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PII: S1001-8417(15)00273-9
DOI: http://dx.doi.org/doi:10.1016/j.cclet.2015.06.017
Reference: CCLET 3374

To appear in: *Chinese Chemical Letters*

Received date: 24-3-2015
Revised date: 13-4-2015
Accepted date: 29-5-2015

Please cite this article as: C.-Y. Sun, Y.-S. Li, A.-L. Shi, Y.-F. Li, R.-F. Cao, H.-W. Ding, Q.-Q. Yin, L.-J. Zhang, H.-C. Zheng, H.-R. Song, Synthesis and antiproliferative activity of novel 4-substituted-phenoxy-benzamide derivatives, *Chinese Chemical Letters* (2015), http://dx.doi.org/10.1016/j.cclet.2015.06.017

Graphical Abstract

Synthesis and antiproliferative activity of novel 4-substituted-phenoxy-benzamide derivatives

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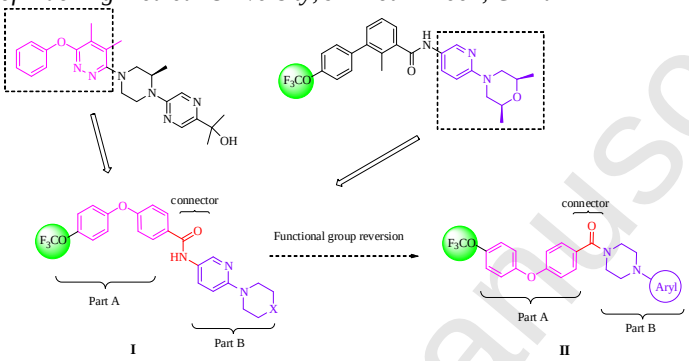

A series of 4-substituted-phenoxy-benzamide derivatives bearing an aryl cycloaliphatic amine moiety were synthesized and evaluated for their antiproliferative activity. Three compounds were further examined for their hedgehog pathway inhibition.

Original article

Synthesis and antiproliferative activity of novel 4-substituted-phenoxy-benzamide derivatives

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ARTICLE INFO

Article history:
Received 24 March 2015
Received in revised form 13 April 2015
Accepted 29 May 2015
Available online

Keywords:
aryl cycloaliphatic amine
antiproliferative activity
hedgehog signaling

ABSTRACT

A series of novel 4-substituted-phenoxy-benzamide derivatives bearing an aryl cycloaliphatic amine moiety were synthesized and evaluated for antiproliferative activity against SW620, HT29 and MGC803 cancer cell lines in vitro. The pharmacological data demonstrated that the majority of target compounds exhibited moderate efficacy in HT29 and MGC803 cell lines. Compound **10c** showed promising inhibition of hedgehog (Hh) signaling pathway in an Hh-related assay. In addition, the superposition pattern of **10c** showed a good fit for a pharmacophoric model generated by Hh inhibitors and provided a basis for further structural optimization.

1. Introduction

The Hedgehog (Hh) protein family, which includes Sonic (Shh), Indian (Ihh), and Desert (Dhh) hedgehogs, regulated cell growth and migration during embryonic development [1-3]. Activation of the Hh signaling pathway is initiated by Shh ligands bound to its receptor Patched (Ptch), which relieves its inhibition of Smoothen (Smo) receptor. Smo activation triggers a series of intracellular events ultimately leads to specific gene expression mediated by the Gli family transcription factors[4,5]. Hh signaling pathway was normally silent in adult tissues, nevertheless aberrant activation of the Hh pathway was associated with certain cancers. Thus, the blockade of Hh pathway had been investigated as a novel strategy in cancer chemotherapy [6,7].

Inhibition of Smo activity has shown some promise in the treatment of cancers driven by activating mutations of the Hh pathway [8-10]. Furthermore, several Hh pathway antagonists have proceeded to clinical development, among which vismodegib (**1**, Fig.1) has obtained marketing authorization in the United States in 2012 [11, 12]. Sonidegib (**2**, Fig.2), a clinical stage Hh inhibitor developed by Novartis, is awaiting for the registration in the U.S. for the treatment of patients with advanced basal cell carcinoma. Sonidegib (**2**) bearing a morpholinopyridine unit suppressed the growth of Hh pathway-dependent tumors by selective inhibition of the positive regulator smoothened (Smo) [13, 14]. LEQ506 (**3a**), a second-generation Hh inhibitor, is currently being investigated in a Phase I clinical trial for patients with solid tumors. SAR studies had demonstrated the replacement of the benzylic methylene linker with an oxygen atom (**3b**) was well tolerated, whereas replacement with an NH group (**3c**) resulted in a 10-fold decrease in inhibition of the Hh pathway [15].

Inspired by the structural characteristics of Sonidegib (**2**) and LEQ506 (**3a**), we envisioned that the merging of these two bioactive components would afford a hybrid structure with the potential for antiproliferative activity. We therefore adopted the biaryl ether (Part A) active fragment from LEQ506 analog (**3b**) and morpholino pyridine (Part B) unit from Sonidegib (**2**) in target compounds, and a carbonyl group or an amide group was selected as the linker between the two parts. Additionally, the order of heteroaryl or aryl cycloaliphatic amine units in the target compounds attaching to the linker was reversed based on the functional group reversion principle. Eventually, a series of 4-substituted-phenoxy-benzamide derivatives (I, II Fig.2) were obtained. In this paper, the synthesis of these benzamide derivatives was reported, and their biological activities were evaluated.

2. Experimental

The key intermediates **7a-d** were synthesized according to the routes outlined in Scheme 1. Aryl iodides were synthesized from aryl amines by a diazotization reaction. The generated diazonium salts were iodinated with KI to give compounds **5a-d** [16]. The

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substituted iodobenzenes **5a-d** were coupled to methylparaben to afford compounds **6a-d**, *via* an *N,N*-dimethyl glycine-promoted Ullmann coupling. Compounds **6a-d** were further hydrolyzed to compounds **7a-d** in excellent yields under reflux [17].

Compounds **10a-f** were synthesized according to the routes outlined in Scheme 2. 2-Chloro-5-nitropyridine was converted to compounds **8a-b** in the presence of morpholine or piperidine [18]. The resulting nitro compounds **8a-b** were reduced to the amino compounds **9a-b** using stannous chloride dihydrate and hydrochloric acid in aqueous ethanol, and then alkalized with sodium hydroxide solution [19]. **7a-d** were treated with thionyl chloride to produce the corresponding acyl chloride. **9a-b** reacted with acyl chloride to give the target compounds **10a-f** [20].

Compounds **14a-n**, **15a-b**, **16a-j** were synthesized according to the routes outlined in Scheme 3. Compound **11** was synthesized from 6-chloronicotinic acid *via* an esterification reaction and then converted to compound **12** using a substitution reaction [21]. Compound **12** was deprotected to give compound **13** under acid conditions [22]. Intermediate **13** was reacted with the corresponding acyl chlorides **7a-d** to give the target compounds **14a-d,** which were further hydrolyzed to compounds **15a-b**. Similarly, compounds **16a-j** were obtained by the reaction of the acyl chloride **7c** and various aryl piperazine [23].

General procedure for preparation of target compounds was given in the Supporting Information.

3. Results and discussion

All the target compounds (**10a-f**, **14a-d, 15a-b and 16a-j**) were evaluated for their antiproliferative activity against human colorectal cancer cell lines (SW620 and HT29), and human gastric cancer cell line (MGC803) using the MTT assay with vismodegib as a positive control. The results expressed as half maximal inhibitory concentration (IC_{50}) values are summarized in Table 1.

Initially, target compounds were divided into two regions: diaryl ether (Part A), aryl cycloaliphatic amine moiety (Part B). In general, most of them displayed high efficacy in HT29 and MGC803 cell lines. At the outset, our focus was on the modifications of Part A, including the substitutions on the para position of phenyl. The para-trifluoromethoxy substituent **10c** (IC_{50} = 1.15 μmol/L [HT29], IC_{50} = 0.56 μmol/L [MGC803]) showed decent activity, however, para-methoxy and para-chlorine led to a significant loss of activity (**10c** *vs* **10a**, **10d** or **14a** *vs* **14b**, **14d**) against MGC803 cells, confirming the beneficial impact of trifluoromethoxy in the R_1 position.

Further investigations focusing on Part B on the antiproliferative activity were performed. On comparing **10d** with **10f**, it was found that morpholino pyridine surrogates were superior to piperidyl pyridine surrogates. Through functional group reversion, pyridyl piperazidine analog **16c** (IC_{50} = 4.33 μmol/L [HT29], IC_{50} = 5.35 μmol/L [MGC803]) was obtained and exhibited moderate potencies. Addition of a polar methoxycarbonyl (**14a,** IC_{50} = 2.36 μmol/L [HT29]) group to pyridine increased the activity while its hydrolysate **15a** lost potency by nearly two-fold. When the pyridyl was changed to a pyrimidyl (**16b,** IC_{50} = 2.14 μmol/L [HT29]), a two-fold increase of activity was observed. However, the trend did not hold in other analogs, as unsubstituted phenyl piperazidine analogue **16j** was less potent. Attempts to increase the antiproliferative activity were made by adding various groups to the phenyl ring (Part B). It was worth noting that the para-fluorine (**16h**, IC_{50} =2.52 μmol/L [HT29]) and meta-methoxy (**16a**, IC_{50} =1.95 μmol/L [HT29]) analogues were much more active than other substituted phenyl piperazidine analogues. Overall, the structure-activity relationships (SAR) study revealed that the antiproliferative activity of **3** compounds (**10a**, **10c**, **10d**) was comparable to that of the positive control vismodegib (**1**) in HT29 and MGC803 cells.

As shown in **Table 2**, the selected compound **10c**, **10d**, **16c** were further tested for their Hh signaling inhibition using a luciferase reporter assay [24, 25] in NIH3T3 cells carrying a stably transfected Gli-reporter construct (NIH3T3-Gli-luc reporter cell line). This assay can effectively identify hedgehog signaling pathway inhibitors. It was suggested that compound **10c** (IC_{50}= 0.082 μmol/L) was less active as compared to the positive control vismodegib (IC_{50}= 0.013 μmol/L). In addition, **10c** and **10d** (IC_{50}= 0.127 μmol/L) showed higher potency than **16c** (IC_{50}= 0.364 μmol/L) in parallel to their antiproliferative activity. Although the hedgehog pathway inhibition of **10c** was less potent than that of vismodegib, these results were encouraging and worthy of further investigation owing to the large structural changes involved.

Then we examined if compound **10c** fit the proposed pharmacophoric model for Hh antagonists. A conformational analysis was performed using the Common feature program in the Discovery studio 3.0 software. This analysis was conducted on the three compounds covering conformers with a range of 20 kcal/mol with respect to the global minimum that was used in building the best pharmacophoric model. According to this, the low energy conformers of vismodegib (**1**), sonidegib (**2**) and LEQ506 (**3a**) showed a very similar orientation and fulfilled all the pharmacophoric features of the model.

This model was built up by two hydrogen bond acceptor (HBA1-2) groups and two hydrophobic (HY1-2) regions. Fitting of vismodegib (**1**), sonidegib (**2**) and LEQ506 (**3a**) to the pharmacophoric model was shown in Fig. 3A. Analysis of the superposition pattern of **10c** showed a good fit between the molecule and the pharmacophoric model (Fig. 3B). For instance, the carbonyl oxygen in the amide moiety of compound **10c** matched HBA1, and the oxygen atom in the morpholine moiety (Part B) corresponded to the HBA2 feature of the model. Moreover, the two phenyl rings in the diaryl ether moiety (Part A) were superimposed to the hydrophobic regions HY1 and HY2 perfectly.

4. Conclusion

In summary, a series of novel and distinctive 4-substituted-phenoxy-benzamide analogues were synthesized and characterized. Furthermore, three human cancer cell lines were used to evaluate their antiproliferative activity; the majority of analogues exhibited moderate activity and high selectivity in HT29 and MGC803 cell lines. In particular, the most promising compound **10c**

(Hh pathway inhibition IC_{50} = 0.082 μmol/L) displayed desirable antiproliferative activity with IC_{50} values of 1.15 μmol/L and 0.56 μmol/L against HT29 and MGC803 cell lines, respectively. The analysis of SAR indicated that compounds with a para-trifluoromethoxyl group on Part A were more potent than those with other substituents. In addition, the morpholino pyridine fragment in Part B exhibited higher efficacy as compared to the aryl piperazidine fragment. Further structural optimization studies are presently in progress and will be reported in due course.

Acknowledgment

The work was supported by Program for Innovative Research Team of the Ministry of Education of China and Program for Liaoning Innovative Research Team in University. Authors wish to thank to the Innovation and entrepreneurship training program for college students in Liaoning Province (No. 201410163009).

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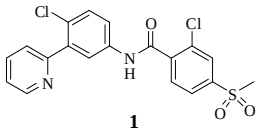

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Fig. 1 The structure of vismodegib (**1**)

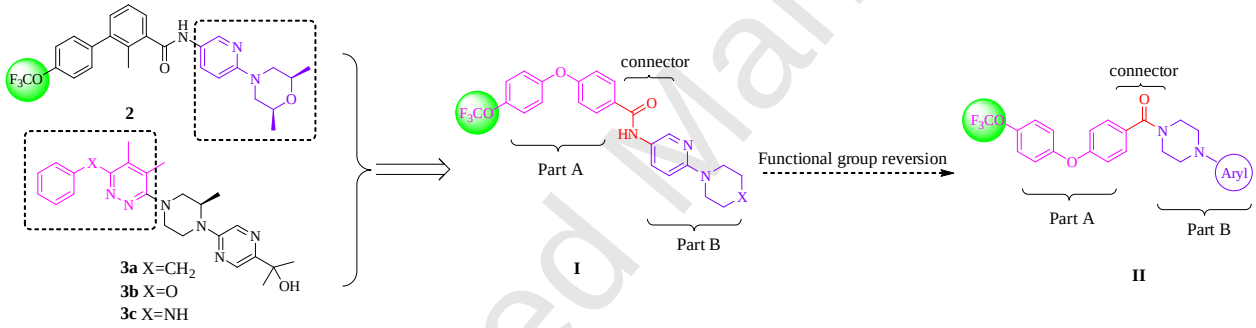
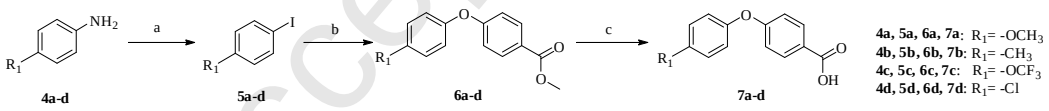
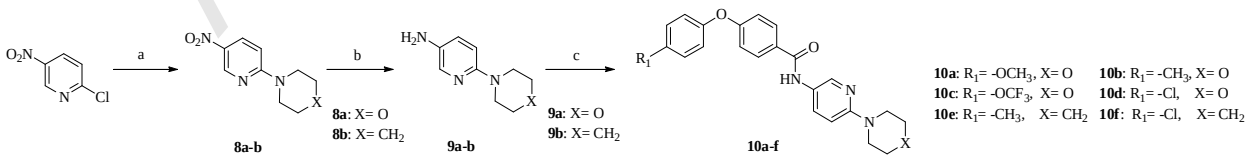


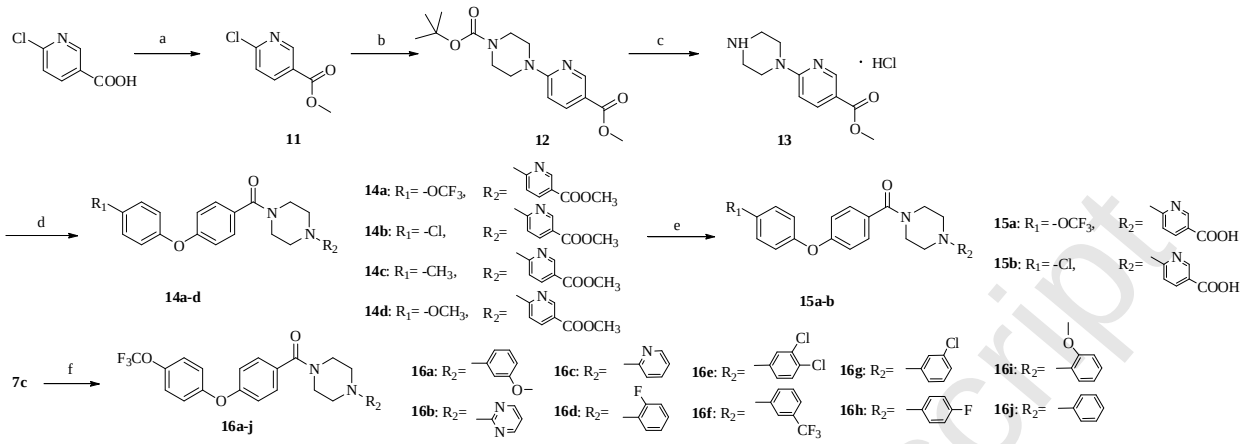
Fig. 2 Sonidegib (**2**), LEQ506 analogues (**3a-c**) and general structure of the target compounds (**I**, **II**).



Scheme 1 Reagents and conditions: (a) (1) $NaNO_2$, H_2SO_4, 0 °C, 2h; (2) KI, dichloromethane, 0 °C, 6h; (b) 4-methyl-1-iodobenzene, CuI, *N*,*N*-dimethylglycine, Cs_2CO_3, 1,4-dioxane, reflux under nitrogen atmosphere, 24h; (c) NaOH, ethanol solution, reflux, 3h



Scheme 2 Reagents and conditions: (a) morpholine or piperidine, K_2CO_3, THF, reflux, 4h; (b) $SnCl_2 \cdot 2H_2O$, ethanol, HCl, reflux, 8h; (c) acyl chloride of **7a-d**, Et_3N, dichloromethane, 0 °C, 12h.



Scheme3 Reagents and conditions: (a) Methanol, concentrated sulfuric acid, 65°C; (b) 1-boc-piperazine, K_2CO_3, DMAP, DMF, 110 °C, 24h; (c) concentrated hydrochloric acid in dioxane, dichloromethane 30 °C, 2h; (d) chloride of **7a-d**, Et_3N, dichloromethane, 0 °C, 4h; (e) NaOH, ethanol solution, reflux, 3h; (f) chloride of **7a-d**, aryl piperazidine or heteroaryl piperazidine, Et_3N, dichloromethane, 0 °C, 4h.

Table 1 *In vitro* cell growth inhibition by the target compounds and vismodegib (**1**).

Compd.	IC_{50} (μmol/L) ± SDa[a]			Compd.	IC_{50} (μmol/L) ± SDa[a]		
	SW620	HT29	MGC803		SW620	HT29	MGC803
10a	26.76 ± 3.56	1.87 ± 0.22	**2.36** ± 0.35	**15b**	26.07 ± 3.64	16.20 ± 4.60	13.29 ± 2.43
10b	79.49 ± 10.28	2.82 ± 0.73	3.08 ± 0.63	**16a**	12.56 ± 2.87	1.95 ± 0.62	2.91 ± 0.57
10c	**7.24** ± 0.80	1.15 ± 0.24	**0.56** ± 0.11	**16b**	10.71 ± 1.98	2.14 ± 0.49	2.63 ± 0.61
10d	23.19 ± 3.03	1.53 ± 0.50	**1.77** ± 0.48	**16c**	19.08 ± 3.62	4.33 ± 1.65	5.35 ± 1.40
10e	>100	4.41 ± 1.35	3.82 ± 1.02	**16d**	46.58 ± 7.41	14.58 ± 3.81	10.84 ± 2.59
10f	48.49 ± 6.17	3.45 ± 0.28	2.80 ± 0.91	**16e**	20.34 ± 3.53	8.26 ± 2.27	6.27 ± 0.82
14a	14.85 ± 3.12	2.36 ± 0.96	3.16 ± 0.87	**16f**	72.35 ± 9.60	34.93 ± 6.54	33.21 ± 5.88
14b	15.96 ± 4.25	3.88 ± 1.05	5.53 ± 1.66	**16g**	65.62 ± 9.75	26.31 ± 4.82	38.54 ± 5.76
14c	>100	16.20 ± 4.65	20.65 ± 2.90	**16h**	14.27 ± 2.82	2.52 ± 0.71	3.43 ± 0.94
14d	**5.19** ± 1.21	11.74 ± 3.03	9.98 ± 1.78	**16i**	51.83 ± 8.36	13.27 ± 2.37	14.18 ± 2.52
15a	57.31 ± 8.65	5.00 ± 1.07	9.19 ± 1.32	**16j**	32.95 ± 4.51	26.08 ± 4.12	25.36 ± 3.35
Vismodegib[b]	10.92 ± 2.76	1.08 ± 0.16	2.39 ± 0.17				

Bold values show the IC_{50} values of target compounds lower than the values of the positive control.
[a] IC_{50}: concentration of the compound (μmol/L) producing 50% cell growth inhibition after 48 h of drug exposure, as determined by the MTT assay. Each experiment was carried out in triplicate.
[b] Used as a positive control.

Table 2 The hedgehog pathway inhibition of compound **10c**, **10d**, **16c** and vismodegib (**1**).

Compd.	NIH3T3-Gli-luc IC_{50} (μmol/L)[a]
10c	0.082
10d	0.127
16c	0.364
vismodegib[b]	0.013

[a] The values are an average of triplicate separate determinations.
[b] Used as a positive control.

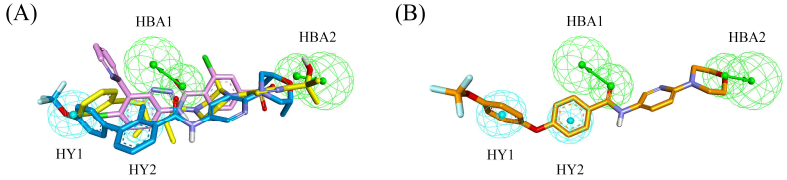


Fig. 3 A. Pharmacophore models for vismodegib, sonidegib and LEQ506. Graphical representation of vismodegib (pink), sonidegib (blue), LEQ506 (yellow) fitted to the proposed pharmacophoric model for hedgehog antagonists. Pharmacophoric features are color coded: green for hydrogen bond acceptor groups (HBA1-2) and cyan for hydrophobic regions (HY1-2). HBA features are constituted by a smaller sphere accommodating the hydrogen bond acceptor group, by a directionality vector represented by an arrow, and by a larger sphere intended to allocate the hydrogen bond donor group of the target macromolecule. B. Pharmacophore model for **10c**. The atoms are color coded: Orange, carbon; white, hydrogen; red, oxygen; blue, nitrogen