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Discovery of small molecule FLT3 inhibitors that are able to overcome drug-resistant mutations

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ABSTRACT

Herein we report the discovery of 1-(5-(tert-butyl)isoxazol-3-yl)-3- (3-fluorophenyl)urea derivatives as new FLT3 inhibitors that are able to overcome the drug resistance mutations: the secondary D835Y and F691L mutations on the basis of the internal tandem duplications (ITD) mutation of FLT3 (FLT3-ITD/D835Y and FLT3-ITD/F691L, respectively). The most potent compound corresponds to 1-(5-(tert-butyl)isoxazol-3-yl)-3-(4-((6,7-dimethoxyquinolin-4-yl)oxy)-3- fluorophenyl)urea (**4d**), which showed IC_{50}s (half maximal inhibitory concentrations) of 0.072 nM, 5.86 nM and 3.48 nM against FLT3-ITD, FLT3-ITD/F691L and FLT3-ITD/D835Y, respectively. Compound **4d** also showed good selectivity for FLT3 in a kinase profiling assay. Collectively, **4d** could be a good lead compound and deserves further in-depth studies.

Acute myeloid leukemia (AML) is a hematological malignancy caused by abnormal proliferation of myeloid hematopoietic progenitor cells in the bone marrow. Clinical data have shown that approximately 30% of patients have activating mutations of FMS-like tyrosine kinase 3 (FLT3), and the most prevalent of which is internal tandem duplications (ITD) mutation in the juxtamembrane domain of FLT3.[1–5] Numerous studies have indicated that FLT3 mutations are associated with a poor prognosis for overall survival.[6–9] FLT3 has thus been considered as a valid target for the treatment of AML.[10] Currently, a number of FLT3 inhibitors have been reported, and three of them, Midostaurin, Gilteritinib and Quizartinib (**AC220**), have been approved to clinical use for the treatment of AML.[11–19] Unfortunately, recent studies have shown that resistance to currently known FLT3 inhibitors emerged due to secondary point mutations in the kinase domain (K_D) of FLT3. The most common mutation sites are phenylalanine at position 691 (F691) and aspartic acid at position 835 (D835).[20–23] Drug discovery targeting the secondary mutations of FLT3-ITD has thus been an urgent task.

In a previous study, we obtained a potent FLT3 inhibitor, 1-(4-((*1H*-pyrazolo [3, 4-d]pyrimidin-4-yl)oxy)-3-fluorophenyl)-3-(5-(tert-butyl) isoxazol-3-yl)urea (Figure 1**a, SKLB-677**). This compound showed an excellent activity against FLT3-ITD mutant with an IC_{50} value of 1.3 nM.[24] It also showed activity against the secondary mutations D835Y and F691L of FLT3-ITD with IC_{50} values of 0.129 μM and 0.108 μM, respectively. Obviously, the potencies against these second mutations of FLT3-ITD are not good and need further improvement. We will in this investigation carry out a structural optimization to this compound to improve its potency against the second mutations of FLT3-ITD.

Our structural optimization will focus on the *1H*-pyrazolo [3,4-d] pyrimidine region (Figure 1 **b**) because 1-(5-(tert-butyl)isoxazol-3-yl)-3-(3-fluoro-4-hydroxyphenyl)urea has been determined as an optimal fragment in our previous study.[24] We synthesized a total of 22 derivatives (**4a**, **11a-o**) of compound **SKLB-677** with *1H*-pyrazolo[3, 4-*d*] pyrimidine substituted by different heteroaryl rings containing a pyridine or pyrimidine ring.

In the first step, we replaced *1H*-pyrazolo[3,4-*d*]pyrimidine with various pyridine (or pyrimidine) -fused five (or six) membered ring fragments and synthesized 7 compounds (**4a-g**). Synthetic routes for compounds **4a-g** are depicted in Scheme 1**.** Commercially available reagents **1a-g** reacted with 4-amino-2-fluorophenol through a nucleophilic substitution to give intermediate **2a-g**. Condensation reaction between **2a-g** and 5-(tert-butyl)-3-isocyanatoisoxazole (**3**) produced target compounds **4a-g**.

Bioactivities of compounds **4a-g** together with **AC220** and **SKLB-**

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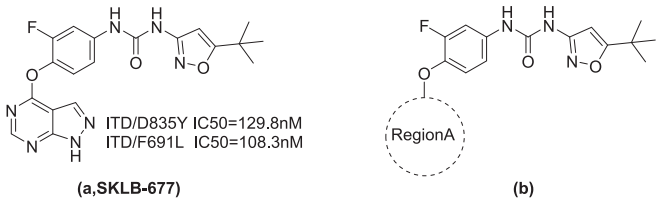


Fig. 1. (a) The chemical structure of compound **SKLB-677**; (b) Schematic showing the region being the focus of structural modification.

677 were then examined. Here cell-based bioactivity assays were adopted, in which Ba/F3 cells expressing FLT3-ITD, FLT3-ITD/D835Y and FLT3-ITD/F691L, as well as the parental Ba/F3 cells were used. The measured bioactivities of these compounds are displayed in Table 1. Compounds **4a, 4b, 4d** and **4 g** showed potent activity against both FLT3-ITD mutation and drug resistance mutations FLT3-ITD/D835Y and FLT3-ITD/F691L. Among them, **4d** and **4 g**, bearing quinoline and quinazoline in region A, respectively, are the most active ones in terms of the potency against drug resistance mutations FLT3-ITD/D835Y and FLT3-ITD/F691L. All of these compounds showed weak or no activity against the parental Ba/F3 cells, indicating that they indeed target the mutated FLT3, and also have a low toxicity. Considering the poor water solubility of **4 g**, we chose **4d** for further structural optimization.

In the second step, we tested the possible influence of 6 and 7-positions of quinoline on the bioactivity. To this end, a total of 15 compounds (**11a-o)** with difference substituents at the 6 or 7-position of quinoline were synthesized. Reaction routes for compounds **11a-o** are summarized in Scheme 2**.** Demethylation of 4-chloro-6,7-dimethoxyquinoline (**5**) under different conditions gave intermediates4-chloro-7-methoxyquinolin-6-ol (**7**) or 4-chloroquinoline-6, 7-diol (**8**). Commercially available 4-chloro-6-methoxyquinolin-7-ol (**6**) and the synthesized intermediates **7** and **8** reacted with various alkyl chloride to provide **9a-o**. Nucleophilic substitution reactions between **9a-o** and 4-amino-2-fluorophenol produced corresponding **10a-o**. Reactions of intermediate **10a-o** and 5-(tert-butyl)-3-isocyanatoisoxazole (**3**) offered final product compounds **11a-o** by condensation reaction.

Bioactivities of compounds **11a-o** are displayed in Table 2**.** Compounds **11a-o** all showed potent activity against FLT3-ITD, ITD/D835Y and ITD/F691L, except **11 g**, which displaced moderate activity. Nevertheless, their potency did not exceed that of compound **4d**.

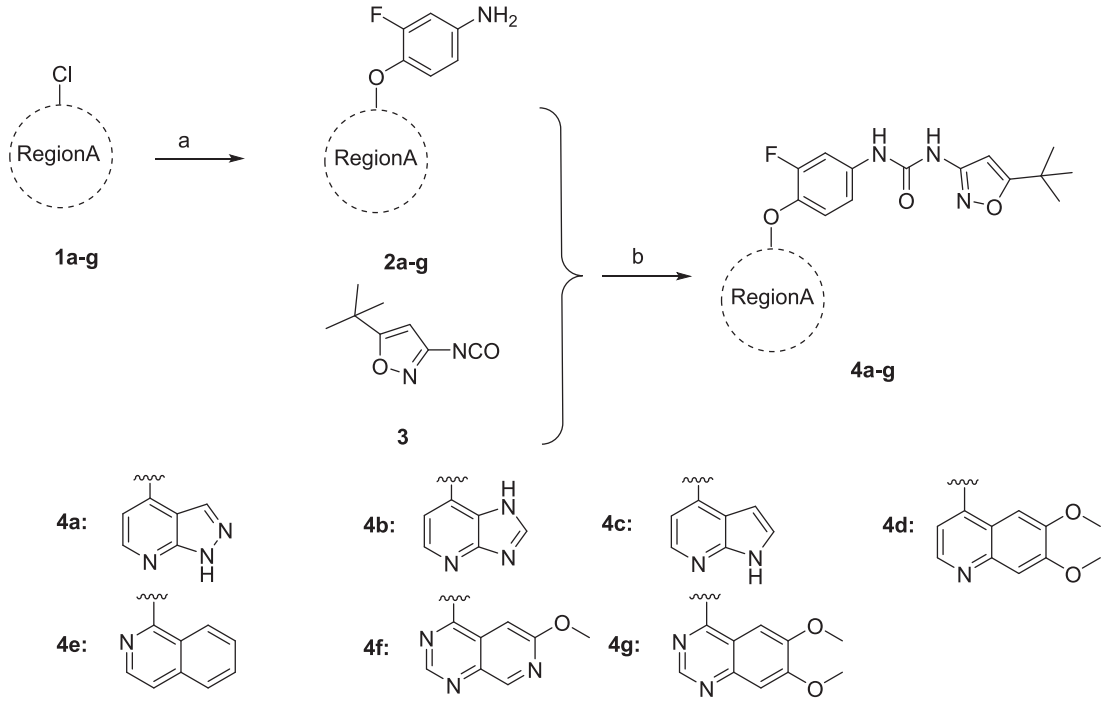
Overall, through the above structural optimization and SAR studies, we obtained a number of new FLT3 inhibitors containing the scaffold 1-(5-(tert-butyl) isoxazol-3-yl)-3-(3-fluoro-4-(quinolin-4-yloxy)phenyl) urea. Among them, **4d** is the most potent one, which exhibited excellent inhibitory activity against FLT3-ITD and drug-resistance mutations FLT3-ITD/D835Y and FLT3-ITD/F691L (Figure 2**)**. Further bioactivity evaluations were then carried out on this compound.

To examine the kinase selectivity of compound **4d,** a kinase profiling assay was performed through DiscoverX KINOMEscan kinase profiling services. The results showed that **4d** has a good kinase selectivity with the calculated selectivity scores, S(1), S(5) and S(10), being 0.082, 0.119 and 0.164, respectively (See Supporting Information Table S1).

Molecular docking was then used to predict the binding model of the most active compound **4d** in the active pocket of FLT3. The receptor structure was taken from the crystal structure of FLT3 (PDB ID: 4RT7). The preparation and preprocessing of the receptor and the ligand were performed on the platform of Discovery Studio 3.1. The program GOLD version 5.1 was adopted for molecular docking. The predicted interaction model between compound **4d** and FLT3 is depicted in Figure 3. **4d** suitably resides in the ATP binding pocket of FLT3. Four hydrogen bonds are formed between **4d** and FLT3: one is between quinoline (1-N) and Cys694; one is between oxygen of urea and ASP829; and the other two are between nitrogen of urea and Glu661. It is also obvious that the benzene ring of **4d** forms good π-π interactions with the benzene rings of residues Phe691 and Phe830.

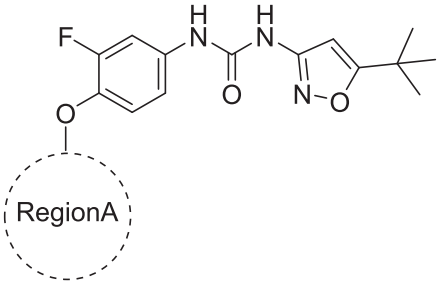
Our previous study has shown that **SKLB-677** could block the Wnt/β-catenin signaling pathway. To examine whether **4d** has a similar effect, we used the same STF3a cell model as before,[24] which stably express both the STF (Super Top Flash) luciferase reporter promoter and the wnt3a gene. The results indicated that **4d** was able to dose-dependently inhibit the Wnt/β-catenin signaling with an IC_{50} value of < 0.5 μM (Figure 4), which is very similar with **SKLB-677**. In the same experiment, AC220 exhibited very weak effect (Figure 4).

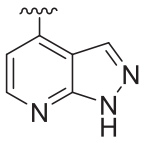
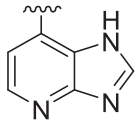
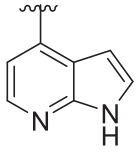
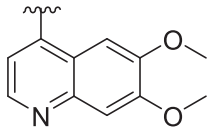
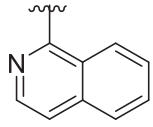
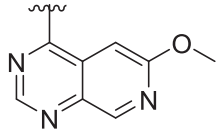
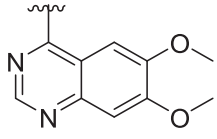
In summary, we obtained a new 1-(5-(tert-butyl)isoxazol-3-yl)-3-(3-



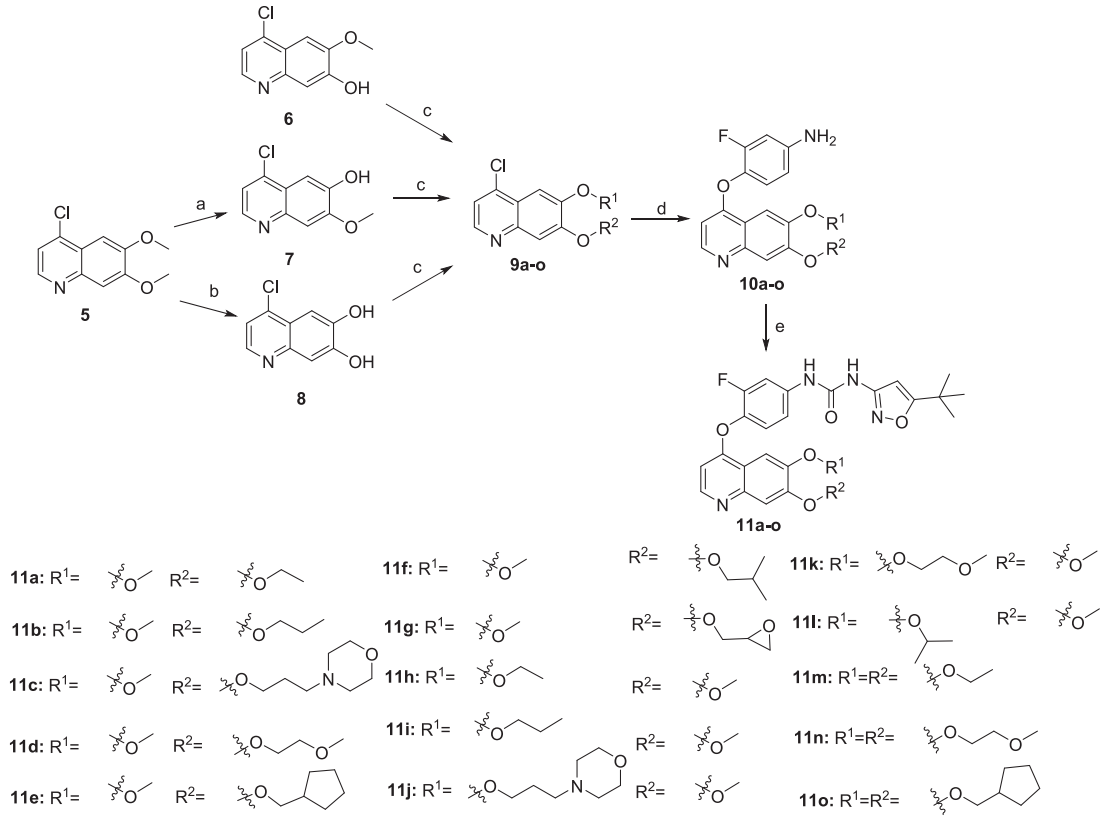
Scheme 1. Synthetic routes for compounds **4a-g**. Reagents and conditions: (a)*t*-BuOK, anhydrous DMF, 100 ˚C, overnight; (b) Et_3N, EtOAc, 80 ˚C, overnight.

Table 1
Inhibitory activities of compounds **4a-g** against Ba/F3 cells expressing FLT3-ITD, FLT3-ITD/D835Y and FLT3-ITD/F691L, as well as the parental Ba/F3 cells.



Cpd	Region A	FLT3-ITD(IC_{50}, nM)	FLT3-ITD/D835Y(IC_{50}, nM)	FLT3-ITD/F691L(IC_{50}, nM)	Ba/F3(IC_{50}, nM)
AC220	–	3.01 ± 0.79	520 ± 27.8	625 ± 31.2	9312.2 ± 16.0
SKLB-677	–	0.038 ± 0.001	130 ± 14.6	108 ± 17.3	3156.3 ± 17.1
4a		0.292 ± 0.054	34.3 ± 7.2	35.0 ± 2.7	2623.2 ± 10.0
4b		0.015 ± 0.004	12.4 ± 5.7	18.6 ± 2.4	2084.0 ± 13.0
4c		140.9 ± 7.3	741.1 ± 35.6	1023.7 ± 9.8	> 10000
4d		0.072 ± 0.02	3.48 ± 0.59	5.86 ± 0.62	1212.4 ± 10.9
4e		1258.0 ± 23.9	> 10000	> 10000	> 10000
4f		1.20 ± 0.24	64.2 ± 11.7	1021.7 ± 6.8	3035.4 ± 12.0
4 g		1.51 ± 0.32	7.93 ± 1.29	13.8 ± 2.8	> 1988.1 ± 13.5

[a]All IC_{50} values were obtained by triplet testing.



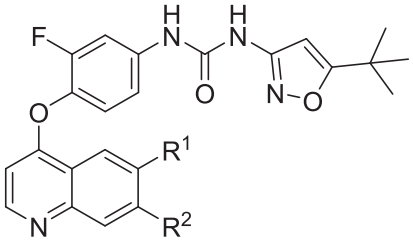
Scheme 2. Synthetic routes for compounds **11a-o**. Reagents and conditions: (a) L-Methionine, Methanesulfonic acid, 120 °C, 12 h; (b) BBr_3, DCM, 0 °C-25 °C, 6 h; (c) K_2CO_3, DMF, 150 °C, 4 h; (d) *t*-BuOK, anhydrous DMF, 100 °C, overnight; (e) Et_3N, EtOAc, 80 °C, overnight.

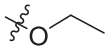
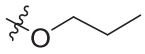
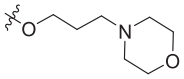
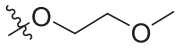
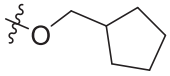
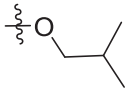
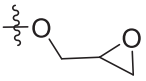
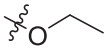
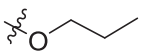
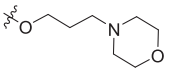
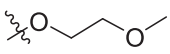
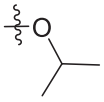
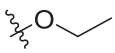
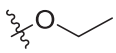
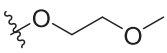
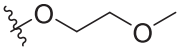
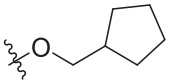
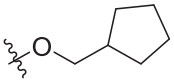
fluorophenyl)urea derivative (**4d**), which showed potent activity against both FLT3-ITD mutation and drug resistance mutations FLT3-ITD/D835Y and FLT3-ITD/F691L. This compound has a good kinase selectivity, and is able to inhibit the Wnt/β-catenin signaling pathway. Nevertheless, we have to mention that more studies, including in vivo anti-AML activity, and pharmacokinetic properties, as well as mechanism of actions, are still needed to evaluate its druggability.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Table 2
Inhibitory activities of compounds **11a-o** against Ba/F3 cells expressing FLT3-ITD, FLT3-ITD/D835Y and FLT3-ITD/F691L, as well as parental Ba/F3 cells.



Cpd	R_1	R_2	FLT3-ITD (IC_{50}, nM)	FLT3-ITD/D835Y (IC_{50}, nM)	FLT3-ITD/F691L (IC_{50}, nM)	Ba/F3 (IC_{50}, nM)
4d			0.072 ± 0.020	3.48 ± 0.59	5.86 ± 0.62	1212.4 ± 10.9
11a			5.20 ± 0.68	5.96 ± 0.57	8.31 ± 1.33	1645.6 ± 9.4
11b			19.2 ± 7.2	14.9 ± 3.2	54.3 ± 5.9	4935.0 ± 15.0
11c			3.22 ± 0.35	5.09 ± 1.18	9.93 ± 2.75	1097.1 ± 17.7
11d			4.73 ± 0.24	5.35 ± 1.20	11.9 ± 1.6	1125.2 ± 13.0
11e			66.5 ± 9.5	104 ± 16.0	189 ± 25.1	5556.1 ± 12.4
11f			36.5 ± 4.5	62.4 ± 11.3	127 ± 13.4	4817.0 ± 7.2
11g			2649.0 ± 11.1	3540.8 ± 21.3	5270.6 ± 15.5	9203.9 ± 28.5
11h			7.25 ± 0.21	10.9 ± 2.9	29.9 ± 6.1	2877.7 ± 10.3
11i			76.1 ± 9.4	160 ± 12.9	296 ± 19.3	7774.1 ± 20.0
11j			5.77 ± 0.30	6.28 ± 0.82	16.8 ± 3.4	1840.0 ± 13.8
11k			4.91 ± 0.53	10.1 ± 3.1	20.8 ± 4.5	3000.2 ± 11.4
11l			6.88 ± 0.74	10.6 ± 4.0	23.1 ± 3.3	2034.8 ± 15.6
11m			3.08 ± 1.16	15.5 ± 2.5	32.6 ± 1.4	3137.5 ± 28.6
11n			5.17 ± 0.55	11.0 ± 3.7	16.0 ± 4.4	1996.3 ± 17.6
11o			37.2 ± 6.3	27.9 ± 7.4	38.9 ± 9.1	6500.1 ± 19.4

[a]All IC_{50} values were obtained by triplet testing.

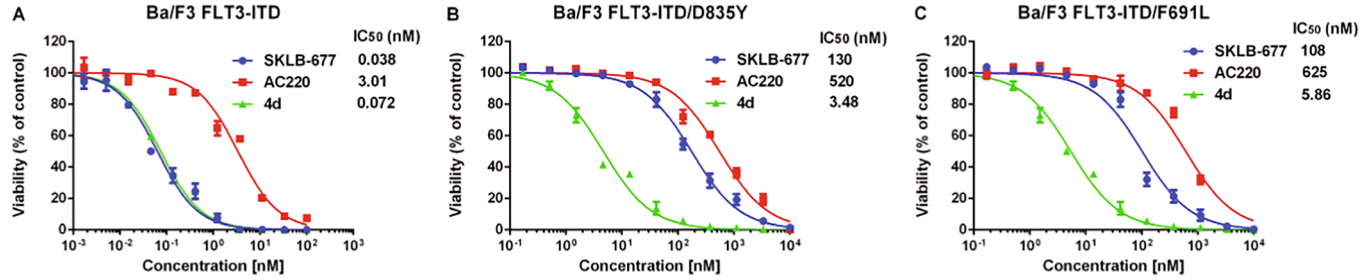


Fig. 2. Dose-response curves of the inhibitory activities of **SKLB-677**, **AC220**, and **4d** against Ba/F3-FLT3-ITD (A), Ba/F3-FLT3-ITD/D835Y (B) and Ba/F3-FLT3-ITD/F691L (C).

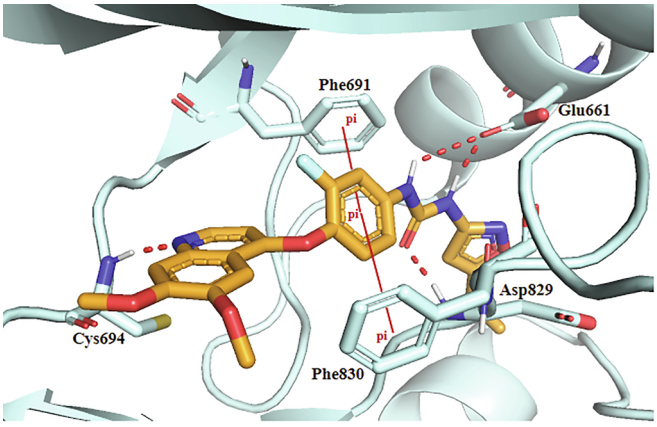


Fig. 3. Prediction binding model of compound **4d** in the active pocket of FLT3(Protein Data Bank: 4RT7). Compound **4d** is color coded with carbon atoms by brown, nitrogen atoms by blue, and oxygen atoms by red. Hydrogen bonds are shown in purple dashed line. The π-π interactions are shown in dashed red line. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

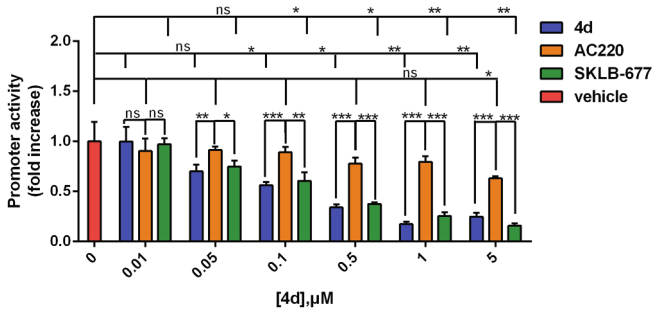


Fig. 4. Inhibitory activities of compounds **4d, SKLB-677** and **AC220** against Wnt/β-catenin pathway were determined using STF3a cells treated with various concentrations of compounds for 24 h before luciferase activity measurements. All data points are means of triplicates ± SD. ns, not statistically significant, $^{**}P < 0.01$, $^{***}P < 0.005$ among groups.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2020.127532.

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