compound. When SKBR 3 cells were treated with 0.5 μM and 5 μM of CSUOH0901, cells were accumulated at G2/M and subG1 phase after 12 h. As for 0.25 μM treatment, this phenomenon was observed after 24 h (Table 2). The results demonstrate that CSUOH0901 was able to inhibit cell mitosis and induce cell apoptosis at similar dosage to inhibit cell growth. JCC76 has been shown in previous studies to induce cell apoptosis via cytochrome c release [39,40]. It is not surprising that the more potent JCC76 analog CSUOH0901 exhibits better potency to induce cell apoptosis. However, JCC76 did not show good potency to cause cell cycle arrest in previous studies [39,40]. CSUOH0901 obviously exhibited the cell G2/M phase arresting activity. Therefore, the anti mitosis potency is significantly increased by the structure modification. 0.1 μM CSUOH0901 clearly induced subG1 cell accumulation after 24 h treatment, but no G2/M cell accumulation was observed, suggesting that the compound at low concentrations was able to induce cell apoptosis without affecting the cell replication. It is difficult to determine if the cell apoptosis and cell growth arrested by the compound are correlated or not, since the specific molecular targets of CSUOH0901 still remain unclear. Further investigation is needed to identify the anti cancer molecular targets of these compounds.

Multi cancer cell lines growth inhibition and animal acute toxicity studies of CSUOH0901

Due to the structure novelty and potency of CSUOH0901, it was submitted to the Developmental Therapeutic Program at the National Cancer Institute (assigned with code NSC751382), and was selected by



1 R= 4-Nitro-phenyl (88%)
2 R= 3-Nitro-phenyl (86%)
3 R= 4-Chloro-3-nitro-phenyl (82%)
4 R= 3,4-Dichloro-phenyl (69%)
5 R= 2-Naphthyl (72%)
6 R= 4-Biphenyl (73%)
7 R= 4-Cyano-phenyl (75%)
8 R= 3-Cyano-phenyl (71%)
9 R= 4-Bromo-phenyl (80%)
10 R= 5-Benzo[1,3]dioxolyl(71%)
11 R= 3,4,5-Trimethoxy-phenyl (93%)
12 R= Propionyl (88%)
13 R= Hexadecanyl (98%)
14 R= 2,4-Dichloro-phenyl (80%)
15 R= 3-Trifluoromethyl-phenyl (93%)
16 R= 3,4-Dimethoxy-phenyl (93%)
17 R= 3,4-Dimethoxy-benzyl (95%)
18 R= 4-Methoxy-phenyl (96%)
19 R= 2-Methoxy-phenyl (97%)
20 R= 3-Methoxy-phenyl (91%)
21 R= 3-Methoxy-2,4,5-trifluorophenyl (98%)
22 R= 3-Pentyl (96%)
23 R= Cyclopentyl (93%)
24 R= Benzyl (99%)
25 R= 1-Naphthyl (83%)
26 R= 3,5-Bis(trifluoromethyl)-phenyl (93%)
27 R= 3-Bromophenyl (99%)
28 R= 4-Chlorophenyl (99%)
29 R= 4-Iodophenyl (96%)
30 R= 4-Methylsulfanyl-phenyl (92%)
31 R= 4- Dimethylamino-phenyl (66%)
32 R= 4-Ethoxy-phenyl (70%)
33 R= 4-Ethyl-phenyl (64%)
34 R= 4-Trifluoromethyl-phenyl (84%)
35 R= 4-Trifluoromethoxy-phenyl(79%)
36 R= 4-Methyl-phenyl (69%)
37 R= 2-Furyl (66%)
38 R= 2-Thiophenyl (72%)
39 R= 5-Isoxazolyl(99%)

Scheme 1. Synthesis of JCC76 analogs.

Table 2
Summary of altered cell cycle distribution in response to treatment with CSUOH0901 (NSC751382). SKBR-3 cells were treated for different time period with the indicated concentrations of compound. Cells were processed for FACS using propidium iodide staining as described. Percent distribution of cells in each cell cycle phase was displayed.

Time	Concentrations of CSUOH0901	Sub-G1%	G1%	S%	G2/M%
3 h	0.10 (μM)	3.12	70.84	17.20	8.83
	0.25 (μM)	3.23	69.63	16.34	10.77
	0.50 (μM)	3.43	67.14	17.76	11.54
	5.0 (μM)	2.81	69.09	16.49	11.57
6 h	0.10 (μM)	3.60	71.43	15.84	9.07
	0.25 (μM)	3.49	72.21	14.95	9.32
	0.50 (μM)	4.03	67.10	14.73	13.90
	5.0 (μM)	3.42	64.76	14.61	17.19
12 h	0.10 (μM)	5.27	73.14	8.17	12.05
	0.25 (μM)	6.85	73.62	6.14	13.35
	0.50 (μM)	6.36	63.15	5.94	24.26
	5.0 (μM)	9.76	63.71	5.99	20.54
18 h	0.10 (μM)	6.04	75.43	8.71	9.71
	0.25 (μM)	8.89	73.42	6.81	10.57
	0.50 (μM)	8.47	61.19	7.28	22.27
	5.0 (μM)	8.31	63.55	6.73	21.80
24 h	0.10 (μM)	11.78	67.90	8.91	11.23
	0.25 (μM)	24.95	41.27	10.87	22.53
	0.50 (μM)	27.94	31.77	11.21	28.14
	5.0 (μM)	27.62	31.87	11.53	28.29

NCIDTP for screening against 60 human tumor cell lines, representing leukemia, melanoma, and cancers of the lung, colon, CNS, ovary, renal, prostate, and breast. After 48 h treatment, CSUOH0901 does dependently inhibited the growth of sixty cell lines from each class of tumor cells. Three dose response parameters are calculated for the experimental agent. Growth inhibition of 50% (GI50) is the drug concentration resulting in a 50% reduction in the net protein increase compared with control cells during the drug incubation. Total growth inhibition (TGI) shows the drug concentration that causes a 100% reduction in the net protein increase during the drug incubation. The LC50 is the concentration of drug resulting in a 50% reduction in protein at the end of the drug treatment as compared to the protein amount present at the time of drug addition. Values are calculated for each of these three parameters if the requisite level of activity is achieved. The values of these parameters among the 60 different cell lines after 48 h treatment are as follows: concentration resulting in 50% growth inhibition (GI50), 0.03 μM−0.5 μM; concentration resulting in total growth inhibition (TGI), 0.2 μM−2.0 μM (in approximately one half of the cell lines); concentrations resulting in a 50% reduction in the measured protein level at the end of drug treatment (LC50), above 20 μM in only 3 cell lines (Table 3).

The compound was also tested for the acute toxicity to determine the maximum tolerated dose in nude mice. After a single dose of 400 mg/kg, 200 mg/kg, or 100 mg/kg, the nude mice were observed for a period of 2 weeks. No body weight lost was found in the three mice tested, indicating that the animals were highly tolerable to CSUOH0901.

In vivo investigation

To determine whether the high activity of our compounds at cell culture would be translated into *in vivo* active anti cancer agents, CSUOH0901 as a representative one was investigated in the tumor xenograft nude mice model. SKBR 3 cells do not readily form xenografts in nude mice, therefore we used a colon cancer HT29 xenograft model to investigate the *in vivo* activity of the compound, since it significantly inhibited HT29 cell growth in the *in vitro* study (Developmental Therapeutics Program at National Cancer Institute) with an IC_{50} of 0.42 μM (Table 3). We confirmed the activity of CSUOH0901 in HT29 cells with an MTT cell proliferation assay, and obtained an IC_{50} of 0.46 μM. HT29 xenograft is a well established *in vivo* tumor model, and has been used to test the activity of many anti cancer drug candidates. Nude mice (three mice per group, two tumors per mouse) bearing HT29 xenografts were given daily (5 times per week) intraperitoneal injections (5 mg/kg) of CSUOH0901 for three weeks after the tumor reached a measurable size.

CSUOH0901 treatment significantly decreased the size of the HT29 tumors compared to the control group (Fig. 2A). Weights of the mice were not affected by the treatment (Fig. 2B), suggesting the low toxicity of the compound. After the treatment, the tumors were removed and weighed. CSUOH0901 significantly decreased the tumor weights as well (Fig. 2C). The results reveal that CSUOH0901 is active *in vivo* and could be a promising anti cancer drug candidate.

Pharmacokinetic study

CSUOH0901 exhibited potent *in vitro* and *in vivo* anti tumor activity. To support future further pharmacological and toxicological study, a pharmacokinetic study of the compound was also performed. CSUOH0901 was administrated to rats intraperitoneally at a dose of 20 mg/kg. Blood samples (150 μL each) were collected from the saphenous veins and femoral veins into heparized tubes at 0 h (before drug administration) and at 0.25, 0.5, 1, 2, 4, 8 and 24 h after dosing. The peripheral blood drug level was then determined

Table 3
Growth inhibitory effect of CSUOH0901 on 60 human tumor cell lines. Growth inhibition of 50% (GI50), total growth inhibition (TGI), and LC50 are listed in the table.

Panel/Cell line	GI50	TGI	LC50
Leukemia			
CCRF-CEM	4.85E-8	>1.00E-4	>1.00E-4
HL-60(TB)	2.69E-8	9.67E-8	>1.00E-4
K-562	4.03E-8	>1.00E-4	>1.00E-4
MOLT-4	1.63E-7	>1.00E-4	>1.00E-4
RPMI-8226	2.90E-7		>1.00E-4
Non-small cell lung cancer			
A549/ATCC	2.78E-7	1.36E-5	>1.00E-4
EKVX	5.98E-7	> 1.00E-4	>1.00E-4
HOP-62	3.29E-7	> 1.00E-4	>1.00E-4
HOP-92	3.22E-6	> 1.00E-4	>1.00E-4
NCI-H226	6.86E-7	> 1.00E-4	>1.00E-4
NCI-H23	1.62E-7	> 1.00E-4	>1.00E-4
NCI-H322M	4.68E-7	> 1.00E-4	>1.00E-4
NCI-H460	1.84E-7	7.44E-7	>1.00E-4
NCI-H522	3.35E-8	2.40E-7	>1.00E-4
Colon cancer			
COLO 205	3.18E-7	1.07E-6	>1.00E-4
HCC-2998	1.20E-7	3.92E-7	2.97E-6
HCT-116	7.53E-8	1.02E-6	>1.00E-4
HCT-15	2.89E-7	>1.00E-4	>1.00E-4
HT29	7.72E-8	4.19E-7	>1.00E-4
KM12	8.55E-8	1.13E-6	>1.00E-4
SW-620	1.12E-7	>1.00E-4	>1.00E-4
CNS cancer			
SF-268	2.88E-7	>1.00E-4	>1.00E-4
SF295	9.83E-8	6.26E-7	>1.00E-4
SF539	1.25E-7		>1.00E-4
SNB-19	5.37E-7	>1.00E-4	>1.00E-4
SNB-75	2.66E-7		>1.00E-4
U251	2.66E-7	2.19E-5	>1.00E-4
Melanoma			
LOXIMVI	6.66E-8	>1.00E-4	>1.00E-4
MALME-3M	2.29E-7	>1.00E-4	>1.00E-4
M14	7.57E-8	6.69E-7	>1.00E-4
MDA-MB-435	2.85E-8	9.14E-8	>1.00E-4
SK-MEL-2	8.97E-8	6.16E-7	>1.00E-4
SK-MEL-28	6.92E-7	>1.00E-4	>1.00E-4
SK-MEL-5	2.53E-7	1.65E-6	3.41E-5
UACC-257	1.44E-7	>1.00E-4	>1.00E-4
UACC-62	4.14E-7	>1.00E-4	>1.00E-4
Ovarian cancer			
IGROV1	2.37E-7	>1.00E-4	>1.00E-4
OVCAR-3	3.96E-8	1.66E-7	
OVCAR-4		>1.00E-4	>1.00E-4
OVCAR-5	5.38E-7	>1.00E-4	>1.00E-4
OVCAR-8	2.65E-7	>1.00E-4	>1.00E-4
NCI/ADR-RES	5.62E-8	4.07E-7	>1.00E-4
SK-OV-3	2.85E-7	>1.00E-4	>1.00E-4
Renal cancer			
786 0	1.91E-7	1.11E-6	>1.00E-4
A498	1.22E-7	8.53E-7	>1.00E-4
ACHN	6.87E-7	>1.00E-4	>1.00E-4
CAKI-1	1.26E-7	>1.00E-4	>1.00E-4
RXF 393	1.28E-7	4.03E-7	>1.00E-4
SN12C	4.72E-7	>1.00E-4	>1.00E-4
TK-10	3.54E-7	>1.00E-4	>1.00E-4
UO-31	3.95E-7	>1.00E-4	>1.00E-4
Prostate cancer			
PC-3	6.77E-8	>1.00E-4	>1.00E-4
DU-145	1.59E-7	4.57E-7	>1.00E-4
Breast cancer			
MCF7	1.20E-7	>1.00E-4	>1.00E-4
MDA-MB-231/ATCC	2.56E-7	9.26E-7	>1.00E-4
HS 578T	8.70E-8		>1.00E-4
BT-549	6.56E-7	>1.00E-4	>1.00E-4
T-47D	6.82E-7	>1.00E-4	>1.00E-4
MDA-MB-468	1.55E-7	4.01E-7	1.99E-5

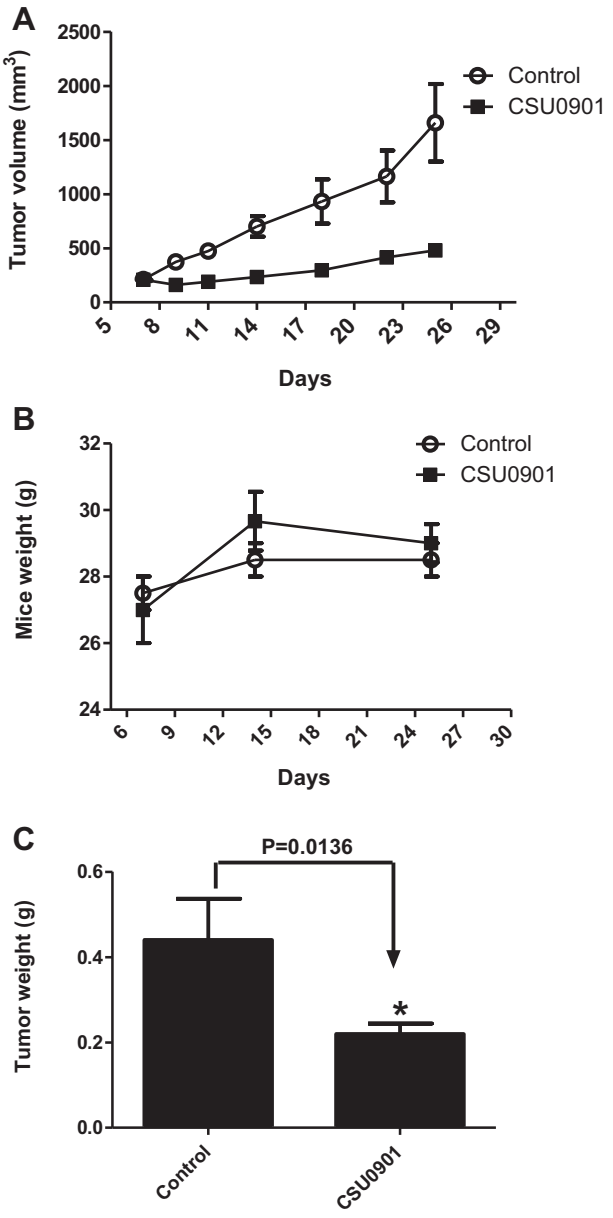


Fig. 2. *In vivo* anti-cancer evaluation of CSUOH0901. A. Nude mice bearing HT29 xenograft after the treatment. The treatment group is significantly smaller than the control group (n 6); B Mice weight changing (n 3); C HT29 tumor weight comparison (n 6). *$P < 0.05$ *vs* control.

with LC MS/MS. The mean CSUOH0901 concentration in plasma versus time profile was presented in Fig. 3. Peak drug concentra tions were observed at 2 h after administration and reached nearly1500 ng/mL. The pharmacokinetic parameters were calcu lated by using non compartmental model. The estimated phar macokinetic parameters including the terminal phase elimination half life ($T_{1/2}$), the area under the plasma concentration time curve (AUC) from time 0 to time of the last measurable concen tration ($AUC_{(0\text{-}t)}$), the volume of distribution ($V_{z/F}$), the total body clearance ($Cl_{/F}$), and the mean residence time (MRT) from time 0 to time of the last measurable concentration ($MRT_{(0\text{-}t)}$) are listed in Table 4. The half life and volume of distribution of the compound is relatively lower compared with more hydrophobic anti cancer drugs such as Taxol [47], suggesting the compound has better drug like characters than Taxol. The good bioavailability of the compound recommends further drug development of these small molecule anti cancer agents.

Conclusion

The structural modifications of nimesulide have been effective in abolishing its COX 2 inhibiting property and reducing its hepa totoxicity. Among the new derivatives synthesised, compounds **5**, **9**, **10** (CSUOH0901), **16**, **18** and **29** have exhibited good growth inhibitory activity against SK BR 3 breast cancer cells at low nanomolar concentrations. CSUOH0901 displayed good potency to inhibit the growth of a broad range of cancer cell lines and demonstrated minor animal toxicity. The *in vivo* tumor suppression activity and pharmacokinetic results of CSUOH0901 suggest that the drug candidate has great clinical application potential.

Experimental section

Chemistry

Chemicals were commercially available and used as received without further purification unless otherwise noted. Moisture sensitive reactions were carried out under a dry argon atmosphere in flame dried glassware. Solvents were distilled before use under argon. Thin layer chromatography was performed on precoated silica gel F254 plates (Whatman). Silica gel column chromatog raphy was performed using silica gel 60A (Merck, 230 400 Mesh), and hexane/ethyl acetate was used as the elusion solvent. Mass spectra were obtained on the Micromass QTOF Electrospray mass spectrometer at Cleveland State University MS facility Center. Melting point was recorded with a Mel Temp melting point appa ratus. All the NMR spectra were recorded on a Varian 400 MHz in either DMSO d_6 or $CDCl_3$. Chemical shifts (δ) for ^1H NMR spectra are reported in parts per million to residual solvent protons. The IR spectra were obtained on a Bruker ALPHA FT IR spectrometer with ATR module.

For the HPLC analysis, a 1.00 mg/mL stock solution of each standard was prepared in either methanol or acetonitrile. The HPLC system consists of two LC 20AD pumps, a DGU $20A_3$ degasser, a SIL 20AC autosampler, and a CBM 20A module (Shimazu, Tokyo, Japan). The chromatographic separation was performed on a Luna C18 column (2.0 mm × 150 mm, 5 μm) with a guard column (2 mm × 40 mm, 5 μm) from Phenomenex (Torrance, CA, USA) at room temperature with a flow rate of 0.2 mL/min. Two mobile phases (10 mM ammonium acetate in 90% methanol or acetonitrile) were employed to run 15 min. An injection volume of 5–15 μL was used. The UV detector was set up at 290 and 256 nM.

Compounds **a**–**e** were prepared as described by Su et al. [41,46].

*General procedure for the preparation of the substituted benzamide **1**–**39***

K_2CO_3 (5 mmol, 5eq) and substituted acyl chloride (1.2 mmol, 1.2 eq) were successively added to a solution of the aniline **e** (1.0 mmol, 1.0 eq) in 3 mL dry 1, 4 dioxane and the mixture was stirred at room temperature overnight. 10 mL H_2O and 3 mL saturated aqueous Na_2CO_3 was added to the mixture and it was stirred at room temperature overnight. The precipitated solid was collected by filtration and purified by silica gel column chromatography.

*N [3 (2,5 dimethyl benzyloxy) 4 (methanesulfonyl methyl amino) phenyl] 4 nitro benzamide (**1**).* 4 Nitro benzoyl chloride was used and it was stirred at room temperature overnight. Pale yellow solid, melting point 206–209 °C, yield 88%: ^1H NMR (400 MHz, DMSO d_6) δ 10.68 (1H, s), 8.41 (2H, d, J 8.0 Hz), 8.21 (2H, d, J 8.2 Hz), 7.75 (1H, s), 7.44 (1H, d, J 8.4 Hz), 7.33(2H, m),

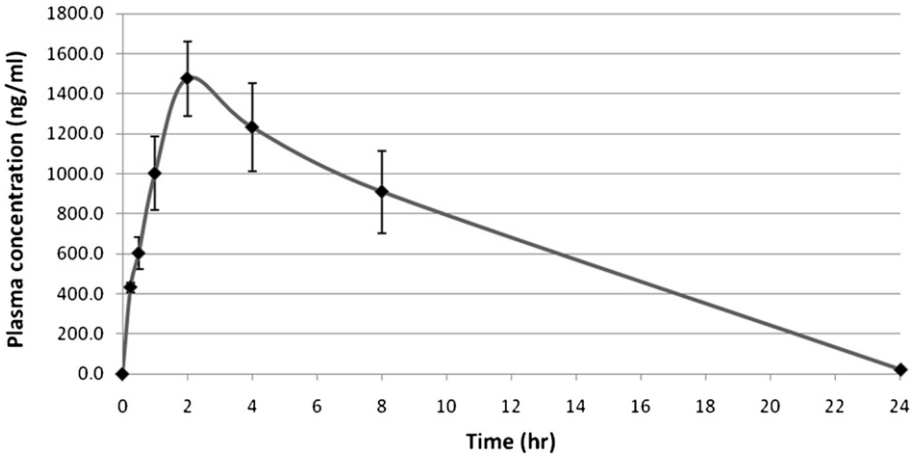


Fig. 3. CSUOH0901 rats blood concentrations *vs* time. CSUOH0901 was administrated to rats (n 3) intraperitoneally with 20 mg/kg. Blood samples of 150 μL each were collected from the saphenous veins and femoral veins into heparized tubes at 0 h (before drug administration) and at 0.25, 0.5, 1, 2, 4, 8 and 24 h after dosing. The blood drug concentration was then determined with LC-MS/MS.

7.15(1H, d, J 7.8 Hz), 7.09(1H, d, J 7.8 Hz), 5.11 (2H, s), 3.12 (3H, s), 2.88 (3H, s), 2.32 (3H, s), 2.28 (3H, s); HRMS calculated for $C_{24}H_{26}N_2NaO_4S$ $[M + Na]^+$ 461.1511, found: 461.1511.

*N [3 (2,5 dimethyl benzyloxy) 4 (methanesulfonyl methyl amino) phenyl] 3 nitro benzamide (**2**).* 3 Nitro benzoyl chloride was used and it was stirred at room temperature overnight. White solid, melting point 163–165 °C, yield 86%: ^{1}H NMR (400 MHz, DMSO d_6) δ 10.70 (1H, s), 8.80 (1H, s), 8.48 (1H, d, J 8.0 Hz), 8.43 (1H, d, J 8.0 Hz),7.89 (1H, dd, J 7.8, 7.8 Hz), 7.75 (1H, s), 7.45 (1H,d, J 8.6 Hz),7.34(2H, m), 7.15(1H, d, J 7.4 Hz), 7.09 (1H, d, J 7.5 Hz), 5.12 (2H, s), 3.12 (3H, s), 2.88 (3H, s), 2.33 (3H, s), 2.28 (3H, s). ESI MS (m/z) calculated for $C_{24}H_{24}N_3O_6S$ $[M + H]^+$: 482.1, found: 481.9.

*4 Chloro N [3 (2,5 dimethyl benzyloxy) 4 (methanesulfonyl methyl amino) phenyl] 3 nitro benzamide (**3**).* 4 Chloro 3 nitro be nzoyl chloride was used and it was stirred at room temperature overnight. White solid, melting point 204–206 °C, yield 82%: ^{1}H NMR (400 MHz, DMSO d_6) δ 10.65 (1H, s), 8.65 (1H, s), 8.28 (1H, d, J 8.4 Hz), 8.01 (1H, d, J 8.4 Hz), 7.72 (1H, s), 7.42 (1H, d, J 8.4 Hz), 7.33(2H, m), 7.15(1H, d, J 7.7 Hz), 7.09 (1H, d, J 7.4 Hz), 5.11 (2H, s), 3.12 (3H, s), 2.87 (3H, s), 2.32 (3H, s), 2.28 (3H, s). ESI MS calculated for $C_{24}H_{23}ClN_3O_6S$ $[M + H]^+$: 516.1, found: 515.8.

*3,4 Dichloro N [3 (2,5 dimethyl benzyloxy) 4 (methanesulf onyl methyl amino) phenyl] benzamide (**4**).* 3,4 Dichloro benzoyl chloride was used and it was stirred at room temperature over night. White solid, melting point 177–179 °C, yield 69%: ^{1}H NMR (400 MHz, DMSO d_6) δ 10.51 (1H, s), 8.23 (1H, s), 7.97 (1H, d, J 8.4 Hz), 7.85 (1H, d, J 8.4 Hz), 7.73 (1H, s), 7.42 (1H, d, J 7.6 Hz), 7.31(2H, m), 7.15(1H, d, J 7.7 Hz), 7.09 (1H, d, J 7.6 Hz), 5.10 (2H, s), 3.12 (3H, s), 2.87 (3H, s), 2.32 (3H, s), 2.28 (3H, s). ESI MS calculated for $C_{24}H_{23}Cl_2N_2O_4S$ $[M + H]^+$: 505.1, found: 504.8.

Table 4
The pharmacokinetic parameters of CSUOH0901.

Parameters	Rat 1	Rat 2	Rat 3	Mean	S.D.
$T_{1/2}$ (h)	2.44	3.71	3.21	3.12	0.64
$AUC_{(0\ t)}$ (ng*h/mL)	16466	22648	9718	16277	6467
$V_{z/F}$ (L/kg)	1.51	1.65	3.33	2.16	1.01
$Cl_{/F}$ (L/h)	0.42	0.32	0.70	0.48	0.20
$MRT_{(0\ t)}$ (h)	5.95	6.43	5.62	6.00	0.40

*Naphthalene 2 carboxylic acid [3 (2,5 dimethyl benzyloxy) 4 (methanesulfonyl methyl amino) phenyl] amide (**5**).* Naphthalene 2 carbonyl chloride was used and it was stirred at room temperature for three days. White solid, melting point 133–136 °C, yield 72%: IR ν 3314 (N–H), 1649 (C=O), 1595, 1509 (aromatic C=C). ^{1}H NMR (400 MHz, DMSO d_6) δ 10.56 (1H, s), 8.60 (1H, s), 8.12 (4H, m), 7.82 (1H, s), 7.67 (2H, m), 7.50 (1H, d, J 8.5 Hz), 7.33(2H, m), 7.16(1H, d, J 7.8 Hz), 7.10 (1H, d, J 7.3 Hz), 5.13 (2H, s), 3.13 (3H, s), 2.88 (3H, s), 2.34 (3H, s), 2.29 (3H, s). ^{13}C NMR (100 MHz, DMSO d_6) δ 165.581, 155.169, 148.112, 140.164, 134.702, 134.229, 134.084, 133.100, 131.925, 130.956, 130.003, 129.133, 128.874, 128.660, 127.989, 127.951, 127.836, 127.600, 126.829, 124.663, 124.297, 112.289, 105.348, 68.106, 37.623, 37.509, 20.520, 17.873. ESI MS calculated for $C_{28}H_{27}N_2O_4S$ $[M + H]^+$: 487.2, found: 486.9.

*Biphenyl 4 carboxylic acid [3 (2,5 dimethyl benzyloxy) 4 (methanesulfonyl methyl amino) phenyl] amide (**6**).* Biphenyl 4 carbonyl chloride was used and it was stirred at room tempera ture for three days. White solid, melting point 136–139 °C, yield 73%: ^{1}H NMR (400 MHz, DMSO d_6) δ 10.42 (1H, s), 8.09 (2H, d, J 7.9 Hz), 7.88 (2H, d, J 7.8 Hz), 7.81 (1H, s), 7.79 (2H, d, J 7.2 Hz), 7.54(4H, m), 7.33 (2H, m), 7.16(1H, d, J 7.7 Hz), 7.10 (1H, d, J 7.5 Hz), 5.12 (2H, s), 3.12 (3H, s), 2.87 (3H, s), 2.33 (3H, s), 2.28 (3H, s). ESI MS calculated for $C_{30}H_{29}N_2O_4S$ $[M + H]^+$: 513.2, found: 512.9.

*4 Cyano N [3 (2,5 dimethyl benzyloxy) 4 (methanesulfonyl methyl amino) phenyl] benzamide (**7**).* 4 Cyano benzoyl chloride was used and it was stirred at room temperature overnight. White solid, melting point 183–187 °C, yield 75%: ^{1}H NMR (400 MHz, DMSO d_6) δ 10.60 (1H, s), 8.13 (2H, d, J 8.4 Hz), 8.06 (2H, d, J 8.2 Hz), 7.75 (1H, d, J 1.7 Hz), 7.43 (1H, dd, J 1.9, 8.5 Hz), 7.32 (2H, m), 7.15(1H, d, J 7.7 Hz), 7.09 (1H, d, J 7.7 Hz), 5.11 (2H, s), 3.12 (3H, s), 2.87 (3H, s), 2.32 (3H, s), 2.28 (3H, s). ESI MS calculated for $C_{25}H_{24}N_3O_4S$ $[M + H]^+$: 462.1, found: 461.9.

*3 Cyano N [3 (2,5 dimethyl benzyloxy) 4 (methanesulfonyl methyl amino) phenyl] benzamide (**8**).* 3 Cyano benzoyl chloride was used and it was stirred at room temperature overnight. White solid, melting point 166–170 °C, yield 71%: ^{1}H NMR (400 MHz, DMSO d_6) δ 10.54 (1H, s), 8.42 (1H, s), 8.27 (1H, d, J 7.8 Hz), 8.10 (1H, d, J 7.7 Hz), 7.80 (2H, m), 7.43 (1H, d, J 8.5 Hz), 7.32(2H, m), 7.15(1H, d, J 7.7 Hz), 7.09 (1H, d, J 7.8 Hz), 5.11 (2H, s), 3.12 (3H,

s), 2.87 (3H, s), 2.32 (3H, s), 2.28 (3H, s). ESI MS calculated for $C_{25}H_{24}N_3O_4S$ $[M + H]^+$: 462.1, found: 461.9.

*4 Bromo N [3 (2,5 dimethyl benzyloxy) 4 (methanesulfonyl methyl amino) phenyl] benzamide (**9**).* 4 Bromo benzoyl chloride was used and it was stirred at room temperature overnight. White solid, melting point 196–197 °C, yield 80%: IR ν 3338 (N–H), 1654 (C=O), 1593, 1507 (aromatic C=C). ^{1}H NMR (400 MHz, DMSO d_6) δ 10.43 (1H, s), 7.93 (2H, d, J 8.2 Hz), 7.79 (2H, d, J 8.5 Hz), 7.75 (1H, s), 7.40 (1H, m), 7.31 (2H, m), 7.15(1H, d, J 7.7 Hz), 7.09 (1H, d, J 7.7 Hz), 5.10 (2H, s), 3.11 (3H, s), 2.87 (3H, s), 2.32 (3H, s), 2.28 (3H, s). ^{13}C NMR (100 MHz, DMSO d_6) δ 164.551, 155.146, 139.874, 134.702, 134.046, 133.657, 133.100, 131.368, 130.948, 130.003, 129.720, 129.141, 128.668, 125.456, 124.815, 112.335, 105.393, 68.114, 37.623, 37.486, 20.520, 17.866. ESI MS calculated for $C_{24}H_{24}BrN_2O_4S$ $[M + H]^+$: 515.1, found: 514.8.

*Benzo[1,3]dioxole 5 carboxylic acid [3 (2,5 dimethyl benzyloxy) 4 (methanesulfonyl methyl amino) phenyl] amide (**10**).* 1,3 Dihydro isobenzofuran 5 carbonyl chloride was used and it was stirred at room temperature overnight. White solid, melting point 159–165 °C (decomposed), yield 71%: IR ν 3354 (N–H), 1653 (C=O), 1596, 1506 (aromatic C=C). ^{1}H NMR (400 MHz, DMSO d_6) δ 10.18 (1H, s), 7.75 (1H, s), 7.60 (1H, d, J 8.1 Hz), 7.52 (1H, s), 7.41 (1H, dd, J 1.5, 8.4 Hz), 7.31 (1H, s), 7.28 (1H, d, J 8.5 Hz), 7.15(1H, d, J 7.6 Hz), 7.09 (2H, d, J 8.0 Hz), 6.15 (2H, s), 5.09 (2H, s), 3.11 (3H, s), 2.86 (3H, s), 2.32 (3H, s), 2.28 (3H, s). ^{13}C NMR (100 MHz, DMSO d_6) δ 164.475, 155.100, 150.088, 147.311, 140.186, 134.686, 134.068, 133.107, 130.857, 129.995, 129.171, 128.660, 128.362, 124.472, 122.832, 112.206, 107.873, 107.598, 105.271, 101.770, 68.076, 37.585, 37.493, 20.513, 17.858. ESI MS calculated for $C_{25}H_{25}N_2O_6S$ $[M + H]^+$: 481.1, found: 480.8.

*N [3 (2,5 dimethyl benzyloxy) 4 (methanesulfonyl methyl amino) phenyl] 3,4,5 trimethoxy benzamide (**11**).* 3,4,5 Trimethoxy benzoyl chloride was used and it was stirred at room temperature overnight. White solid, melting point 187–189 °C, yield 93%: ^{1}H NMR (400 MHz, DMSO d_6) δ 10.26 (1H, s), 7.76 (1H, s), 7.36 (5H, m), 7.13(1H, d, J 7.7 Hz), 7.09 (1H, d, J 7.7 Hz), 5.11 (2H, s), 3.89 (6H, s), 3.75 (3H, s), 3.12 (3H, s), 2.87 (3H, s), 2.32 (3H, s), 2.28 (3H, s). ESI MS calculated for $C_{27}H_{31}N_2O_7S$ $[M + H]^+$: 527.2, found: 526.9.

*N [3 (2,5 dimethyl benzyloxy) 4 (methanesulfonyl methyl amino) phenyl] propionamide (**12**).* Propionoyl chloride was used and it was stirred at room temperature overnight. White solid, melting point 168–170 °C, yield 88%: ^{1}H NMR (400 MHz, DMSO d_6) δ 10.00 (1H, s), 7.58 (1H, s), 7.28 (1H, s), 7.23 (1H, d, J 8.5 Hz), 7.16(1H, s), 7.14(1H, d, J 7.6 Hz), 7.08 (1H, d, J 7.6 Hz), 5.06 (2H, s), 3.08 (3H, s), 2.84 (3H, s), 2.36 (2H, dd, J 7.5, 6.4 Hz), 2.30 (3H, s), 2.27 (3H, s), 1.10 (3H, dd, J 7.5, 7.6 Hz). ESI MS calculated for $C_{20}H_{25}N_2O_4S$ $[M + H]^+$: 389.2, found: 388.9.

*Hexadecanoic acid [3 (2,5 dimethyl benzyloxy) 4 (methanesulfonyl methyl amino) phenyl] amide (**13**).* Hexadecanoyl chloride was used and it was stirred at room temperature overnight. White solid, melting point 134–140 °C(decomposed), yield 98%: ^{1}H NMR (400 MHz, DMSO d_6) δ 10.03 (1H, s), 7.59 (1H, s), 7.28 (1H, s), 7.20 (4H, m), 5.06 (2H, s), 3.08 (3H, s), 2.84 (3H, s), 2.30 (3H, s), 2.27 (3H, s), 1.58 (2H, br), 1.23 (26H, br), 0.87 (3H, dd, J 5.1, 6.6 Hz).

*2,4 Dichloro N [3 (2,5 dimethyl benzyloxy) 4 (methanesulfonyl methyl amino) phenyl] benzamide (**14**).* 2,4 Dichloro benzoyl chloride was used and it was stirred at room temperature overnight. White solid, melting point 163–166 °C, yield 80%: ^{1}H NMR (400 MHz, DMSO d_6) δ 10.68 (1H, s), 7.79 (1H, s), 7.61 (1H, s), 7.64 (1H, d, J 8.2 Hz), 7.59 (1H, d, J 8.4 Hz), 7.29 (3H, br), 7.14(1H, d, J 7.4 Hz), 7.08 (1H, d, J 7.4 Hz), 5.09 (2H, s), 3.12 (3H, s), 2.87 (3H, s), 2.31 (3H, s), 2.27 (3H, s). ESI MS calculated for $C_{24}H_{23}Cl_2N_2O_4S$ $[M + H]^+$: 505.1, found: 504.8.

*N [3 (2,5 dimethyl benzyloxy) 4 (methanesulfonyl methyl amino) phenyl] 3 trifluoromethyl benzamide (**15**).* 3 Trifluoromethyl benzoyl chloride was used and it was stirred at room temperature overnight. White solid, melting point 179–181 °C, yield 93%: ^{1}H NMR (400 MHz, DMSO d_6) δ 10.61 (1H, s), 8.30 (2H, m), 8.00 (1H, d, J 7.6 Hz), 7.81 (2H, m), 7.44 (1H, d, J 8.5 Hz), 7.32(1H, s), 7.32 (1H, d, J 8.4 Hz), 7.15(1H, d, J 7.6 Hz), 7.09 (1H, d, J 7.6 Hz), 5.11 (2H, s), 3.12 (3H, s), 2.87 (3H, s), 2.32 (3H, s), 2.28 (3H, s). ESI MS calculated for $C_{25}H_{24}F_3N_2O_4S$ $[M + H]^+$: 505.1, found: 504.9.

*N [3 (2,5 dimethyl benzyloxy) 4 (methanesulfonyl methyl amino) phenyl] 3,4 dimethoxy benzamide (**16**).* 3,4 Dimethoxy benzoyl chloride was used and it was stirred at room temperature overnight. White solid, melting point 184–186 °C, yield 93%: IR ν 3352 (N–H), 1649 (C=O), 1599, 1505 (aromatic C=C). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.025 (2H, m), 7.510 (1H, J 2 Hz), 7.434 (1H, dd, J 8.4, 2 Hz), 7.309 (1H, J 8.8 Hz), 7.162 (1H, s), 7.109 (2H, m), 6.919 (1H, d, J 8.4 Hz), 6.832 (1H, dd, J 2, 8.4 Hz), 5.067 (2H, s), 3.957 (3H, s), 3.952 (3H, s), 3.195 (3H, s), 2.707 (3H, s), 2.335 (3H, s), 2.320 (3H, s). ^{13}C NMR (100 MHz, DMSO d_6) δ 164.933, 155.138, 151.682, 148.242, 140.278, 134.709, 134.099, 133.130, 130.865, 130.010, 129.194, 128.683, 126.616, 124.457, 120.948, 112.312, 110.955, 110.749, 105.355, 68.114, 55.588, 55.527, 37.608, 37.516, 20.520, 17.873. ESI MS calculated for $C_{26}H_{29}N_2O_6S$$[M + H]^+$: 497.2, found: 496.9.

*N [3 (2,5 dimethyl benzyloxy) 4 (methanesulfonyl methyl amino) phenyl] (3,4 dimethoxyphenyl) acetamide (**17**).* (3,4 Dimethoxy phenyl) acetyl chloride was used and it was stirred at room temperature overnight. White solid, melting point 149–152 °C, yield 95%: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.844 (1H, d, J 2.4 Hz), 7.240 (2H, m), 7.141 (1H, s), 7.103 (2H, m), 6.893 (2H, m), 6.825 (1H, d, J 1.6 Hz), 6.555 (1H, dd, J 2.4, 8.4 Hz), 5.029 (2H, s), 3.907 (3H, s), 3.898 (3H, s), 3.696 (2H, s), 3.157 (3H, s) 2.683 (3H, s), 2.322 (3H, s), 2.312 (3H, s). ESI MS calculated for $C_{27}H_{31}N_2O_6S$ $[M + H]^+$: 511.2, found: 510.9.

*N [3 (2,5 dimethyl benzyloxy) 4 (methanesulfonyl methyl amino) phenyl] 4 methoxy benzamide (**18**).* 4 Methoxy benzoyl chloride was used and it was stirred at room temperature overnight. White solid, melting point 175–178 °C, yield 96%: IR ν 3345 (N–H), 1649 (C=O), 1602, 1506 (aromatic C=C). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.022 (1H, d, J 2.4 Hz), 7.864 (3H, m), 7.330 (1H, d, J 8.4 Hz), 7.175 (1H, s), 7.113 (2H, m), 6.995 (2H, m), 6.823 (1H, dd, J 2.4, 8.4 Hz), 5.086 (2H, s), 3.886 (3H, s), 3.198 (3H, s), 2.712 (3H, s), 2.345 (3H, s), 2.325 (3H, s). ^{13}C NMR (100 MHz, DMSO d_6) δ 164.887, 161.920, 155.107, 140.324, 134.694, 134.091, 133.107, 130.857, 129.995, 129.552, 129.171, 128.660, 126.600, 124.380, 113.541, 112.190, 105.264, 68.083, 55.351, 37.593, 37.509, 20.513, 17.866. ESI MS calculated for $C_{25}H_{27}N_2O_5S$ $[M + H]^+$: 467.2, found: 466.9.

*N [3 (2,5 dimethyl benzyloxy) 4 (methanesulfonyl methyl amino) phenyl] 2 methoxy benzamide (**19**).* 2 Methoxy benzoyl chloride was used and it was stirred at room temperature overnight. White solid, melting point 147–149 °C, yield 97%: ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.932 (1H, s), 8.283 (1H, dd, J 2, 8 Hz), 8.202 (1H, d, J 2.4 Hz), 7.535 (1H, m), 7.342 (1H, d, J 8.4 Hz), 7.106 (5H, m), 6.759 (1H, dd, J 2, 8.4 Hz), 5.119 (2H, s), 4.079 (3H, s), 3.201

(3H, s), 2.706 (3H, s), 2.361 (3H, s), 2.329 (3H, s). ESI MS calculated for $C_{25}H_{27}N_2O_5S$ $[M + H]^+$: 467.2, found: 466.9.

N [3 (2,5 dimethyl benzyloxy) 4 (methanesulfonyl methyl amino) phenyl] 3 methoxy benzamide (***20***). 3 Methoxy benzoyl chloride was used and it was stirred at room temperature over night. White solid, melting point 143–144 °C, yield 91%: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.021 (1H, d, J 2.4 Hz), 7.938 (1H, s), 7.445 (1H, m), 7.406 (2H, m), 7344 (1H, d, J 8.4 Hz), 7.177 (1H, s), 7.121 (3H, m), 6.848 (1H, dd, J 2.4, 8.4 Hz), 5.093 (2H, s), 3.885 (3H, s), 3.201 (3H, s), 2.714 (3H, s), 2.349 (3H, s), 2.327 (3H, s). ESI MS calculated for $C_{25}H_{27}N_2O_5S$ $[M + H]^+$: 467.2, found: 466.9.

N [3 (2,5 dimethyl benzyloxy) 4 (methanesulfonyl methyl amino) phenyl] 3 methoxy 2,4,5 trifluorobenzamide (***21***). 3 Meth oxy 2,4,5 trifluorobenzoyl chloride was used and it was stirred at room temperature overnight. White solid, melting point 166–169 °C, yield 98%: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.381 (1H, d, J 14.8 Hz), 7.927 (1H, s), 7.680 (1H, m), 7.362 (1H, d, J 8.4 Hz), 7.180 (1H, s), 7.115 (2H, m), 6.880 (1H, d, J 8.4 Hz), 5.094 (2H, s), 4.106 (3H, s), 3.199 (3H, s), 2.719 (3H, s), 2.358 (3H, s), 2.328 (3H, s). ESI MS calculated for $C_{25}H_{24}F_3N_2O_5S$ $[M + H]^+$: 521.1, found: 520.9.

N [3 (2,5 dimethyl benzyloxy) 4 (methanesulfonyl methyl amino) phenyl] 2 ethyl butyramide (***22***). 2 Ethyl butyryl chloride was used and it was stirred at room temperature overnight. White solid, melting point 151–154 °C, yield 96%: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.049 (1H, d, J 2.4 Hz), 7.296 (1H, s), 7.275 (1H, s), 7.157 (1H, s), 7.108 (2H, m), 6.673 (1H, dd, J 2.4, 8.8 Hz), 5.064 (2H, s), 3.172 (3H, s), 2.691(3H, s), 2.339 (3H, s), 2.318 (3H, s), 2.08 (1H, m), 1.70 (4H, m), 0.970 (6H, t, J 7.6 Hz). ESI MS calculated for $C_{23}H_{31}N_2O_4S$ $[M + H]^+$: 431.2, found: 430.9.

N [3 (2,5 dimethyl benzyloxy) 4 (methanesulfonyl methyl amino) phenyl] cyclopentanecarboxamide (***23***). Cyclopentane carb onyl chloride was used and it was stirred at room temperature overnight. White solid, melting point 170–172 °C, yield 93%: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.998 (1H, d, J 2 Hz), 7.357 (1H, s), 7.265 (1H, d, J 8.4 Hz), 7.149 (1H, s), 7.106 (2H, m), 6.641 (1H, dd, J 2.4, 8.4 Hz), 5.045 (2H, s), 3.169 (3H, s), 2.703 (1H, m), 2.690 (3H, s), 2.327 (3H, s), 2.318 (3H, s), 1.921 (4H, m), 1.80 (2H, m), 1.641(2H, m). ESI MS calculated for $C_{23}H_{29}N_2O_4S$ $[M + H]^+$: 429.2, found: 428.9.

N [3 (2,5 dimethyl benzyloxy) 4 (methanesulfonyl methyl amino) phenyl] 2 phenyl acetamide (***24***). Phenylacetyl chloride was used and it was stirred at room temperature overnight. White solid, melting point 185–186 °C, yield 99%: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.867 (1H, d, J 2.4 Hz), 7.399 (5H, m), 7.239 (1H, d, J 8.4 Hz), 7.187 (1H, s), 7.102 (3H, m), 6.535 (1H, dd, J 3, 9 Hz), 5.026 (2H, s), 3.757 (2H, s), 3.156 (3H, s), 2.679 (3H, s), 2.320 (3H, s), 2.312 (3H, s). ESI MS calculated for $C_{25}H_{27}N_2O_4S$ $[M + H]^+$: 451.2, found: 450.9.

N [3 (2,5 dimethyl benzyloxy) 4 (methanesulfonyl methyl amino) phenyl] 1 naphthalenecarboxyamide (***25***). 1 Naphthoyl chloride was used and it was stirred at room temperature over night. White solid, melting point 164–168 °C, yield 83%: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.350 (1H, d, J 8 Hz), 8.105 (1H, s), 7.992 (1H, d, J 8.4 Hz), 7.921 (1H, m), 7.865 (1H, s), 7.741 (1H, d, J 6.4 Hz), 7.543 (3H, m), 7.345 (1H, d, J 8.4 Hz), 7.199 (1H, s), 7.116 (2H, m), 6.845 (1H, dd, J 2, 8.4 Hz), 5.129 (2H, s), 3.208 (3H, s), 2.711 (3H, s), 2.365 (3H, s), 2.336 (3H, s). ESI MS calculated for $C_{28}H_{29}N_2O_4S$ $[M + H]^+$: 489.2, found: 489.1.

N [3 (2,5 dimethyl benzyloxy) 4 (methanesulfonyl methyl amino) phenyl] 3,5 bis(trifluoro methyl)benzamide (***26***). 3,5 Bis (trifluoro methyl)benzoyl chloride was used and it was stirred at room temperature overnight. White solid, melting point 191–192 °C, yield 93%: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.348 (3H, s), 8.067 (1H, s), 7.892 (1H, s), 7.289 (1H, d, J 10.8 Hz), 7.173 (1H, s), 7.108 (2H, m), 6.908 (1H, d, J 8.4 Hz), 5.051 (2H, s), 3.193(3H, s), 2.754 (3H, s), 2.342 (3H, s), 2.309 (3H, s). ESI MS calculated for $C_{26}H_{23}F_6N_2O_4S$ $[M + H]^+$: 573.1, found: 572.9.

N [3 (2,5 dimethyl benzyloxy) 4 (methanesulfonyl methyl amino) phenyl] 3 bromobenzamide (***27***). 3 Bromobenzoyl chloride was used and it was stirred at room temperature overnight. White solid, melting point 176–178 °C, yield 99%: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.022 (2H, m), 7.947 (1H, d, J 2.4 Hz)), 7.810 (1H, d, J 8 Hz), 7.698 (1H, d, J 8 Hz), 7.404 (1H, t, J 8 Hz), 7.320 (1H, d, J 8.4 Hz), 7.172 (1H, s), 7.114 (2H, m), 6.867 (1H, dd, J 2.4, 8.4 Hz), 5.072 (2H, s), 3.199 (3H, s), 2.726 (3H, s), 2.343 (3H, s), 2.322 (3H, s). ESI MS calculated for $C_{24}H_{24}BrN_2O_4S$ $[M + H]^+$: 515.1, found: 514.8.

N [3 (2,5 dimethyl benzyloxy) 4 (methanesulfonyl methyl amino) phenyl] 4 chlorobenzamide (***28***). 4 Chloro benzoyl chlo ride was used and it was stirred at room temperature overnight. White solid, melting point 171–174 °C, yield 99%: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.967 (1H, d, J 2.4 Hz), 7.951 (1H, s), 7.838 (2H, m), 7.491 (2H, m), 7.335 (1H, d, J 8.4 Hz), 7.176 (1H, s), 7.111 (2H, m), 6.850 (1H, dd, J 2.4, 8.4 Hz), 5.084 (2H, s), 3.204 (3H, s), 2.725 (3H, s), 2.348 (3H, s), 2.326 (3H, s). ESI MS calculated for $C_{24}H_{24}ClN_2O_4S$ $[M + H]^+$: 471.1, found: 470.8.

N [3 (2,5 dimethyl benzyloxy) 4 (methanesulfonyl methyl amino) phenyl] 4 iodobenzamide (***29***). 4 Iodo benzoyl chloride was used and it was stirred at room temperature overnight. White solid, melting point 207–209 °C, yield 96%: IR ν 3308 (N–H), 1653 (C=O), 1594, 1518 (aromatic C=C). ^{1}H NMR (400 MHz, DMSO d_6) δ 10.408 (1H, s), 7.939 (2H, m), 7.748 (3H, m), 7.398 (1H, dd, J 2.4, 8.8 Hz), 7.291 (2H, m), 7.124 (2H, m), 5.090 (2H, s), 3.105(3H, s), 2.858 (3H, s), 2.311 (3H, s), 2.270 (3H, s). ^{13}C NMR (100 MHz, DMSO d_6) δ 164.811, 155.130, 139.912, 137.204, 134.686, 134.046, 133.954, 133.092, 130.926, 129.995, 129.514, 129.133, 128.660, 124.762, 112.328, 105.386, 99.474, 68.106, 37.615, 37.486, 20.513, 17.858. ESI MS calculated for $C_{24}H_{24}IN_2O_4S$ $[M + H]^+$: 563.1, found: 562.8.

N [3 (2,5 dimethyl benzyloxy) 4 (methanesulfonyl methyl amino) phenyl] 4 methylsulfanyl benzamide (***30***). 4 Methylsulfanyl benzoyl chloride was used and it was stirred at room temperature overnight. White solid, melting point 195–197 °C, yield 92%: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.017 (1H, d, J 2.4 Hz), 7.868 (1H, s), 7.799 (2H, m), 7.327 (3H, m), 7.177 (1H, s), 7.116 (2H, m), 6.834 (1H, dd, J 2, 8.4 Hz), 5.093 (2H, s), 3.200 (3H, s), 2.715 (3H, s), 2.543 (3H, s), 2.348 (3H, s), 2.326 (3H, s). ESI MS calculated for $C_{25}H_{27}N_2O_4S_2$ $[M + H]^+$: 483.1, found: 483.0.

N [3 (2,5 dimethyl benzyloxy) 4 (methanesulfonyl methyl amino) phenyl] 4 dimethylamino benzamide (***31***). 4 Dimethylamino benzoyl chloride was used and it was stirred at room temperature overnight. White solid, melting point 171–172 °C, yield 66%: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.086 (1H, d, J 2.4 Hz), 7.823 (1H, s), 7.789 (2H, d, J 9.2 Hz), 7.321 (1H, d, J 8.4 Hz), 7.174 (1H, s), 7.110 (2H, m), 6.790 (1H, dd, J 2, 8.4 Hz), 6.720 (2H, d, J 9.2 Hz), 5.089 (2H, s), 3.194 (3H, s), 3.064 (6H, s), 2.703 (3H, s), 2.343 (3H, s), 2.324 (3H, s). ESI MS calculated for $C_{26}H_{30}N_3O_4S$ $[M + H]^+$: 480.2, found: 479.9.

N [3 (2,5 dimethyl benzyloxy) 4 (methanesulfonyl methyl amino) phenyl] 4 ethoxy benzamide (***32***). 4 Ethoxy benzoyl chlo ride was used and it was stirred at room temperature overnight. White solid, melting point 184–185 °C, yield 70%: ^{1}H NMR

(400 MHz, $CDCl_3$) δ 8.014 (1H, d, J 2 Hz), 7.925 (1H, s), 7.847 (2H, m), 7.316 (1H, d, J 8.4 Hz), 7.168 (1H, s), 7.110 (2H, m), 6.972 (2H, m), 6.822 (1H, dd, J 2.4, 8.4 Hz), 5.074 (2H, s), 4.111 (2H, q, J 7.2 Hz), 3.195 (3H, s), 2.708 (3H, s), 2.340 (3H, s), 2.320 (3H, s), 1.455 (3H, t, J 7.2 Hz). ESI MS calculated for $C_{26}H_{29}N_2O_5S$ $[M + H]^+$: 481.2, found: 480.9.

*N [3 (2,5 dimethyl benzyloxy) 4 (methanesulfonyl methyl amino) phenyl] 4 ethyl benzamide (**33**).* 4 Ethyl benzoyl chloride was used and it was stirred at room temperature overnight. Pale yellow solid, melting point 178–181 °C, yield 64%: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.038 (1H, d, J 2.4 Hz), 7.987 (1H, s), 7.814 (2H, m), 7.327 (3H, m), 7.173 (1H, s), 7.112 (2H, m), 6.834 (1H, dd, J 2.4, 8.4 Hz), 5.083 (2H, s), 3.197 (3H, s), 2.733 (2H, q, J 7.6 Hz), 2.708 (3H, s), 2.343 (3H, s), 2.324 (3H, s), 1.276 (3H, t, J 7.6 Hz). ESI MS calculated for $C_{26}H_{29}N_2O_4S[M + H]^+$: 465.2, found: 464.9.

*N [3 (2,5 dimethyl benzyloxy) 4 (methanesulfonyl methyl amino) phenyl] 4 trifluoromethyl benzamide (**34**).* 4 Trifluorome thyl benzoyl chloride was used and it was stirred at room temperature overnight. White solid, melting point 215–218 °C, yield 84%: ^{1}H NMR (400 MHz, DMSO d_6) δ 10.590 (1H, s), 8.157 (2H, d, J 8 Hz), 7.941 (2H, d, J 8 Hz), 7.753 (1H, d, J 2.4 Hz), 7.419 (1H, dd, J 2.4, 8.8 Hz), 7.307 (2H, m), 7.129 (2H, m), 5.107 (2H, s), 3.116 (3H, s), 2.871 (3H, s), 2.319 (3H, s), 2.275 (3H, s). ESI MS calculated for $C_{25}H_{24}F_3N_2O_4S$ $[M + H]^+$: 505.1, found: 504.9.

*N [3 (2,5 dimethyl benzyloxy) 4 (methanesulfonyl methyl amino) phenyl] 4 trifuoromethoxy benzamide (**35**).* 4 Trifluorome thoxy benzoyl chloride was used and it was stirred at room temperature overnight. White solid, melting point 194–197 °C, yield 79%: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.954 (4H, m), 7.350 (3H, m), 7.177 (1H, s), 7.117 (2H, m), 7.848 (1H, dd, J 2.4, 8.4 Hz), 5.088 (2H, s), 3.204 (3H, s), 2.724 (3H, s), 2.349 (3H, s), 2.324 (3H, s). ESI MS calculated for $C_{25}H_{24}F_3N_2O_5S$ $[M + H]^+$: 521.1, found: 520.9.

*N [3 (2,5 dimethyl benzyloxy) 4 (methanesulfonyl methyl amino) phenyl] 4 methyl benzamide (**36**).* 4 Methyl benzoyl chlo ride was used and it was stirred at room temperature overnight. White solid, melting point 170–173 °C, yield 69%: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.032 (1H, d, J 2 Hz), 7.956 (1H, s), 7.786 (2H, m), 7.339 (1H, s), 7.309 (2H, d, J 7.6 Hz), 7.175 (1H, s), 7.113 (2H, m), 6.834 (1H, dd, J 2, 8.4 Hz), 5.084 (2H, s), 3.198 (3H, s), 2.710 (3H, s), 2.438 (3H, s), 2.344 (3H, s), 2.324 (3H, s). ESI MS calculated for $C_{25}H_{27}N_2O_4S$ $[M + H]^+$: 451.2, found: 450.9.

*N [3 (2,5 dimethyl benzyloxy) 4 (methanesulfonyl methyl amino) phenyl] furan 2 carboxamide (**37**).* 2 Furoyl chloride was used and it was stirred at room temperature overnight. White solid, melting point 156–157 °C, yield 66%: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.159 (1H, s), 8.004 (1H, d, J 2.4 Hz), 7.551 (1H, m), 7.352 (1H, d, J 8.4 Hz), 7.268 (1H, m), 7.180 (1H, s), 7.119 (2H, m), 6.875 (1H, dd, J 2.4, 8.8 Hz), 6.595 (1H, dd, J 2, 3.6 Hz), 5.091 (2H, s), 3.202 (3H, s), 2.720 (3H, s), 2.351 (3H, s), 2.328 (3H, s). ESI MS calculated for $C_{22}H_{23}N_2O_5S$ $[M + H]^+$: 427.1, found: 426.8.

*N [3 (2,5 dimethyl benzyloxy) 4 (methanesulfonyl methyl amino) phenyl] thiophene 2 carboxamide (**38**).* 2 Thiophene carbonyl chloride was used and it was stirred at room temperature overnight. White solid, melting point 163–164 °C, yield 72%: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.986 (1H, d, J 2.4 Hz), 7.877 (1H, s), 7.668 (1H, dd, J 1.2, 3.6 Hz), 7.589 (1H, dd, J 1.2, 5.2 Hz), 7.323 (1H, d, J 8.4 Hz), 7.144 (4H, m), 6.816 (1H, dd, J 2.4, 8.8 Hz), 5.072 (2H, s), 3.197 (3H, s), 2.718 (3H, s), 2.341(3H, s), 2.324 (3H, s). ESI MS calculated for $C_{22}H_{23}N_2O_4S_2[M + H]^+$: 443.1, found: 442.8.

*N [3 (2,5 dimethyl benzyloxy) 4 (methanesulfonyl methyl amino) phenyl] isoxazole 5 carboxamide (**39**).* Isoxazole 5 carbonyl chloride was used and it was stirred at room temperature over night. White solid, melting point 130–138 °C(decomposed), yield 99%: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.418 (1H, d, J 2 Hz), 8.360 (1H, s), 7.849 (1H, dd, J 2.4 Hz), 7.391 (1H, d, J 8.4 Hz), 7.181 (1H, s), 7.126 (2H, m), 7.060 (1H, d, J 2 Hz), 7.005 (1H, dd, J 2.4, 8.4 Hz), 5.095 (2H, s), 3.211 (3H, s), 2.741 (3H, s), 2.357 (3H, s), 2.329 (3H, s). ESI MS calculated for $C_{21}H_{22}N_3O_5S$ $[M + H]^+$: 428.1, found: 427.8.

Pharmacological studies

Cell culture

SKBR 3, HT29 cells were obtained from ATCC (Rockville, MD). All cells were maintained in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mmol/L ʟ Glutamine, 1 mmol/L sodium pyruvate, 100 U/mL penicillin streptomycin. FBS was heat inactivated for 30 min in a 56 °C water bath before use. Cell cultures were grown at 37 °C, in a humidified atmosphere of 5% CO_2 in a Hereaus CO_2 incubator.

Cell viability analysis

The effect of nimesulides derivatives on SKBR 3 and HT29 cell viability was assessed by using the 3 (4,5 dimethylthiazol 2 yl) 2,5 diphenyl 2H tetrazolium bromide assay in six replicates. Cells were grown in RPMI1640 medium in 96 well, flat bottomed plates for 24 h, and were exposed to various concentrations of JCC76 derivatives dissolved in DMSO (final concentration $\leq$0.1%) in media for 48 h. Controls received DMSO vehicle at a concentration equal to that in drug treated cells. The medium was removed, replaced by 200 μl of 0.5 mg/mL of 3 (4,5 dimethylthiazol 2 yl) 2,5 diphenyl 2H tetrazolium bromide in fresh media, and cells were incubated in the CO2 incubator at 37 °C for 2 h. Supernatants were removed from the wells, and the reduced 3 (4,5 dimethylthiazol 2 yl) 2,5 diphenyl 2H tetrazolium bromide dye was solubilized in 200 μl/ well DMSO. Absorbance at 570 nm was determined on a plate reader.

Flow cytometry analysis

For all the assays, cells were treated for the indicated time. To analyze the cell cycle profile, treated cells were fixed overnight with 70% EtOH at 20 °C and stained with propidium iodide buffer [38 mM sodium citrate (pH 7.5), 69 μM propidium iodide, and 120 μg/mL RNase A]. Samples were mixed gently and incubated at room temperature in the dark for 15 min. Immediately before analysis by flow cytometry, 400 μL binding buffer was added to each sample. A total of 1.2×10^4 cells were acquired for each sample and a maximum of 1×10^4 cells within the gated region were analyzed.

In vivo xenograft studies

Five to six week old BALB/c *nu/nu*, athymic, mice were purchased from Charles River Laboratories, Inc. (Wilmington, MA). Subconfluent HT29 cells were harvested from monolayer culture and resuspended in an equal volume of Matrigel (BD Biosciences, San Jose, CA) to a final concentration of 1×10^7/0.2 mL. At 10 weeks of age, each animal received s.c. inoculations in two sites per flank with 200 μL of HT29 cell suspension. Six animals were randomly grouped into two. Tumors were measured twice weekly with calipers, and tumor volume was calculated by the following formula: $(\text{width})^2 \times \text{length}/2$. Treatments began when the tumors reached a measurable size ($\sim$200 mm^3). Treated group was intra peritoneal administrated 5 mg/kg/d of CSUOH0901 five times a week (1%DMSO, sesame oil as vehicle). The control group received the vehicle treatment. Body weights were monitored weekly as an indicator of the animals' overall health. After three weeks of

treatment, the mice were euthanized and the tumors were removed, weighed.

Preparation of calibration standards and quality controls (QC) samples

The stock standard solutions of CSUOH0901 were prepared by dissolving in DMSO it at 1 mg/mL and stored at −20 °C. One set of CSUOH0901 working solutions at 3, 10, 20, 100, 200 and 1000 ng/mL, prepared by serial diluting stock solution with 50% acetonitrile in water, was used for calibration standards. Another set of JCC76 working solutions at 3, 9, 90, and 900 ng/mL was made in the similar way and used for QC samples. All of the working solutions were freshly prepared before use. Calibration standards were prepared by spiking 5 μL different CSUOH0901 working solutions into 50 μL blank rat plasma to give the final concentration of CSUOH0901 at 0.3, 1, 2, 10, 2 and 100 ng/mL. The QC samples were prepared in same way as the calibration standards at four different levels of 0.3, 0.9, 9 and 90 ng/mL, representing lower limit of quantitation (LLOQ), low QC (LQC), middle QC (MQC) and high QC (HQC) of CSUOH0901 in plasma. All of the calibration standards and QC samples were further treated in the same preparation procedure.

Pharmacokinetic study

Male Sprague–Dawley rats (each weight 300–350 g) were purchased from Charles River Laboratories International (Spencer ville, OH, USA). Animals were housed in a 12 h light/dark cycle room with free access to food and water for at least 7 days to adapt the environment. All the animal experiment procedures were per formed under the guideline approved by Institutional Animal Care and Use Committee at Cleveland State University.

Before the intraperitoneal administration of CSUOH0901 at a single dose of 20 mg/kg (PBS with 01% Tween80, 20% DMSO as vehicle), animals were fasted overnight but with free access of water. Blood samples of 150 μL each were collected from the saphenous veins and femoral veins into heparized tubes at 0 h (before drug administration) and at 0.25, 0.5, 1, 2, 4, 8 and 24 h after dosing. The blood samples were centrifuged immediately at 10,000 rpm for 5 min in room temperature. The plasma samples were separated and store at −20 °C until analysis.

Plasma concentrations of CSUOH0901 were determined by an LC–MS/MS method developed and validated for this study. The LC MS/MS method consisted of a Shimazu HPLC system ((Shimazu, Tokyo, Japan) and an AB Sciex QTrap 5500 mass spectrometer equipped with an electrospray ionization source (ABI Sciex, Tor onto, Canada). The chromatographic separation was achieved using a C 18 column (2.0 mm × 150 mm, 5 μm) together with 0.5 mM ammonium formate in 90% methanol for isocratic elution. The eluates were detected using multiple reaction monitoring (MRM) mode for CSUOH0901 (m/z 483.3 to 404.3) and the internal stan dard JCC76 (m/z 445 to 366.3). The liquid–liquid extraction method using the mixture of *tert* butyl methyl ether and *n* hexane (1:1, v/v) was optimized for plasma sample pretreatment. This analytical method was validated over the concentration range of 0.1–100 ng/mL ($r2 \geq 0.999$). The intra and inter assay variability (% coefficient of variation) and mean bias (% relative error) were evaluated and less than 15%. Plasma quantified above the highest concentration of the standard curve were diluted with blank plasma and reanalyzed.

The concentration of CSUOH0901 in rat plasma versus time profiles were analyzed to estimate pharmacokinetics parameters using WinNonlin® software version 5.2 (Pharsight Corporation, Mountain View, CA, USA). The estimated pharmacokinetic param eters including the terminal phase elimination half life ($T_{1/2}$), and the area under the plasma concentration time curve (AUC) from time 0 to time of the last measurable concentration ($AUC_{(0\text{-}t)}$), the volume of distribution ($V_{z/F)}$, the total body clearance ($Cl_{/F}$), and the mean residence time (MRT) from time 0 to time of the last measurable concentration ($MRT_{(0\text{-}t)}$) were determined.

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Appendix. Supplementary material

Supplementary data related to this article can be found online at doi:10.1016/j.ejmech.2011.11.012.

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