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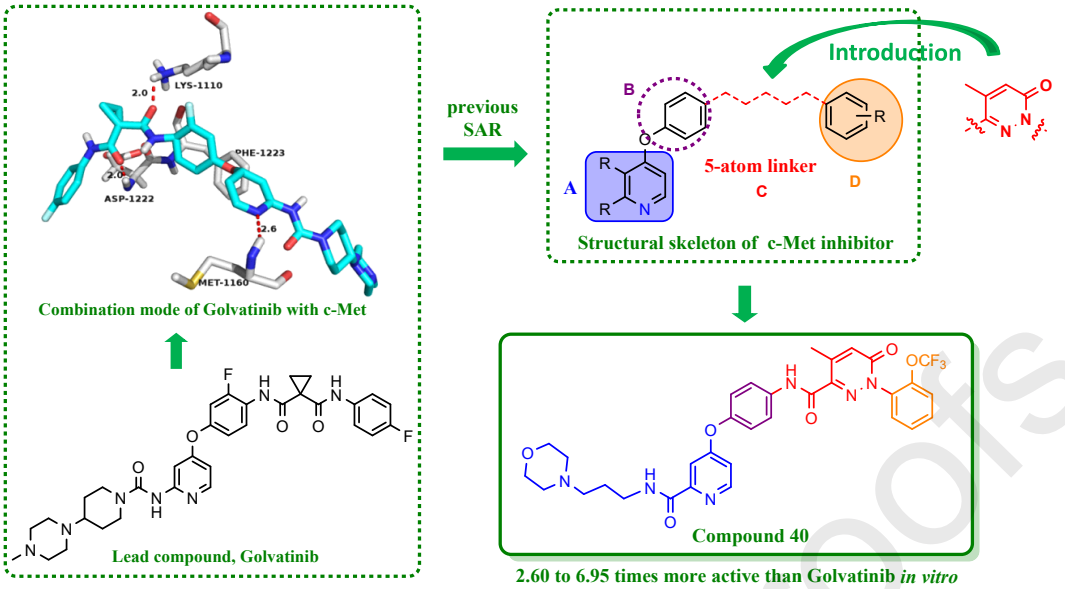


# Design, synthesis and biological evaluation of 4-(pyridin-4-yloxy)benzamide derivatives bearing a 5-methylpyridazin-3(2*H*)-one fragment

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**Abstract:** A series of 4-(pyridin-4-yloxy)benzamide derivatives bearing a 5-methylpyridazin-3(2*H*)-one fragment were designed, synthesized, and evaluated for their biological activity. Most compounds showed effective inhibitory activity against cancer cell lines of A549, HeLa and MCF-7. Among them, the most promising compound **40** showed excellent activity against A549, HeLa and MCF-7 cell lines with $IC_{50}$ values of 1.03, 1.15 and 2.59 μM, respectively, which was 2.60-6.95 times more active than that of Golvatinib. The structure-activity relationships (SARs)

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showed that the introduction of 5-methylpyridazin-3(2*H*)-one to "5-atom linker" and the modification of the amide with morpholine group were beneficial for enhancing the inhibitory activity of compounds. In addition, the further research on compound **40** mainly include c-Met kinase activity, concentration dependence, apoptosis (acridine orange staining), and molecular docking.

**Key words**: Synthesis; 4-(pyridin-4-yloxy)benzamide derivatives; 5-methylpyridazin-3(2*H*)-one; Inhibitors; c-Met

Cancer has become the primary factor in killing people besides cardiovascular diseases.[1] Receptor tyrosine kinases are closely related to cell growth, reproduction and metastasis. However, the aberrant activation of receptor tyrosine kinases usually leads to the occurrence, invasion and metastasis of various cancer diseases.[2-3] In recent years, small molecule inhibitors have become a research hotspot because they can block the transformation of signal pathways by targeting specific sites (including receptor tyrosine kinases), and then induce the apoptosis and necrosis of cancer cells.[4-5]

With the development of molecular pharmacology and molecular oncology, the intracellular mechanism of cancer cells has been gradually clarified, which accelerated the discovery of c-Met inhibitors.[6] Notably, Cabozantinib (**1**, **Fig. 1**) and Crizotinib (**2**, **Fig. 1**) belong to c-Met inhibitors of type II and type I, respectively, which were approved by FDA in 2012 and 2011.[7-8] The other four representative type II c-Met inhibitors in clinical trials are listed in **Fig. 1**, including Golvatinib (**3**), TAS-115 (**4**), BMS-777607 (**5**) and Altratinib (**6**).[9-12]

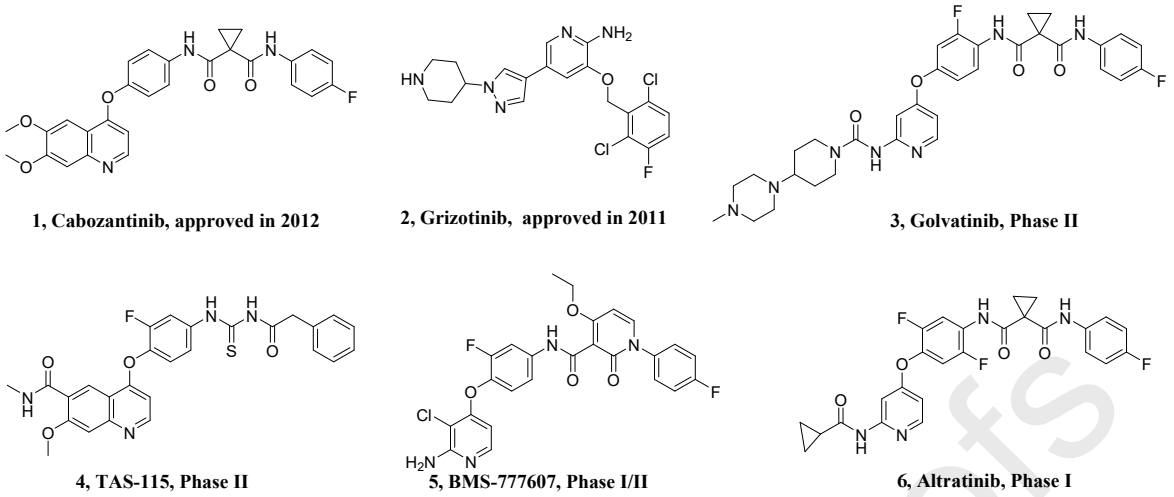



**Fig. 1.** The representative small-molecule c-Met kinase inhibitors.

Type II c-Met inhibitors have the characteristics of low toxicity, strong efficacy and little drug resistance. However, the SAR study on pyridylamide derivatives of c-Met inhibitors[13-15] is not enough to guide the development of drugs. In this study, Golvatinib was used as a lead compound, and a series of modifications and optimization were carried out on it to expand the SAR of type II pyridylamide c-Met inhibitors. First of all, according to our previous research,[16] the skeleton of type II c-Met inhibitors was divided into four moieties of A, B, C, and D, on which our design was based, as shown in **Fig. 2**. Subsequently, docking simulation of Golvatinib with c-Met (PDB code: 3LQ8) was performed to guide our modification. According to the docking results we found that moieties A and C have the potential to enhance the binding affinity through forming strong hydrogen boding interactions with residues Met1160, Asp1222 and Lys1110. Among them, moiety C has two obvious structural characteristics: 5-atom regulation and hydrogen bond receptor/donor regulation,[16] which means that the structure of moiety C not only needs to maintain the distance of six chemical bonds between moieties B and D, but also needs to contain hydrogen bond acceptor/donor atoms to interact with c-Met. In addition, the space around moieties A and C was large enough for further optimization. On the contrary, only minor modifications were suitable for implementation in moieties B and D.

In moiety A, the pyridine ring was retained, it was an important structure to maintain the biological activity by forming hydrogen bond with residue Met1160. On this basis, various flexible or hydrophilic amides, such as alkyl chains, morpholine and thiophene groups, were introduced into moiety A to improve the solubility of the target compounds. As reported, pyridazinone fragment possessing good biological activity was widely used in the design of anticancer agents (**Fig. 3**).[17-19] Therefore, 5-methylpyridazin-3(2*H*)-one fragment was embedded into moiety C to explore its effect on biological activity, based on the "5-atom regulation" and "hydrogen bond donor or acceptor" which had been explained clearly in our previous research.[19] Considering the steric clash problem involved in binding process, we introduced various small substituents, such as halogen atoms, $CH_3$, $CF_3$ and $OCF_3$ to moieties B and D in order to explore their effect on inhibitory activity. Ultimately, a series of 4-(pyridin-4-yloxy)benzamide derivatives containing a 5-methylpyridazin-3(2*H*)-one fragment were designed.

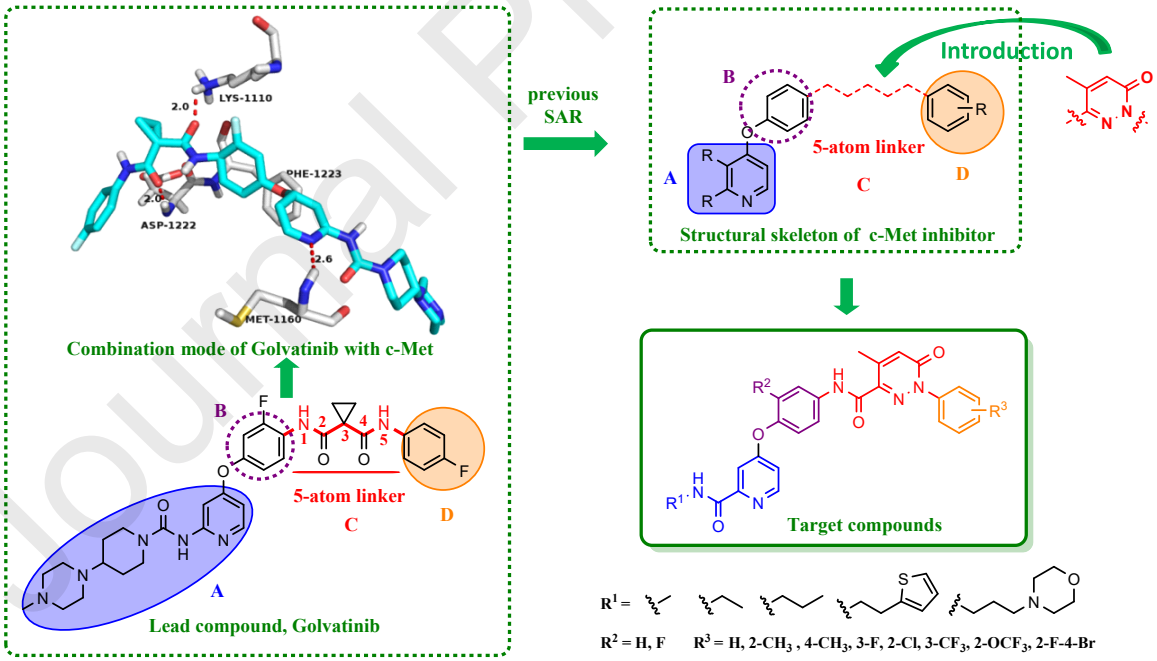
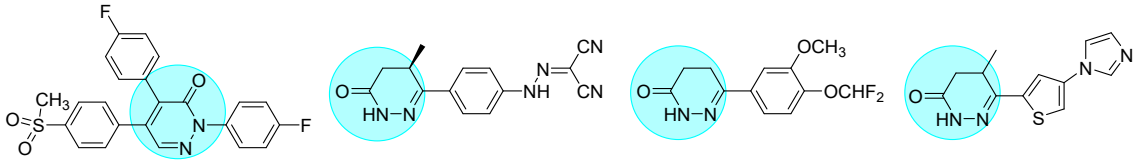


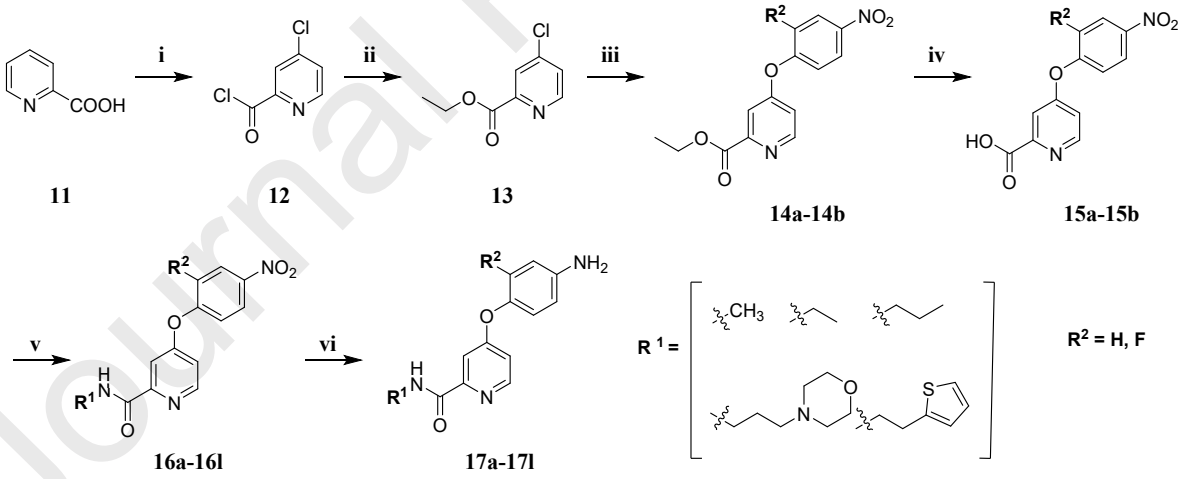

**Fig. 2.** Design strategy for the target compounds using Golvatinib as a lead compound.



**7, A-241611** **8, Levosimendan** **9, Zardavenine** **10, Motapizone**

**Fig. 3.** Some drugs containing pyridazinone fragment.

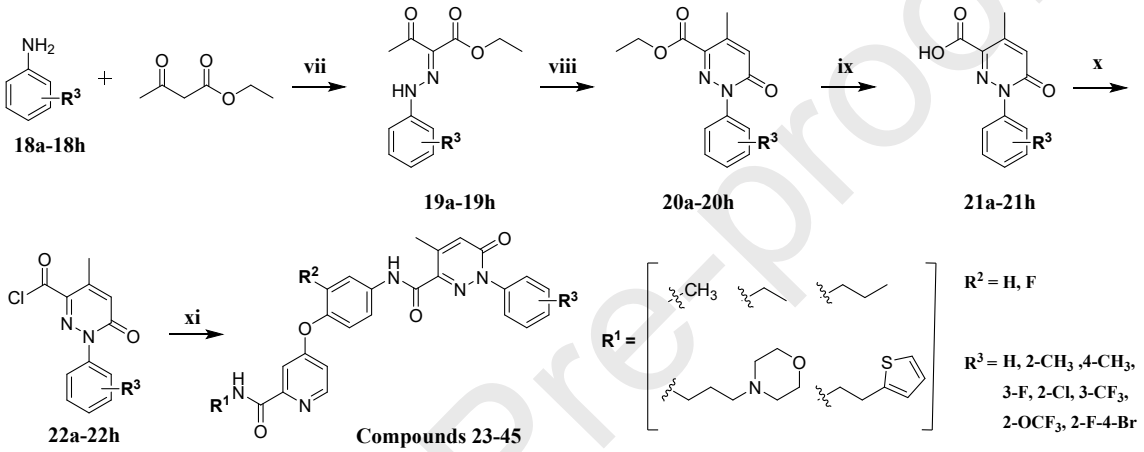
The key intermediates **17a-17l** were prepared according to the sequence outlined in **Scheme 1**. The commercially available picolinic acid **11** was converted into the corresponding acid chloride **12** in thionyl chloride ($SOCl_2$) by an acylation reaction. Substitution of compound **12** with ethanol yielded **13**, which was then reacted with *p*-nitrophenol or 2-fluoro-4-nitrophenol to get **14a-14b**. Subsequently, **14a-14b** were hydrolyzed under the mixture of 1,4-dioxane/sodium hydroxide (NaOH, 10%) = 10:1 to produce carboxyl analogues **15a-15b,** which were acylated and then nucleophilic substituted with various amines to obtain intermediates **16a-16l**. And then **16a-16l** were reduced *via* catalytic hydrogenation to generate the corresponding amines **17a-17l**.



**Scheme 1.** Reagents and conditions: (i) $SOCl_2$, *N,N*-Dimethylformamide (DMF), NaBr, 85 °C, 20 h; (ii) EtOH, trimethylamine ($Et_3N$), dichloromethane (DCM), 25 °C, 0.5 h; (iii) chlorobenzene (PhCl), *p*-nitrophenol or 2-fluoro-4-nitrophenol, 130 °C, 4.5 h; (iv) 1,4-dioxane/NaOH (10%) = 10:1, 25 °C, 0.5 h; (v) $SOCl_2$, DMF, amines, $Et_3N$, DCM, 25 °C, 1 h; (vi) EtOH, $FeCl_3 \cdot 6H_2O$, activated carbon, hydrazine hydrate (80%), 85 °C, 4 h.

The target compounds **23-45** were prepared according to the route of **Scheme 2**. Analog anilines **18a-18h** were diazotized with $NaNO_2$ and then nucleophilic

substituted with ethyl 3-oxobutanoate to gain **19a-19h,** which were reacted with ethyl (triphenylphosphoranylidene)acetate ($Ph_3P$=$CHCOOC_2H_5$) to give pyridazinones **20a-20h**. Then **20a-20h** were hydrolyzed with NaOH solution to get carboxyl analogues **21a-21h**. Ultimately, oxalyl chloride was reacted with **21a-21h** in the presence of DMF in DCM to yield the intermediates **22a-22h**. Nucleophilic substitution of the key intermediates **17a-17l** and **22a-22h** with *N,N*-Diisopropylethylamine (DIPEA) under DCM at 0 °C obtained the target compounds **23-45**.



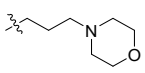
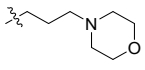
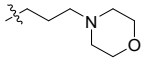

**Scheme 2.** Reagents and conditions: (vii) sodium nitrite ($NaNO_2$), HCl, ethyl 3-oxobutanoate, EtOH/$H_2O$ (2:1), 0 °C, 4 h; (viii) $Ph_3P$=$CHCOOC_2H_5$, dioxane, 90 °C, 10 h; (ix) 1,4-dioxane/NaOH (10%) = 10:1, 25 °C, 0.5 h; (x) DCM, oxalyl chloride, 0 °C, 0.5 h; (xi) intermediates **17a-17l**, DCM, DIPEA, 0 °C, 0.5 h.

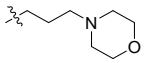
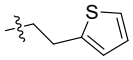
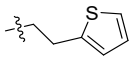
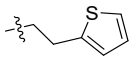
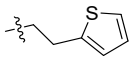
The cytotoxic activities of all synthesized compounds against A549, HeLa and MCF-7 cell lines were determined through MTT method.[20] As shown in **Table 1**, most of the compounds showed moderate to potent inhibitory activities on these three cell lines, especially A549 and HeLa. It is worth noting that compounds **30**, **36**, **38**, **39** and **40** showed stronger cytotoxic activities than Golvatinib. Among them, the most promising compound **40**[21] showed excellent activity against A549, HeLa and MCF-7 cell lines with $IC_{50}$ values of 1.03, 1.15 and 2.59 μM, respectively, which was 2.60-6.95 times more active than that of Golvatinib ($IC_{50}$ values were 6.89, 4.14 and 20.61 μM, respectively).

According to the results of cytotoxic activity (**Table 1**), the SARs were summarized as follows. Firstly, the longer alkyl amide chains have stronger cytotoxic

activity (propyl > ethyl > methyl), such as compounds **35** > **29** > **25**. Secondly, embedding of morpholine group with strong hydrophilicity in the terminal of alkylamide chain was beneficial to the improvement of inhibition activity. For example, the $IC_{50}$ values of compounds **38-40** were lower than that of Golvatinib. The introduction of F/$OCF_3$/$CF_3$ (electron absorption substituent) to the benzene ring of moiety D had a great contribution to the inhibition activity, as represented compounds **28-30** and **34-36**. Conversely, the introduction of H/$CH_3$ or two substituents to the same region reduced the inhibitory activity, such as compounds **23**, **26-27**, **31-33** and etc. Overall, the introduction of propylmorpholine and $OCF_3$ to moieties A and D, respectively, led to the best conformation (compound **40**), which possessed the best cytotoxic activity.

**Table 1:** Cytotoxic activities of the target compounds **23-45** and Golvatinib against the A549, HeLa and MCF-7 cell lines.

| Compd. | $R^1$ | $R^2$ | $R^3$ | $IC_{50}$ (μM) ± SD | | |
|---|---|---|---|---|---|---|
| | | | | A549 | HeLa | MCF-7 |
| **23** | Me | F | H | 40.80 ± 2.11 | NA [b] | NA |
| **24** | Me | F | 2-Cl | 11.80 ± 1.32 | 9.87 ± 0.36 | 135.74 ± 3.27 |
| **25** | Me | F | 3-$CF_3$ | 10.24 ± 0.51 | 11.77 ± 0.44 | 45.78 ± 3.36 |
| **26** | Et | H | 2-$CH_3$ | 34.84 ± 0.27 | 27.34 ± 5.56 | 44.27 ± 4.56 |
| **27** | Et | H | 4-$CH_3$ | 11.55 ± 0.38 | 10.74 ± 0.11 | 51.25 ± 7.44 |
| **28** | Et | H | 3-F | 7.41 ± 0.30 | 6.87 ± 0.14 | **18.88 ± 2.65** [a] |
| **29** | Et | H | 3-$CF_3$ | 10.04 ± 0.31 | 10.73 ± 0.22 | 40.57 ± 2.20 |
| **30** | Et | H | 2-$OCF_3$ | **5.28 ± 0.16** | **3.99 ± 0.10** | **15.45 ± 0.45** |
| **31** | Et | H | 2-F-4-Br | 34.49 ± 1.55 | NA | 35.14 ± 12.80 |
| **32** | n-Pr | F | 2-$CH_3$ | 25.69 ± 0.35 | 24.55 ± 0.53 | 39.59 ± 2.82 |
| **33** | n-Pr | F | 4-$CH_3$ | 17.97 ± 2.14 | 14.24 ± 2.27 | 34.55 ± 3.53 |
| **34** | n-Pr | F | 3-F | 7.02 ± 0.21 | 6.66 ± 0.65 | **13.34 ± 1.92** |
| **35** | n-Pr | F | 3-$CF_3$ | 9.24 ± 0.51 | 8.00 ± 0.44 | 30.02 ± 2.25 |
| **36** | n-Pr | F | 2-$OCF_3$ | **4.22 ± 0.09** | **3.69 ± 0.10** | **7.42 ± 0.18** |
| **37** | n-Pr | F | 2-F-4-Br | 234.92 ± 12.82 | 53.54 ± 3.43 | NA |
| **38** |  | H | 4-$CH_3$ | **2.94 ± 0.26** | **1.92 ± 0.14** | **3.57 ± 0.11** |
| **39** |  | H | 3-$CF_3$ | **2.50 ± 0.22** | **1.65 ± 0.19** | **5.21 ± 0.22** |
| **40** |  | H | 2-$OCF_3$ | **1.03 ± 0.14** | **1.15 ± 0.06** | **2.59 ± 0.09** |

| | | | | | | |
|---|---|---|---|---|---|---|
| **41** |  | H | 2-F-4-Br | **6.83 ± 0.17** | 4.25 ± 0.36 | **8.23 ± 1.10** |
| **42** |  | H | 4-$CH_3$ | 965.49 ± 22.37 | 660.36 ± 10.99 | NA |
| **43** |  | H | 3-$CF_3$ | 722.36 ± 23.45 | 885.20 ± 14.90 | NA |
| **44** |  | H | 2-F-4-Br | 585.46 ± 11.99 | 387.21 ± 15.76 | 969.43 ± 12.86 |
| **45** |  | H | 2-$OCF_3$ | 876.23 ± 15.67 | 433.89 ± 14.38 | NA |
| **Golvatinib** [c] | | | | 6.89 ± 0.45 | 4.14 ± 0.17 | 20.61 ± 0.30 |

[a] Bold values show the $IC_{50}$ values of the target compounds lower than the values of the positive control.

[b] NA: Low inhibitory activity.

[c] Used as the positive control.

Inspired by the results of cytotoxic activity, compounds **38**-**41** were selected to evaluate their c-Met kinase activity based on Mobility Shift Assay.[22] Staurosporine was used as positive control to insure the reliability of experimental data. Among them, compound **40** showed potent c-Met kinase activity with $IC_{50}$ value of 0.807 μM, as shown in **Table 2**.

**Table 2**: c-Met kinase activity of selected compounds **38**, **39**, **40**, **41** and Staurosporine.

| Compd. | $IC_{50}$ on c-Met (μM) |
|---|---|
| **38** | >10 |
| **39** | >10 |
| **40** | 0.807 |
| **41** | >10 |
| Staurosporine | 0.057 |

Next, HeLa cells were treated with seven different concentrations of compound **40** for 72 hours by MTT method,[20] and the relationship between concentration and inhibition rate was discussed. As shown in **Fig. 4**, compound **40** was observed in a concentration-dependent manner. The inhibition rate of cells was more than 50% after the treatment with compound **40** at the concentration of 1.23 μmol/L, while it was around 20% when treated the cells with Golvatinib at same concentration.

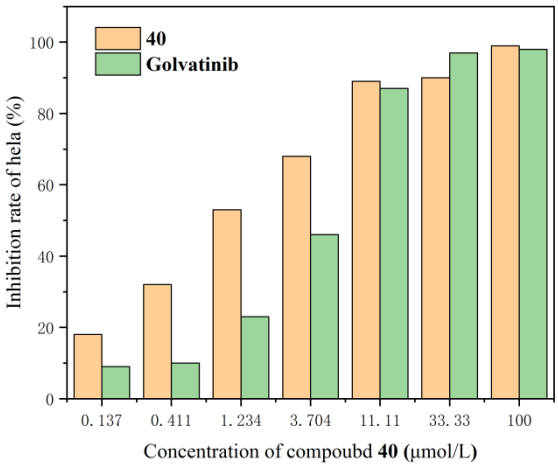



**Fig. 4.** Concentration-dependent test of compound **40** against HeLa cells.

To further investigate the ability of compound **40** to induce cells apoptosis, we performed AO (acridine orange) staining experiment[23] with HeLa cells. In the control group, the cells were arranged densely and orderly, with uniform and regular morphology, as shown in **Fig. 5**. After treated with compound **40** at the concentrations of 1 and 5 μg/L for 12 hours, a series of apoptotic phenomena appeared, such as cytoplasmic contraction, membrane swelling and apoptotic bodies increasing. Generally, compound **40** induced apoptosis of HeLa cells in a concentration-dependent manner.

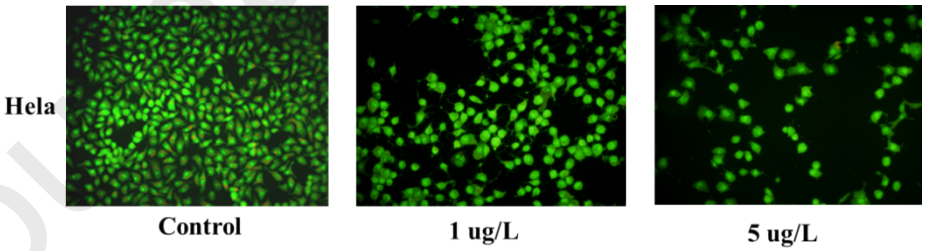



**Fig. 5.** Morphological study of HeLa cells after treated with compound **40**.

In order to explore the interaction mode of compound **40** with c-Met, we performed the docking study[24] of compound **40** with apoenzyme (c-Met without endogenous ligand, PDB code: 3LQ8) and holoenzyme (c-Met with endogenous ligand, PDB code: 3LQ8) by Autodock 4.0 software, and the binding energy were calculated as -10.80 and -11.23 kcal/mol, respectively. In the binding mode,

compound **40** was completely bound into the internal cavity of c-Met in an extended conformation (**Fig. 6A)**. Interestingly, pyridylamide structure formed a bidentate hydrogen bond with the residue Met1160. Meanwhile, the amide and 5-methylpyridazin-3(2*H*)-one segments of moiety C formed two hydrogen bonds with residues Lys1110 and Asp1222 (**Fig. 6A)**. On the other hand, the binding of endogenous ligand to c-Met formed three hydrogen bonds (**Fig. 6B)**. Generally, the docking configuration of compound **40** was basically consistent with that of endogenous ligand in **Fig. 6C**, and compound **40** has strong interaction with c-Met.

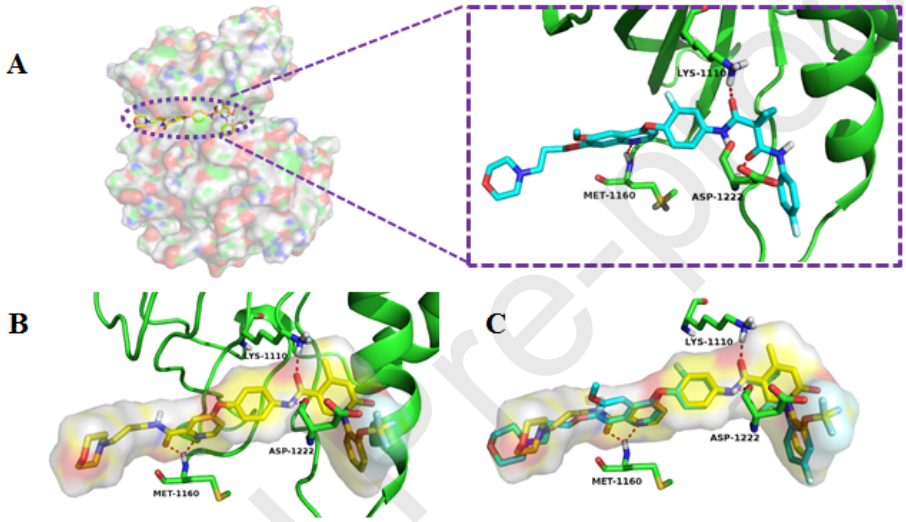



**Fig. 6.** (A) The binding mode of compound **40** with apoenzyme (c-Met, PDB code: 3LQ8). (B) The binding mode of endogenous ligand belonged with c-Met. (C) The binding mode of compound **40** with holoenzyme (c-Met with endogenous ligand). The protein and compound **40** were showed as cartoon and sticks, respectively, and hydrogen bonding interactions were indicated with dashed lines in red.

In conclusion, a series of 4-(pyridin-4-yloxy)benzamide derivatives bearing a 5-methylpyridazin-3(2*H*)-one fragment were designed, synthesized, and their biological activity was evaluated. By analyzing the cytotoxic activity and structural characteristics of the compounds, we have obtained some new SARs of pyridinamide derivatives. The SARs revealed that the introduction of 5-methylpyridazin-3(2*H*)-one structure and morpholine group to the "5-atom linker" and hydrophilic region, respectively, played an important role in the enhancement of inhibitory activity. Among them, the inhibitory activity of the promising compound **40** was 2.60-6.95 times higher than that of Golvatinib, and the $IC_{50}$ values were 1.03, 1.15 and 2.59 μM

against A549, HeLa and MCF-7 cell lines, respectively. Moreover, compound **40** shown excellent biological activities in pharmacological experiments such as c-Met kinase activity, concentration dependence and apoptosis. Compound **40** will be further studied in our laboratory in the near future.

## Acknowledgments

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20. *Cytotoxic activity assay*: The cancer cells lines were cultured in DMEM/1640 medium containing 10% fetal bovine serum (FBS). Approximate $4\times10^3$ suspended cells were plated into 96-well plates and then incubated in 5% $CO_2$ at 37 °C for 24 h. Compounds being diluted to the appropriate concentrations with medium or DMSO were added to 96-well plates and the cells were incubated continually for 72 h. Fresh MTT (5 μg/mL) was added to each well and incubated continually for 3.5 hours at 37 °C. The formazan crystals reduced by MTT were dissolved in DMSO (150 μL), and the absorbance at 492 nm (for absorbance of MTT formazan) and 630 nm (for the reference wavelength) was measured with an ELISA reader. All of the target compounds were tested in triplicate in each cell line. The results, demonstrating as $IC_{50}$ values, were tested evenly by three times and calculated by using the Bacus Laboratories Incorporated Slide Scanner (Bliss) software.
21. *Analytic data of potent inhibitor **40***: The commercial pyridinic acid (0.041 mol), DMF (0.32 mL), NaBr (0.004 mol) were dissolved in $SOCl_2$ (25 mL) and stirred at 85 °C for 20 h. The mixture was then concentrated in vacuum, dissolved in DCM, added dropwise to the mixture of EtOH (2 mL), $Et_3N$ (1.61 mL), DCM (25 mL), and stirred at room temperature for 0.5 h. After that, the reaction solution was adjusted with hydrochloric acid (aq) to pH 6-7, extracted with DCM, concentrated to obtain ethyl 4-chloroacetate (0.022 mol), which was reacted with *p*-nitrophenol (0.033 mol) at 130 °C in PhCl for 4.5 h. The mixture was concentrated, extracted with DCM/NaOH mixture, and the organic layer was concentrated to obtain solid (0.026 mol), then it was dissolved in the mixture of 1,4-dioxane (50 mL), NaOH (0.033 mol), $H_2O$ (1 mL), and stirred at 25 °C for 0.5 h. The reaction solution was concentrated, dissolved in saturated salt water, adjusted pH to 2-3 to obtain light yellow solid

4-(4-nitrophenoxy)picolinic acid (0.014 mol), which was reacted with $SOCl_2$ (30 mL) at 85 °C for 0.5 h. The mixture was concentrated, dissolved in DCM (5 mL), added dropwise to a mixture of DCM (30 mL), $Et_3N$ (2.91 mL) and 4-(3-aminopropyl)morpholine (0.021 mol) to react at 25 °C for 0.5 h. Subsequently, the reaction solution was concentrated and recrystallized to obtain a brown solid, which was reduced by hydrazine hydrate to get the intermediate 4-(4-aminophenoxy)-*N*-(3-morpholinopropyl)picolinamide. The commercially available 2-(trifluoromethoxy)aniline (0.028 mol), 37.5% HCl (0.5 mL), $NaNO_2$ (0.056 mol), 3-oxobutanoate (0.036 mol) were dissolved in EtOH (30 mL), and stirred at 0 °C for 4 h. The solution was then concentrated and extracted with solution of $H_2O$/DCM to obtain ethyl (*E*)-3-oxo-2-(2-(2-(trifluoromethoxy)phenyl)hydrazono)butanoate (0.020 mol), which was dissolved in the mixture of dioxane (50 mL) and $Ph_3P{=}CHCOOC_2H_5$ (0.022 mol), and stirred at 90 °C for 10 h. The mixture was then concentrated and recrystallized to a yellow solid, which was performed the same hydrolysis method as above to get the corresponding acid analogue (0.0016 mol). Then it was dissolved in a mixture of oxalyl chloride (0.0016 mol) and DCM (10 mL), and reacted at 0 °C for 0.5 h. The mixture was then added dropwise to a solution of DIPEA (one drop), DCM (30 mL), 4-(4-aminophenoxy)-*N*-(3-morpholinopropyl)picolinamide (0.0016 mol), and stirred at 0 °C for 0.5 h. The solution was coagulated under vacuum to obtain a yellow solid, which was further purified by a chromatographic column with PE/EA to get compound **40**. Purity: 97.99%; $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 10.81 (s, 1H), 9.08 (s, 1H), 8.51 (d, $J$ = 4.9 Hz, 1H), 7.86 (d, $J$ = 7.3 Hz, 3H), 7.65 (d, $J$ = 7.4 Hz, 1H), 7.59 (d, $J$ = 7.2 Hz, 2H), 7.39 (s, 1H), 7.23 (d, $J$ = 8.0 Hz, 2H), 7.15 (s, 1H), 7.09 (s, 1H), 3.61 (s, 4H), 3.33 (s, 2H), 2.41 (s, 9H), 1.68 (s, 2H); $^{13}$C NMR (100 MHz, DMSO-$d_6$) $\delta$ 166.25, 163.56, 161.95, 158.67, 152.95, 150.86, 149.73, 143.84, 142.74, 136.47, 133.36, 131.49 (2C), 130.40, 130.52, 128.45 (2C), 122.58 (2C), 121.83 (2C), 121.15, 114.63, 109.39, 66.40 (2C), 56.95, 53.67 (2C), 38.48, 25.70, 18.77; TOF MS ES+ (*m/z*): $[M + H]^+$, calcd for $C_{30}H_{29}F_4N_7O_5$: 653.2345, found, 653.2344.


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