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Discovery of a new class of valosine containing protein (VCP/P97) inhibitors for the treatment of non-small cell lung cancer

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ABSTRACT

Valosine containing protein (VCP/p97) is a member of the AAA ATPase family involved in several essential cellular functions and plays an important role in the ubiquitin-mediated degradation of misfolded proteins. P97 has a significant role in maintaining the cellular protein homeostasis for tumor cell growth and survival and has been found overexpressed in many tumor types. No new molecule entities based on p97 target were approved in clinic. Herein, a series of novel pyrimidine structures as p97 inhibitors were designed and synthesized. After enzymatic evaluations, structure-activity relationships (SAR) were discussed in detailed. Among the screened compounds, derivative **35** showed excellent enzymatic inhibitory activity (IC_{50}, 36 nM). The cellular inhibition results showed that compound **35** had good antiproliferative activity against the non-small cell lung cancer A549 cells (IC_{50}, 1.61 μM). Liver microsome stability showed that the half-life of compound **35** in human liver microsome was 42.3 min, which was more stable than the control **CB-5083** (25.8 min). The in vivo pharmacokinetic results showed that the elimination phase half-lives of compound **35** were 4.57 h for ig and 3.64 h for iv, respectively and the oral bioavailability was only 4.5%. These results indicated that compound **35** could be effective for intravenous treatment of non-small cell lung cancer.

1. Introduction

Valosin-containing protein (VCP/p97) is a member of the AAA (ATPase associated with various cellular activities) ATPase family. P97 promotes several biological processes, including ubiquitin-dependent protein degradation, endoplasmic reticulum-associated degradation (ERAD), nuclear membrane fusion after completion of mitosis, Golgi reassembly, activation of transcription factors and autophagy, where it supplies the mechanical force required for extracting proteins by ATP hydrolysis.[1–4] As most AAA-ATPases, p97 structurally adopts a ring-shaped homohexamer structure comprising six identical 90 kDa subunits arranged in a ring, with each protomer containing three domains: two ATPase domains (D1 and D2) and one *N*-terminal domain.[5–10] The D1 domain has low basal hydrolytic activity, due in part to a very low off rate of ADP.[1] The D2 domain is responsible for the major ATPase activity of p97 under physiological conditions. The D2 ATPase region has been displayed to have both a higher K_m for ATP and a faster hydrolysis of ATP to ADP.[10] The *N*-terminal domain binds various cofactors that interact with a variety of substrate proteins. Many studies have revealed p97′s role in promoting ERAD in cooperation with the ubiquitin-proteasome system (UPS). P97 serves as a force-generating machine to remove misfolded poly-ubiquitinated proteins from the ER into the cytosol and then transports them to the proteasome for degradation[11,12].

P97 plays an important role in maintaining the cellular protein homeostasis for tumor cell growth and survival, which was found to be overexpressed in several malignancies including non-small cell lung cancer and associated with malignancy.[13–18] For instance, it had been

Abbreviations: VCP, valosine containing protein; AAA, ATPases associated with various cellular activities; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum-associated degradation; SAR, structure-activity relationship; UPS, ubiquitin-proteasome system; IC_{50}, half-maximum inhibitory concentration; AUC, area under the curve; $T_{1/2}$, halftime; SD, Sprague-Dawley; PK, pharmacokinetics; F, absolute bioavailability in %; HPLC, high performance liquid chromatography; HRMS, high-resolution mass spectra; ESI, electrospray source; DCM, dichloromethane; DMSO, dimethyl sulfoxide; THF, tetrahydrofuran; LDA, lithium diisopropylamide

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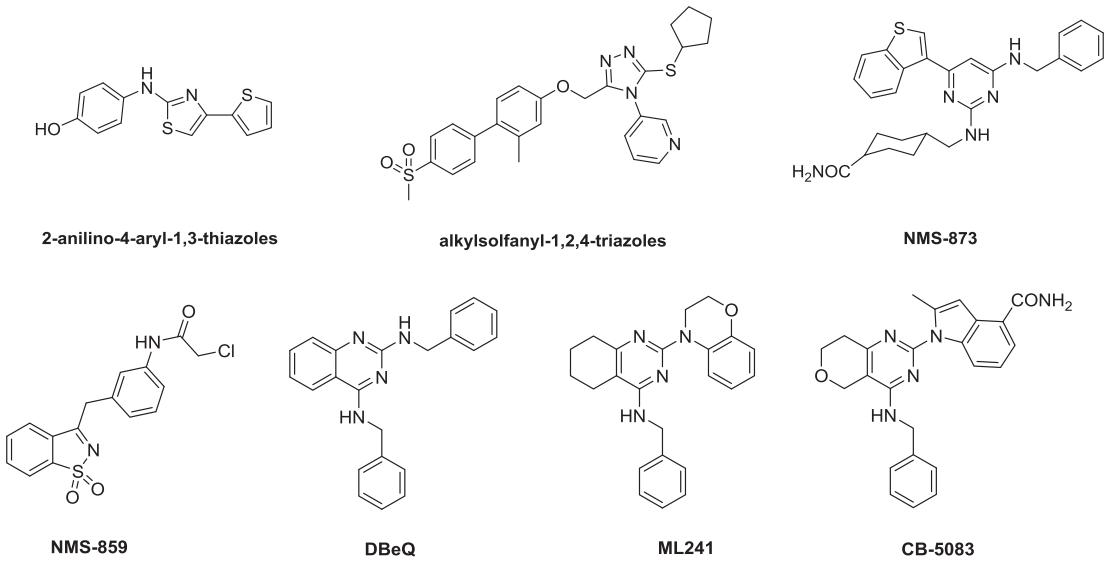


Fig. 1. The p97 inhibitors reported in the literatures.

shown that RNA interference or overexpression of ATPase deficient protein in tumor cell lines could disrupt the function of p97 to cause cell death.[19,20] Besides, gene knockout of p97 in mice was found to be embryonic lethal,[21] while mutations of the p97 gene were thought to be associated with the neurodegenerative diseases.[22] So in recent years, p97 had drawn great attention as a potential drug target for developing small molecules for cancer therapy. Up to now, a number of p97 inhibitors had been reported (Fig. 1).[23–30] Among them, compound **CB-5083** was the first selective p97 inhibitor with the requisite pharmacological properties that showed promising preclinical activities.[31] Besides, two phase I clinical trials (NCT02243917 and NCT02223598) of **CB-5083** had been completed in 2017. However, due to the toxicities of **CB-5083**, the clinical trial was discontinued. So there is a necessity to develop new compounds to meet the unmet medical need. Herein, we described the design, synthesis and structure-activity relationships (SAR) of a series of novel p97 inhibitors. Among the inhibitors, compound **35** exhibited good in vitro activities and microsomal stabilities. Furthermore, compound **35** showed good pharmacokinetic results, which indicated that compound **35** might be effective for the treatment of non-small cell lung cancer.

2. Results and discussion

2.1. Chemistry

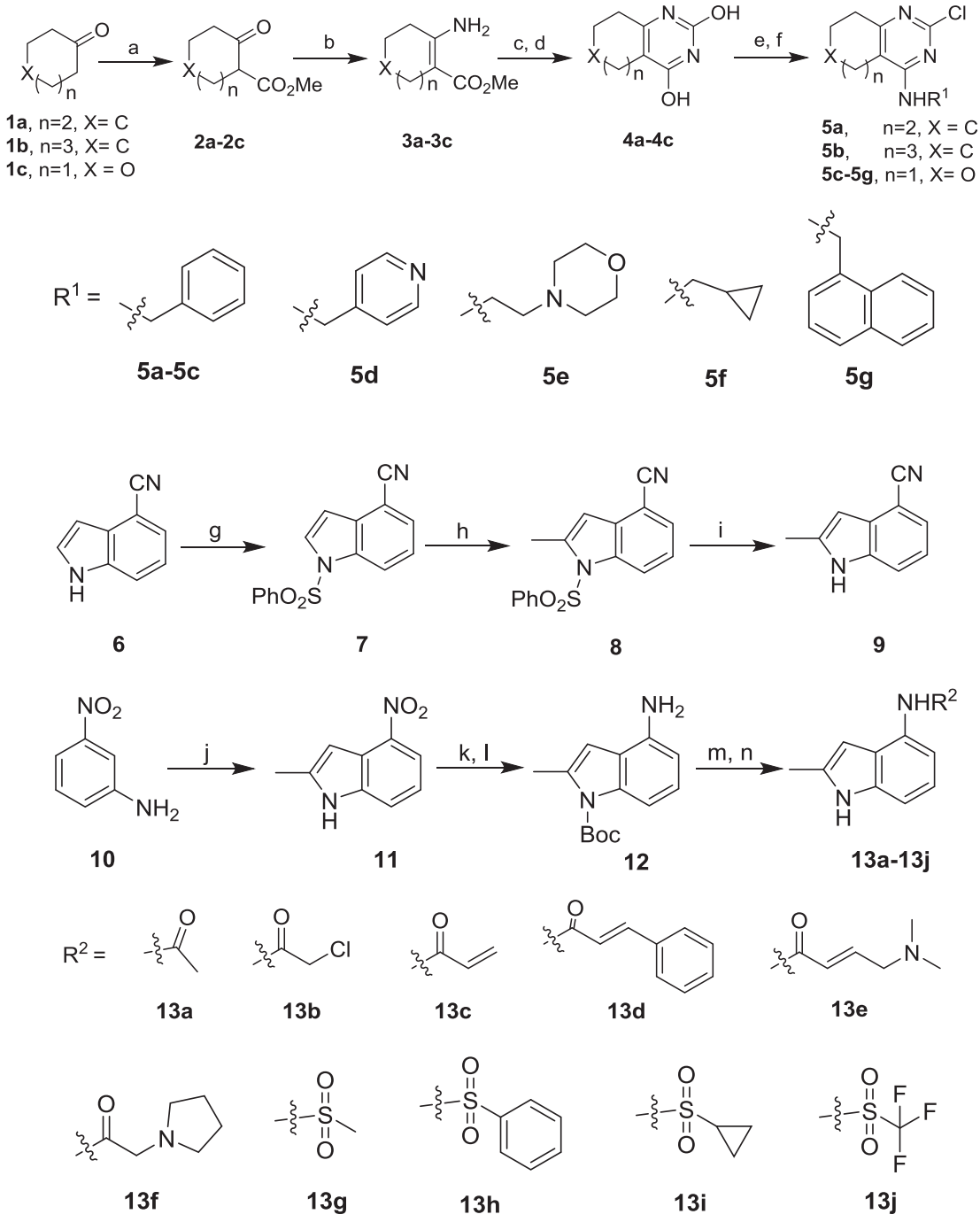
The synthesis of key intermediates **5a-5g**, **9** and **13a-13j** was outlined in Scheme 1. Compounds **1a-1c** reacted with dimethyl carbonate in the presence of NaH to produce methyl esters **2a-2c** (yields 29%-35%). And then the carbonyl groups of intermediates **2a-2c** were ammonified by ammonium acetate to give amines **3a-3c** (yields 83%-90%). Subsequently, compounds **3a-3c** reacted with 2,2,2-trichloroacetyl isocyanate and ammonia to form 2,4-diol pyrimidyl heterocyclic rings **4a-4c**, which reacted with $POCl_3$ and then were ammonified by different amines R^1NH_2 to produce key intermediates **5a-5g** (yields 19%-26%). 1H-indole-4-carbonitrile **6** reacted with benzenesulfonyl chloride to afford *N*-protected intermediate **7** with the yield of 75%. Intermediate **7** reacted with lithium diisopropylamide at −40 °C followed by methyl iodide to yield indole derivative **8** (yield 69%). Then the *N*-terminal protected group benzenesulfonyl was removed with sodium hydroxide solution to form 2-methyl-4-cyano indole **9** (yield 90%). 3-Nitroaniline **10** condensed with acetone under strongly basic conditions to give 2-methyl-4-nitro indole **11** (yield 62%). Then Boc-protecting group was introduced to the N atom of indole ring of **11** and the nitro group was reduced to amino group by Fe to give amine **12** (yield 71%). Finally, amine **12** reacted with different R^2Cl and deprotected the Boc groups to yield **13a-13j** (yields 75%-85%).

The synthesis of target molecules **CB-5083** and **17–20** was summarized in Scheme 2. Intermediate **5c** was coupled with cyanoindole **9** in the presence of $Pd_2(dba)_3$ as the catalyst and Cs_2CO_3 as base to get the compound **16** (yield 74%). Intermediate **16** reacted with acetaldehyde oxime in the presence of palladium acetate and triphenylphosphine to convert the nitrile group into the primary carboxamide to yield target molecule **CB-5083** (yield 70%). And then **CB-5083** reacted with Lawesson's reagent to change the O atom into S one to give molecule **17** (yield 81%). Intermediate **5c** reacted with commercially available N-(1H-indol-4-yl)acetamide in the presence of $Pd_2(dba)_3$, X-Phos and cesium carbonate to give target molecule **18** (yield 72%). Similar reaction between **5c** and 3-aminobenzonitrile produced the target molecule **19** (yield 66%).

The synthesis of target molecules **20–25** was illustrated in Scheme 3. Cyanoindole **9** was coupled with intermediates **5a, 5b, 5d-5f**, respectively and then the nitrile groups were hydrolyzed to the primary carboxamides to give target molecules **20–24** (yields 60%-72%). The target molecule **25** was prepared from commercially available 2,4-dichloro pyrimidine **14**, which firstly reacted with benzylamine to form compound **15** (yield 85%). Intermediate **15** was coupled with **9** and then the nitrile substituent was converted into the carboxamide to get target molecule **25** (yield 60%). Similar substitution reactions were performed between **5c** and indole derivatives **13a-13j** to give the target molecules **26–31** and **35–38** (yields 60%–78%) and intermediate **13c** reacted with **5d, 5f** and **5g** to produce target molecules **32–34** (yields 64%-74%) as showed in Scheme 4.

2.2. Biological evaluation

The kinase inhibitory activities of the target compounds were evaluated via ADP-Glo assay (Promega) against purified human p97 enzyme. Cell-based assay included a 72 h Cell Counting Kit-8 (CCK8) viability assay. Metabolic stabilities were investigated in five liver microsomes, mouse, rat, dog, monkey and human. Absolute bioavailability (F %) was determined by the pharmacokinetic assessment of areas under the plasma concentration versus time curves following iv and ig administrations.

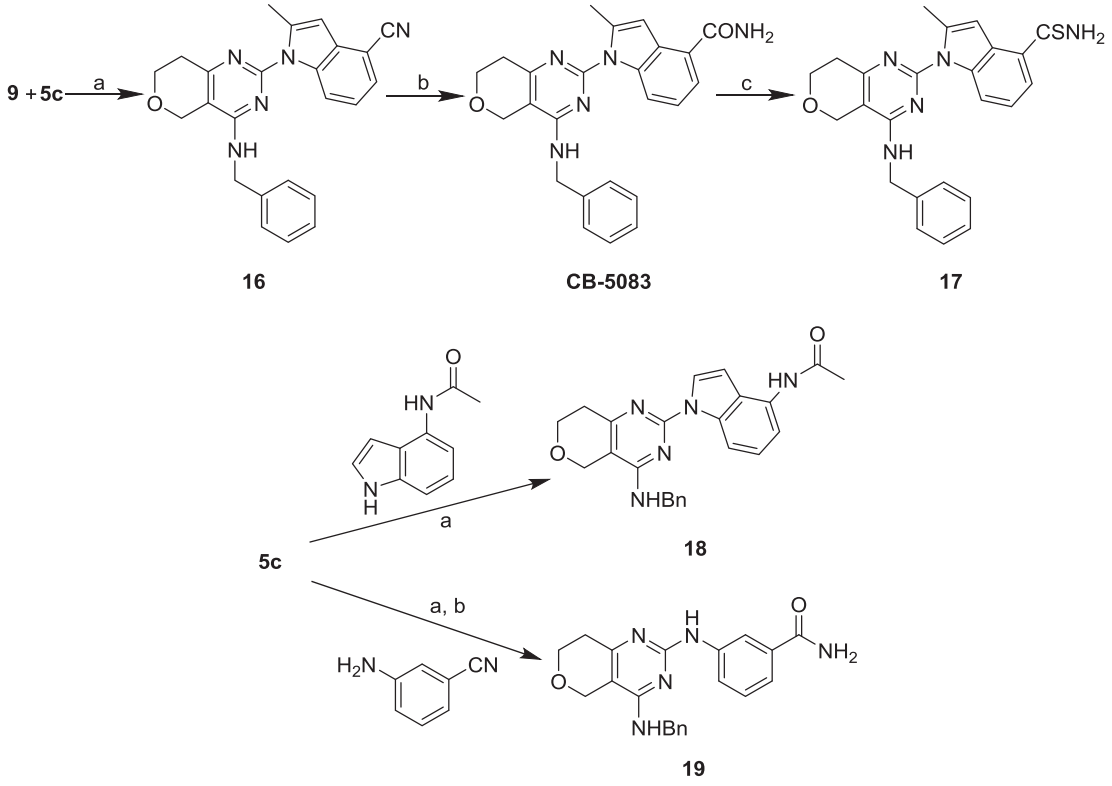


Scheme 1. Synthesis of key intermediates **5a-5g**, **9** and **13a-13j**.[a] [a]Reagents and conditions: (a) NaH, $(MeO)_2CO$, THF, 45 °C; (b) NH_4OAc, MeOH, rt; (c) $Cl_3CCONCO$, MeCN, rt; (d) NH_3, MeOH, 70 °C; (e) $POCl_3$, 120 °C; (f) MeCN, R^1NH_2 rt; (g) $PhSO_2Cl$, NaH, THF, rt; (h) MeI, LDA, THF, −40 °C; (i) NaOH, MeOH, H_2O, 40 °C; (j) Acetone, *t*-BuOK, DMSO; (k) $(Boc)_2O$, Et_3N, DCM, rt; (l) Fe, NH_4Cl, EtOH, 60 °C; (m) R^2Cl, Et_3N, rt; (n) TFA, DCM, rt.

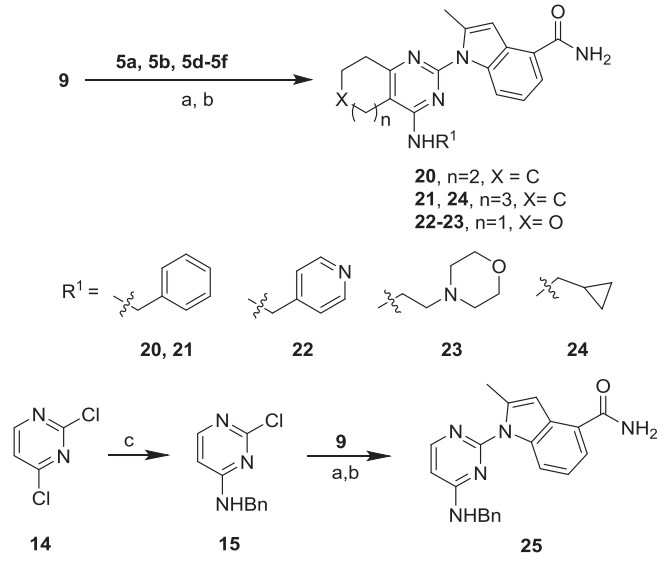
2.3. Discussion

Enzymatic. The enzymatic results of compounds **17**–**25** and **CB-5083** were showed in Table 1. While the O atom (compound **CB-5083**, IC_{50} 27.0 nM) on the formamide was substituted by S one (**17**, IC_{50} 192.6 nM), it led to the activity decrease by more than seven folds. When the 4-indol-formamide group of **CB-5083** was changed to acetyl substituted 4-indol-amine and 2-methyl was removed at the same time, the activity of compound **18** (IC_{50} 922.1 nM) was greatly reduced. Replacement of indol ring with benzyl one resulted in an inactive compound **19**. Subsequently, the replacement of the phenyl ring on benzylamino group by aromatic heterocycle (pyridyl **20**), aliphatic heterocycle (morpholinyl **21**), aliphatic ring (cyclopropyl **22**) led to the complete loss of p97 activities. In addition, the replacement of pyran ring (**CB-5083**) with oxepane (**23**) showed potent p97 activity (IC_{50} 82.8 nM). However, when the pyran ring (**CB-5083**) was replaced by oxecane (**24**, 119.7 nM) or was removed (**25**, 119.7 nM), all failed to increase the p97 potencies.

Attention was next focused on the modification of the R^2 groups of 2-methylindole and R^1 groups of pyrimidin-4-amine in **CB-5083** as illustrated in Table 2. When the formamide of **CB-5083** was changed to acetyl substituted 4-indol-amine, the activity of compound **26** (129.4 nM) was reduced greatly. The introduction of 2-chloroacetyl group at the R^2 position (**27**, 308.1 nM) also failed to increase the p97



Scheme 2. Synthesis of the target molecules **CB-5083** and **17–19**. [a] [a]Reagents and conditions: (a) Cs_2CO_3, $Pd_2(dba)_3$, X-Phos, dioxane, 100 °C; (b) $Pd(OAc)_2$, Ph_3P, MeC = NOH, EtOH, H_2O, 90 °C; (c) Lawesson's reagent, THF, 80 °C; (d) MeCN, $BnNH_2$, rt.



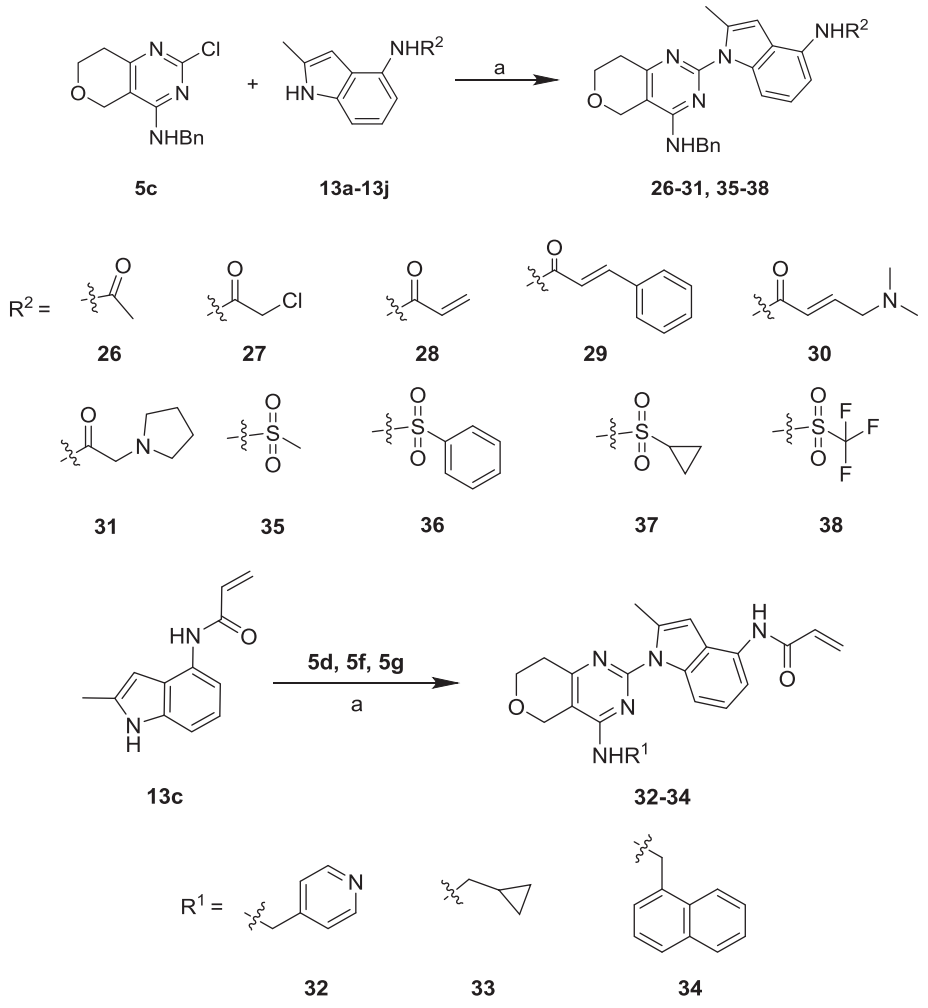
Scheme 3. Synthesis of the target molecules **20–25**. [a] [a]Reagents and conditions: (a) Cs_2CO_3, $Pd_2(dba)_3$, X-Phos, dioxane, 100 °C; (b) $Pd(OAc)_2$, Ph_3P, MeC = NOH, EtOH, H_2O, 90 °C; (c) MeCN, $BnNH_2$, rt.

potency. Conversely, vinyl group at the R^2 position (**28**, 58.3 nM) significantly increased the activity. Furthermore, compared with compounds **26–28**, larger sterically hindered groups (**29–31**) at R^2 position led to the decreased activities, which indicated that smaller groups at the R^2 position were more favorable for the improvement of activity. Since the introduction of vinyl substituent to compound **28** showed good activity, we changed the benzylamine moiety at the R^1 position into pyridyl (**32**), cyclopropyl (**33**) and naphthyl (**34**) groups. However, the biological results indicated that the generated compounds were inactive. Subsequently, we replaced the acrylamide of compound **28** with different sulfamide groups to give compounds **35–38** (Table 3). Compared with acrylamide substituent (**28**, IC_{50} 58.3 nM), methanesulfonamide (**35**, 36.4 nM) and cyclopropyl (**37**, 34.6 nM) substituents showed better activities. However, benzenesulfonamide (**36**, 134.9 nM) and trifluoromethanesulfonamide groups (**38**, 100.9 nM) resulted in decreased potencies in a certain extent.

Cellular. The compounds with the IC_{50} values of enzymatic inhibition less than 0.1 μM were further evaluated their potential antitumor effects in A549 cells. The biological results were listed in Table 4. These compounds showed significant inhibitory potency against A549 cell lines with the IC_{50} values less than 5 μM. Especially compound **35** inhibited the cell proliferation at 1 μM level, which was nearly as active as **CB-5083**. Therefore, compound **35** was used for further evaluation

Microsome stabilities. The metabolic stabilities of the promising compound **35** were determined with various species of liver microsomes, such as human, mouse, rat, dog and monkey. And the compound **CB-5083** was selected as the standard. The half-life ($T_{1/2}$) and intrinsic clearance (CL_{int}) parameters were used to evaluate their metabolic stabilities. It could give a good indication of the in vivo hepatic clearance when the overall clearance mechanism was metabolic and when oxidative metabolism dominates (i.e., $CL_{metabolic} \gg CL_{renal} + CL_{biliary} + CL_{other}$.[32,33] The data were showed in Table 5. The results revealed that both compounds **35** and **CB-5083** displayed good metabolic stabilities in human, mouse and dog species. While for the rat and money liver microsomes, the two compounds were metabolized too rapidly. The half-life of compound **35** in human liver microsome was 42.3 min, which was more stable than the control **CB-5083** (25.8 min).

Pharmacokinetic. With these encouraging in vitro data, candidate **35** was further investigated by profiling ig and iv PK in male Sprague-Dawley (SD) rats. The results were illustrated in Table 6. It indicated that the elimination phase half-lives of compound **35** were 4.57 h for ig and 3.64 h for iv, respectively. The oral bioavailability of compound **35** was only 4.2%, which was not suitable for developing an oral drug. However, the data of C_{max}, $T_{1/2}$ and AUC reflected that compound **35**



Scheme 4. Synthesis of the target molecules **26–38**. [a] [a]Reagents and conditions: (a) Cs_2CO_3, $Pd_2(dba)_3$, X-Phos, dioxane, 100 °C.

could be a good candidate for iv injection. Nowadays, the in vivo efficacy of this compound was being carried out to evaluate the drug-availability.

3. Conclusion

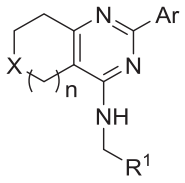
A novel series of p97 inhibitors were designed and synthesized. The structure activity relationship (SAR) was discussed in detail and the results demonstrated that the benzylamine linked to the pyrimidine structure and the pyran ring were necessary for maintaining good activities. Besides, hydrophilic and smaller groups in the indole structure showed better cellular activities. From the optimized results, compound **35** was screened and showed nanomolar level in the inhibition of p97 activity. Further cellular assay indicated that compound **35** could inhibit the proliferation of non-small cell lung cancer lines A549 with the IC_{50} value of 1.61 μM. Candidate **35** exhibited good liver microsomal stabilities in mice, dog and human. For human liver microsome, the half-life of compound **35** was 42.3 min, which was more stable than the control **CB-5083** (25.8 min). The in vivo pharmacokinetic results showed that the elimination phase half-lives of compound **35** were 4.57 h for ig and 3.64 h for iv, respectively. However, the oral bioavailability was only 4.5%, which needs to be improved in the next work. These results showed that compound **35** might be an effective candidate for intravenous treatment of non-small cell lung cancer. The in vivo efficacy of this compound was being performed to evaluate the drug-availability.

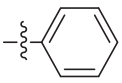
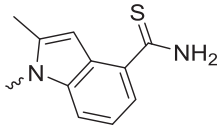
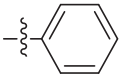
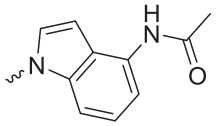
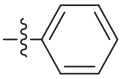
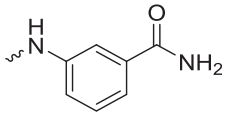
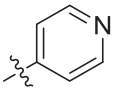
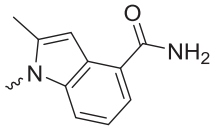
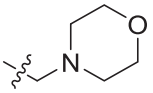
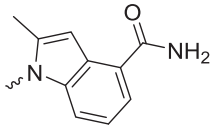
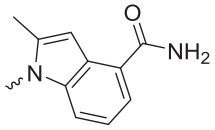
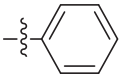
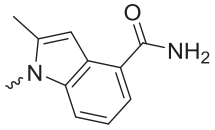
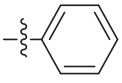
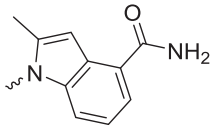
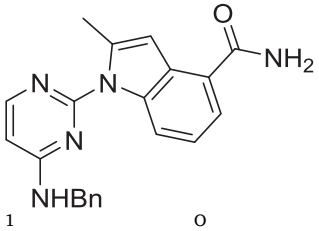
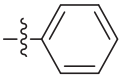
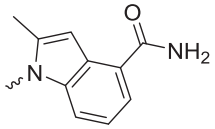
4. Experimental section

4.1. General methods

Unless otherwise indicated, chemicals, solvents and reagents were purchased from commercial suppliers and they were used without any purification. Absolutely anhydrous solvents (CH_2Cl_2, THF, DMF, etc.) were purchased from Energy packaged under nitrogen in Sure/Seal bottles. All reactions involving air or moisture-sensitive reagents were performed under an argon atmosphere. All reactions were detected by thin layer chromatography on silica gel 60 plate coated with 0.25 mm layer and spotted with UV light or iodine. All final products were purified to > 95% purity. The purity of the final products was determined by HPLC (Thermo) on an Agilent Poroshell 120 EC-C18 column (50 mm × 4.6 mm, 2.7 μm) with 0.1% FA/ACN (gradient eluted program: 0–5 min 90/10–5/95 v/v; 5–11.9 min 5/95 v/ v; 11.9–12.1 min 5/95–90/10 v/v; 12.1–15 min 90/10 v/ v) at 0.3 mL/min flow rate and 254 nm detector wavelength. ^1H and ^{13}C spectra were acquired in $CDCl_3$, DMSO-d_6 or CD_3OD at room temperature on a Bruker Avance 400 spectrometer with chemical shift (δ, ppm) reported relative to TMS as an internal standard. High-resolution mass spectra (HRMS) were recorded on a ZAB-HS instrument using an electrospray source (ESI).

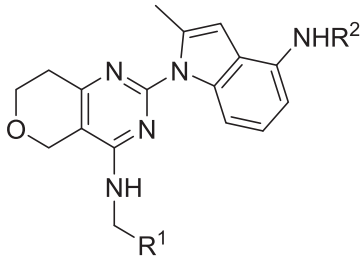
Table 1
P97 inhibitory activities of **CB-5083** and **17–25**.[a]

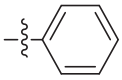
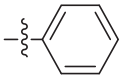
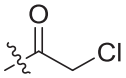
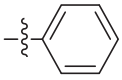
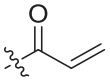
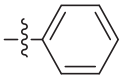
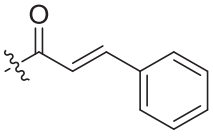
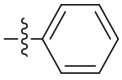
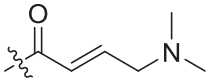
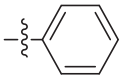
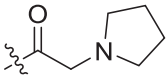
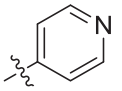
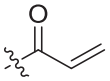
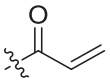
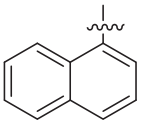
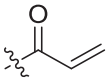


Compd.	n	X	R^1	Ar	IC_{50} (nM)
17	1	O			192.6
18	1	O			922.1
19	1	O			NA
20	1	O			NA[b]
21	1	O			NA[b]
22	1	O			NA[b]
23	2	CH_2			82.8
24	3	CH_2			119.7
25					160.9
CB-5083	1	O			27.0

[a] All experiments were repeated three times.
[b] NA: No activity.

Table 2
P97 inhibitory activities of compounds **26–34**.[a]



Compd.	R^1	R^2	IC_{50} (nM)
26			129.4
27			308.1
28			58.3
29			447.2
30			387.1
31			448.7
32			5104.5
33			NA[b]
34			NA[b]

[a] All experiments were repeated three times.
[b] NA: No activity.

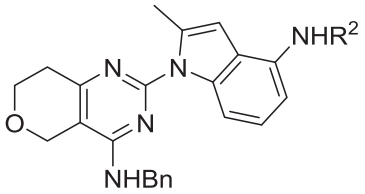
4.2. Chemistry

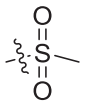
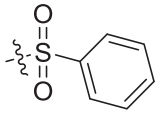
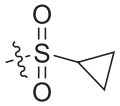
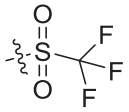
4.2.1. N-benzyl-2-chloro-7,8-dihydro-5H-pyrano[4,3-d]pyrimidin-4-amine (5c)

To a 0 °C tetrahydro-4H- pyran-4-one (1c) (10 g, 100 mmol) in THF (300 mL) was added NaH (6 g, 250 mmol). The mixture was stirred for 30 min, and dimethyl carbonate (23 g, 250 mmol) was then added and stirred for an additional 30 min. The resulting mixture was then stirred at 45 °C for 12 h and then poured into a 0 °C HCl solution (0.4 N, 300 mL). The aqueous phase was separated and extracted with ethyl acetate (100 mL × 3); the combined organic layers were washed with water (100 mL) and brine (100 mL), dried over Na_2SO_4, filtered and evaporated in vacuo. The residue was purified by column chromatography (petroleum ether/EtOAc = 25:1) to give methyl-4-oxotetrahydro-2H-pyran-3- carboxylate (**2c**) (5.5 g, yield 35%, purity 98%) as a colorless liquid. MS (ESI) *m/z* 159.1 $[M+H]^+$.

2c (5.5 g, 34.97 mmol) and ammonium acetate (8.1 g, 104.9 mmol) in methanol (100 mL) were stirred at room temperature overnight. The mixture was concentrated in vacuo, and diluted with water (50 mL) and extracted with DCM (50 mL × 3). The separated organic layer was dried over Na_2SO_4 and concentrated in vacuo. The resulting crude methyl 4-amino-5,6-dihydro-2H-pyran-3-carboxylate **3c** was then dissolved in acetonitrile (50 mL), and 2,2,2-trichloroacetyl isocyanate (7.2 g, 38.46 mmol) was added. The resulting mixture was stirred for 30 min, and the precipitated solids were collected and dissolved in a solution of ammonia in methanol (10 mL, 7 N). Then the resulting

Table 3
P97 inhibitory activities of compounds **32–35**.[a]



Compd.	R^2	IC_{50} (nM)
35		36.4
36		134.9
37		34.6
38		100.9

[a] All experiments were repeated three times.

Table 4
Cell viabilities of representative compounds against A549 cancer cell lines.[a]

Compd	IC_{50} (μM)
23	2.6
28	4.6
35	1.6
37	2.1
CB-5083	1.3

[a] All experiments were repeated at least three times.

Table 5
Stabilities of compounds **35** and **CB-5083** in liver microsomes of various species.[a]

Liver microsomes	**35**		**CB-5083**	
	$T_{1/2}$ (min)	CL_{int} (μL/min/kg)	$T_{1/2}$ (min)	CL_{int} (μL/min/kg)
Human	42.3	29.5	25.8	48.4
Mouse	47.8	114.2	141.4	38.6
Rat	16.8	148.7	17.0	146.5
Dog	36.3	55.0	36.5	54.7
Monkey	12.3	152.3	14.7	127.4

[a] All experiments were repeated at least three times.

Table 6
Single-dose pharmacokinetic parameters of compound **35** following iv and oral in SD rats.[a]

PK parameters	C_{max} (ng/mL)	T_{max} (h)	$T_{1/2}$ (h)	AUC_{0-t} (ng·h/mL)	AUC_{0-inf} (ng·h/mL)	MRT(h)	F(%)
iv (5 mg/kg)	3376.3	0.05	3.64	6947	7422	4.51	
ig (30 mg/kg)	190.9	2.67	4.57	1770	2564	7.08	4.2

[a] iv and ig dose formulations: solution in PEG400/ Castor oil/EtOH/water (40:10:5:45, v/v/v/v). n = 3 animals per study.

mixture was heated at 70 °C for 1 h. The reaction was cooled down and the precipitated solids were collected and dried to afford the diol (**4c**) (3.8 g, yield 65%, purity 97%) as a white solid. MS (ESI) *m/z* 169.2 $[M+H]^+$.

A solution of **4c** (3.8 g, 22.7 mmol) in $POCl_3$ (20 mL) was refluxed and stirred for 3 h. After being cooled to room temperature, the mixture was concentrated in vacuo. The residue was diluted with water (100 mL) and extracted with DCM (50 mL × 3). The combined organic layers were washed with brine (50 mL), dried over Na_2SO_4, filtered and concentrated in vacuo. The residue was purified by column chromatography (petroleum ether/EtOAc = 25:1) to give intermediate 2,4-dichloro-7,8-dihydro-5H-pyrano[4,3-*d*]pyrimidine (1.4 g, yield 32%, purity 97%). MS (ESI) *m/z* 203.4 $[M+H]^+$.

To a solution of 2,4-dichloro-7,8-dihydro-5H-pyrano[4,3-*d*]pyrimidine (1.4 g, 6.83 mmol) in acetonitrile (50 mL) was added phenylmethanamine (2.2 g, 20.5 mmol) and triethylamine (2.1 g, 20.5 mmol). The resulting solution was then stirred at room temperature for 12 h and concentrated in vacuo. The residue was purified by column chromatography (petroleum ether/EtOAc = 5:1) to afford compound **5c** (1.6 g, yield 86%, purity 98%). MS (ESI) *m/z* 276.6 $[M+H]^+$; ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.39–7.18 (m, 5H, Ph), 4.55 (d, J = 5.9 Hz, 2H, OCH_2), 4.44 (s, 2H, CH_2Ph), 3.88 (t, J = 5.6 Hz, 2H, $OC\underline{H_2}CH_2$), 2.62 (t, J = 5.0 Hz, 2H, $OCH_2C\underline{H_2}$).

4.2.2. 2-methyl-1H-indole-4-carbonitrile (9).

To a solution of 1H-indole-4-carbonitrile (**6**) (5 g, 35.17 mmol) in THF (100 mL) was added NaH (1.3 g, 52.75 mmol) at 0 °C. The mixture was stirred for 5 min and benzenesulfonyl chloride (6.5 g, 42.21 mmol) was then added. The reaction was allowed to room temperature and stirred for an additional 30 min and then poured into a precooled saturated aqueous NH_4Cl solution (300 mL). The aqueous phase was separated and extracted with ethyl acetate (100 mL × 3); the combined organic layers were washed with water (100 mL) and brine (100 mL), dried over Na_2SO_4, filtered and evaporated in vacuo. The residue was recrystallized (EtOAc) to give the 1-(phenylsulfonyl)-1H-indole-4-carbonitrile (**7**) (7.4 g, yield 75%, purity 98%). MS (ESI) *m/z* 283.1 $[M+H]^+$.

At −40 °C, to the solution of **7** (7.4 g, 26.38 mmol) in THF (100 mL) was slowly added LDA (2.0 M in THF, 26.4 mL, 52.8 mmol). The mixture was stirred for an additional 1 h and then MeI (7.5 g, 52.76 mmol) was added. The resulting mixture was then allowed to warm to room temperature and stirred for an extra 12 h. The mixture was poured into a precooled saturated aqueous NH_4Cl solution (200 mL) and extracted with ethyl acetate (100 mL × 3); the combined organic layers were washed with water (100 mL) and brine (100 mL), dried over Na_2SO_4, filtered and evaporated in vacuo. The residue was purified by column chromatography (petroleum ether/EtOAc = 20:1) to give 2-methyl-1-(phenylsulfonyl)-1H-indole-4-carbonitrile (**8**) (5.4 g, yield 69%, purity 98%) as a white solid. MS (ESI) *m/z* 297.4 $[M+H]^+$.

At room temperature, to a solution of intermediate (**8**) (5.4 g, 18.19 mmol) in ethanol (100 mL) was added aqueous sodium hydroxide solution (4 M, 18.2 mL, 72.76 mmol). Then the mixture was stirred at 40 °C for 12 h. The resulting solution was concentrated in vacuo and diluted with water (50 mL) and extracted with ethyl acetate (50 mL × 3); the combined organic layers were washed with water (50 mL) and brine (50 mL), dried over Na_2SO_4, filtered and evaporated in vacuo. The residue was purified by column chromatography (petroleum ether/EtOAc = 20:1) to give 2-methyl-1H-indole-4-carbonitrile (**9**) (2.6 g, yield 90%, purity 98%) as a yellow solid. MS (ESI) *m/z* 157.3 $[M+H]^+$; ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.61 (m, 1H, Ph), 7.43 (m, 1H, Ph), 7.13 (m, 1H, Ph), 6.30 (m, 1H, 3-H of indole), 2.44 (s, 3H, CH_3).

4.2.3. N-(2-methyl-1H-indol-4-yl)acetamide (13a)

At room temperature, to a solution of 3-nitroaniline (1 g, 7.25 mmol) in DMSO (20 mL) was added acetone (0.8 g, 14.5 mmol). The mixture was stirred for 5 min, and *t*-BuOK (1.2 g, 10.87 mmol) was then added. The reaction mixture was stirred at room temperature for 24 h and then was added water (50 mL) and the pH was adjusted to 4 and extracted with DCM (50 mL × 3); the combined organic layers were washed with water (50 mL) and brine (50 mL), dried over Na_2SO_4, filtered and evaporated in vacuo. The residue was purified by column chromatography (petroleum ether/EtOAc = 10:1) to give 2-methyl-4-nitro-1H-indole (**11**) (0.8 g, yield 62%, purity 98%) as yellow solid. MS (ESI) *m/z* 175.4 $[M-H]^-$.

At room temperature to a solution of **11** (0.8 g, 4.48 mmol) in DCM (30 mL) were added $(Boc)_2O$ (1.5 g, 6.72 mmol) and DMAP (109 mg, 0.89 mmol). Then the mixture was stirred for 3 h. The reaction was washed with water (50 mL) and brine (50 mL). The separated organic layer was concentrated in vacuo to give the crude tert-butyl 2-methyl-4-nitro-1H-indole-1-carboxylate (1.1 g, yield 91%, purity 95%) as yellow solid, which was used in the next step without further purification. MS (ESI) *m/z* 275.3 $[M-H]^-$, ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.47 (d, J = 8.3 Hz, 1H, Ph), 8.14 (dd, J_1 = 0.8 Hz, J_2 = 8.2 Hz, 1H, Ph), 7.44 (t, J = 8.2 Hz, 1H, Ph), 7.09 (s, 1H, 3-H of indole), 2.64 (s, 3H, CH_3), 1.65 (s, 9H, CH_3).

At room temperature, to a solution of tert-butyl 2-methyl-4-nitro-1H-indole-1-carboxylate (1.1 g, 4.05 mmol) in ethanol (40 mL) was added Fe (1.1 g, 20.26 mmol) and saturated ammonium chloride solution (8 mL). Then the mixture was stirred at 60 °C for 3 h. The resulting solution was filtered and concentrated in vacuo and then the resulting solid was diluted with water (50 mL) and extracted with DCM (50 mL × 3); the combined organic layers were washed with water (50 mL) and brine (50 mL), dried over Na_2SO_4, filtered and evaporated in vacuo. The residue was purified by column chromatography (petroleum ether/EtOAc = 10:1) to give tert-butyl 4-amino-2-methyl-1H-indole-1-carboxylate (**12**) as yellow solid (0.8 g, yield 78%, purity 98%). MS (ESI) *m/z* 247.2 $[M+H]^+$.

At room temperature, to a solution of **12** (0.8 g, 3.17 mmol) in DCM (30 mL) were added acetyl chloride (298 mg, 3.8 mmol) and Et_3N (0.96 g, 9.51 mmol). Then the mixture was stirred for 3 h. The reaction solution was concentrated in vacuo to give the crude tert-butyl 4-acetamido-2-methyl-1H-indole-1-carboxylate (813 mg, yield 89%, purity 95%), which was used in the next step without further purification. MS (ESI) *m/z* 289.5 $[M+H]^+$.

To a 0 °C solution of tert-butyl 4-acetamido-2-methyl-1H-indole-1-carboxylate (813 mg, 2.82 mmol) in DCM (15 mL) was added trifluoroacetic acid (3.2 g, 28.2 mmol). Then the reaction was stirred at room temperature for an additional 3 h and evaporated in vacuo. The residue was purified by column chromatography (petroleum ether/EtOAc = 3:1) to give N-(2-methyl-1H-indol-4-yl)acetamide (**13a**) as brown solid (477 mg, yield 90%, purity 98%). MS (ESI) *m/z* 189.0 $[M+H]^+$; ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.50 (m, 1H, Ph), 6.99 (m, 1H, Ph), 6.89 (t, J = 7.9 Hz, 1H, Ph), 6.37 (s, 1H, 3-H of indole), 2.37 (s, 3H, CH_3), 2.11 (s, 3H, CH_3).

*4.2.4. 1-(4-(benzylamino)-7,8-dihydro-5H-pyrano[4,3-d]pyrimidin-2-yl)-2-methyl-1H-indole-4-carbonitrile **(16)***

At room temperature, to the solution of *N*-benzyl-2-chloro-7,8-dihydro-5H-pyrano [4,3-d]pyrimidin-4-amine (**5c**) (200 mg, 0.725 mmol) and 2-methyl-1H-indole-4-carbonitrile (**9**) (113 mg, 0.725 mmol) in 1,4-dioxane (20 mL) was added cesium carbonate (354 mg, 1.09 mmol), $Pd_2(dba)_3$ (100 mg, 0.11 mmol) and X-Phos (52 mg, 0.11 mmol). The mixture was degassed and refilled with nitrogen three times. The resulting mixture was stirred at 105 °C for 12 h and cooled to room temperature. The volatiles were evaporated in vacuo, and the resulting residue was dissolved in DCM (50 mL), washed with water (50 mL) and brine (30 mL), dried over Na_2SO_4, filtered, and evaporated in vacuo. The residue was purified by column chromatography (petroleum ether/EtOAc = 4:1) to give intermediate **16** (212 mg, yield 74%, purity 98%). MS (ESI) *m/z* 396.2 $[M+H]^+$; ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.92 (d, J = 8.4 Hz, 1H, Ph), 7.52 (dd, J_1 = 0.9 Hz, J_2 = 7.5 Hz, 1H, Ph), 7.37–7.23 (m, 5H, Ph), 7.02 (m, 1H, Ph), 6.53 (s, 1H, 3-H of indole), 4.63 (d, J = 5.9 Hz, 2H, OCH_2), 4.61 (s, 2H, CH_2Ph) , 3.98 (t, J = 5.6 Hz, 2H, $OC\underline{H}_2CH_2$), 2.76 (t, J = 5.7 Hz, 2H, $OCH_2C\underline{H}_2$), 1.31–1.20 (m, 3H, CH_3).

*4.2.5. 1-(4-(benzylamino)-7,8-dihydro-5H-pyrano[4,3-d]pyrimidin-2-yl)-2-methyl-1H-indole-4-carboxamide **(CB-5083)***

At room temperature, to the solution of the **16** (262 mg, 0.663 mmol) in ethanol (12 mL) was added PPh_3 (34 mg, 0.13 mmol), $Pd(OAc)_2$ (29.2 mg, 0.13 mmol), acetaldehyde oxime (35.4 mg, 0.60 mmol) and water (1.5 mL). The resulting mixture was refluxed for 2 h, cooled down to room temperature and concentrated in vacuo. The residue was purified by flash chromatography (petroleum ether/EtOAc = 2:1) to afford **CB-5083** (191 mg, yield 70%, purity 99%). ^{1}H NMR (400 MHz, CD_3OD) δ 7.75–7.70 (m, 1H, Ph), 7.59–7.52 (m, 1H, Ph), 7.48–7.44 (m, 1H, Ph), 7.36–7.28 (m, 5H, Ph), 6.78 (t, J = 1.0 Hz, 1H, 3-H of indole), 4.71 (s, 2H, OCH_2), 4.64 (s, 2H, CH_2Ph), 4.05 (t, J = 5.7 Hz, 2H, $OC\underline{H}_2CH_2$), 2.82 (m, 2H, $OCH_2C\underline{H}_2$), 2.45 (s, 3H, CH_3). ^{13}C NMR (100 MHz, CD_3OD) δ 174.08, 160.60, 156.40, 140.56, 138.89, 133.80, 133.08, 129.99, 128.59, 129.58, 127.87, 125.74, 125.25, 121.96, 117.40, 109.80, 105.42, 65.67, 64.08, 45.19, 31.78, 15.56. m.p.: 71–73 °C. HRMS calcd for $C_{24}H_{23}N_5O_2$ $[M+Na]^+$ 436.1743, found 436.1747.

*4.2.6. 1-(4-(benzylamino)-7,8-dihydro-5H-pyrano[4,3-d]pyrimidin-2-yl)-2-methyl-1H -indole-4-carbothioamide **(17)***

At room temperature, to the solution of **CB-5083** (100 mg, 0.24 mmol) in THF (20 mL) were added Lawesson's reagent (146.7 mg, 0.36 mmol). The resulting solution was then refluxed for overnight and concentrated in vacuo. The residue was dissolved in DCM (50 mL), washed with water (50 mL) and brine (30 mL), dried over Na_2SO_4, filtered, and evaporated in vacuo. The residue was purified by column chromatography (petroleum ether/EtOAc = 2:1) to give **17** (83 mg, yield 81%, purity 98%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.85 (s, 1H, NH), 9.31 (s, 1H, NH), 7.78 (d, J = 8.3 Hz, 1H, Ph), 7.36 (d, J = 7.5 Hz, 1H, Ph), 7.34–7.19 (m, 5H, Ph), 6.94–6.89 (m, 1H, Ph), 6.69 (s, 1H, 3-H of indole), 4.64 (d, J = 5.8 Hz, 2H, OCH_2), 4.61 (s, 2H, CH_2Ph), 3.99 (t, J = 5.6 Hz, 2H, $OC\underline{H}_2CH_2$), 2.77 (t, J = 5.4 Hz, 2H, $OCH_2C\underline{H}_2$), 2.47 (s, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6) δ 201.42, 159.09, 158.75, 154.67, 139.70, 138.00, 136.49, 132.91, 128.42, 126.71, 126.54, 125.00, 120.68, 120.04, 115.22, 108.34, 104.44, 63.97, 62.74, 43.65, 30.72, 15.66. m.p.: 72–74 °C. HRMS calcd for $C_{24}H_{23}N_5OS$ $[M+H]^+$ 430.1696, found 430.1687.

*4.2.7. N-(1-(4-(benzylamino)-7,8-dihydro-5H-pyrano[4,3-d]pyrimidin-2-yl)-1H-indol −4-yl)acetamide **(18)***

Compound **18** was synthesized from **5c** and N-(1H-indol-4-yl)acetamide according to the procedure for preparing **16**. 72% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.68 (s, 1H, NH), 8.25 (d, J = 8.4 Hz, 1H, Ph), 8.10 (d, J = 3.7 Hz, 1H, Ph), 7.65 (dd, J_1 = 4.1 Hz, J_2 = 7.2 Hz, 1H, 2-H of indole), 7.43–7.37 (m, 2H, Ph), 7.33 (dd, J_1 = 6.9 Hz, J_2 = 8.4 Hz, 2H, Ph), 7.25–7.18 (m, 1H, Ph), 7.05 (t, J = 8.1 Hz, 1H, Ph), 6.91 (d, J = 3.7 Hz, 1H, 3-H of indole), 4.72 (d, J = 5.8 Hz, 2H, OCH_2), 4.55 (s, 2H, CH_2Ph), 3.96 (t, J = 5.6 Hz, 2H, $OC\underline{H}_2CH_2$), 2.74 (t, J = 5.6 Hz, 2H, $OCH_2C\underline{H}_2$), 2.13 (s, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6) δ 168.56, 158.91, 158.54, 154.81, 139.78, 135.28, 130.85, 128.36, 126.93, 126.75, 124.86, 123.04, 113.27, 111.92, 107.04, 102.98, 64.00, 62.76, 43.92, 31.20, 23.89. m.p.: 88–90 °C. HRMS calcd for $C_{24}H_{23}N_5O_2$ $[M+Na]^+$ 436.1743, found 436.1747.

*4.2.8. 3-((4-(benzylamino)-7,8-dihydro-5H-pyrano[4,3-d]pyrimidin-2-yl) amino) benzamide **(19)***

Compound **19** was synthesized from **5c** and 3-aminobenzonitrile according to the procedure for preparing **CB-5083**. 66% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.83–7.74 (m, 1H, Ph), 7.40–7.11 (m, 8H, Ph), 4.64 (d, J = 5.9 Hz, 2H, OCH_2), 4.46 (s, 2H, CH_2Ph), 3.89 (t, J = 5.6 Hz, 2H, $OC\underline{H}_2CH_2$), 2.56 (t, J = 5.7 Hz, 2H, $OCH_2C\underline{H}_2$). ^{13}C NMR (100 MHz, DMSO-d_6) δ 174.59, 168.53, 158.18, 140.20, 134.87, 134.84, 128.20, 128.00, 127.09, 126.57, 120.95, 119.09, 117.99, 102.53, 64.06, 62.89, 43.49, 31.38. m.p.: 224–226 °C. HRMS calcd for $C_{21}H_{21}N_5O_2$ $[M+Na]^+$ 398.1587, found 398.1591.

*4.2.9. 1-(4-(benzylamino)-6,7,8,9-tetrahydro-5H-cyclohepta[d]pyrimidin-2-yl)-2- methyl-1H-indole-4-carboxamide **(20)***

Compound **20** was synthesized from **5a** and **9** according to the procedure for preparing **CB-5083**. 65% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.87–7.83 (m, 1H, Ph), 7.64–7.60 (m, 1H, Ph), 7.58–7.54 (m, 1H, Ph), 7.34–7.22 (m, 5H, Ph), 6.82 (s, 1H, 3-H of indole), 4.63 (d, J = 5.8 Hz, 2H, CH_2Ph), 2.89–2.74 (m, 4H, CH_2), 2.47 (s, 3H, CH_3), 1.90–1.83 (m, 2H, CH_2), 1.69–1.58 (m, 4H, CH_2). ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.16, 169.48, 160.67, 154.52, 140.03, 138.97, 137.73, 128.90, 128.70, 128.58, 127.64, 127.60, 127.35, 124.09, 117.34, 113.58, 104.46, 45.75, 38.16, 26.51, 25.74, 25.46, 16.30. m.p.: 62–64 °C. HRMS calcd for $C_{26}H_{27}N_5O$ $[M+H]^+$ 426.2288, found 426.2256.

*4.2.10. 1-(4-(benzylamino)-5,6,7,8,9,10-hexahydrocycloocta[d] pyrimidin-2-yl)-2- methyl-1H-indole-4-carboxamide **(21)***

Compound **21** was synthesized from **5b** and **9** according to the procedure for preparing **CB-5083**. 60% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.84–7.77 (m, 1H, Ph), 7.63–7.59 (m, 1H, Ph), 7.57–7.53 (m, 1H, Ph), 7.45–7.14 (m, 5H, Ph), 6.83 (d, J = 4.5 Hz, 1H, 3-H of indole), 4.63 (d, J = 5.9 Hz, 2H, CH_2Ph), 2.85–2.74 (m, 4H, CH_2), 2.46 (s, 3H, CH_3), 1.79–1.63 (m, 4H, CH_2), 1.51–1.36 (m, 4H, CH_2). ^{13}C NMR (100 MHz, DMSO-d_6) δ 169.89, 169.53, 160.76, 140.24, 138.37, 136.98, 136.89, 132.10, 131.58, 131.48, 128.87, 128.76, 128.38, 126.34, 119.04, 118.79, 113.25, 111.39, 100.47, 43.83, 34.02, 31.19, 29.08, 25.97, 25.81, 13.49. m.p.: 50–52 °C. HRMS calcd for $C_{27}H_{29}N_5O$ $[M+Na]^+$ 462.2264, found 462.2266.

*4.2.11. 2-methyl-1-(4-((pyridin-4-ylmethyl)amino)-7,8-dihydro-5H-pyrano[4,3-d] pyrimidin-2-yl)-1H-indole-4-carboxamide **(22)***

Compound **22** was synthesized from **5d** and **9** according to the procedure for preparing **CB-5083**. 72% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.61 (d, J = 5.9 Hz, 2H, Py), 7.97–7.86 (m, 1H, Ph), 7.52 (d, J = 5.1 Hz, 2H, Ph), 7.45 (d, J = 7.4 Hz, 1H, Py), 7.40–7.36 (m, 1H, Py), 6.83 (d, J = 4.5 Hz, 1H, 3-H of indole), 4.71 (d, J = 5.7 Hz, 2H, CH_2Py), 4.65 (s, 2H, OCH_2), 4.00 (t, J = 5.6 Hz, 2H, $OC\underline{H}_2CH_2$), 2.78 (t, J = 5.7 Hz, 2H, $OCH_2C\underline{H}_2$), 2.18 (t, J = 7.3 Hz, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6) δ 169.51, 159.35, 158.74, 154.66, 149.53, 138.33, 136.88, 127.00, 125.31, 121.80, 120.60, 115.88, 108.52, 105.23, 64.01, 62.71, 42.92, 30.73, 15.59. m.p.: 76–78 °C. HRMS calcd for $C_{23}H_{22}N_6O_2$ $[M+Na]^+$ 437.1696, found 437.1727.

*4.2.12. 2-methyl-1-(4-((2-morpholinoethyl)amino)-7,8-dihydro-5H-pyrano[4,3-d] pyrimidin-2-yl)-1H-indole-4-carboxamide **(23)**.*

Compound **23** was synthesized from **5e** and **9** according to the procedure for preparing **CB-5083**. 70% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.22 (d, J = 8.2 Hz, 1H, Ph), 7.51 (dd, J_1 = 0.9 Hz, J_2 = 7.5 Hz, 1H, Ph), 7.12 (dd, J_1 = 7.4 Hz, J_2 = 8.3 Hz, 1H, Ph), 6.92–6.89 (m, 1H, 3-H of indole), 4.47 (s, 2H, CH_2), 3.95 (t, J = 5.6 Hz, 2H, CH_2), 3.55 (t, J = 4.7 Hz, 6H, CH_2), 2.74 (t, J = 5.7 Hz, 2H, CH_2), 2.65 (d, J = 1.1 Hz, 2H, CH_2), 2.40 (s, 4H, CH_2), 1.75 (s, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6) δ 169.52, 158.89, 158.79, 154.81, 138.39, 137.02, 127.11, 125.39, 120.67, 116.24, 108.09, 105.28, 66.28, 63.98, 62.67, 55.01, 53.41, 31.38, 14.05. m.p.: 58–60 °C. HRMS calcd for $C_{23}H_{28}N_6O_3$ $[M+Na]^+$ 459.2115, found 459.2118.

*4.2.13. 1-(4-((cyclopropylmethyl)amino)-7,8-dihydro-5H-pyrano[4,3-d] pyrimidin-2-yl) −2-methyl-1H-indole-4-carboxamide **(24)***

Compound **24** was synthesized from **5f** and **9** according to the procedure for preparing **CB-5083**. 65% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.77 (s, 1H, Ph), 7.51 (dd, J_1 = 0.9 Hz, J_2 = 7.5 Hz, 1H, Ph), 7.45–7.34 (m, 1H, Ph), 6.91 (t, J = 1.0 Hz, 1H, 3-H of indole), 4.50 (s, 2H, OCH_2), 3.96 (t, J = 5.6 Hz, 2H, $OC\underline{H}_2CH_2$), 3.30 (t, J = 6.2 Hz, 2H, CH_2Ph), 2.74 (t, J = 5.7 Hz, 2H, $OCH_2C\underline{H}_2$), 2.65 (d, J = 1.0 Hz, 3H, CH_3), 0.88–0.81 (m, 1H, CH), 0.46–0.40 (m, 2H, CH_2), 0.25–0.20 (m, 2H, CH_2). ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.56, 169.54, 158.80, 147.16, 138.37, 137.06, 127.10, 125.38, 120.62, 116.24, 107.91, 105.24, 63.98, 62.80, 55.00, 31.21, 14.05, 10.91, 3.49. m.p.: 50–52 °C. HRMS calcd for $C_{21}H_{23}N_5O_2$ $[M+Na]^+$ 400.1743, found 400.1747.

*4.2.14. 1-(4-(benzylamino)pyrimidin-2-yl)-2-methyl-1H-indole-4-carboxamide **(25)***

Compound **25** was prepared from commercially available 2,4-dichloro pyrimidine **14**, which firstly reacted with benzylamine to form compound **15** and then coupled with **9** via the similar procedure for preparing **CB-5083**. 60% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.06 (d, J = 6.0 Hz, 1H, CH), 7.77 (s, 1H, Ph), 7.62–7.53 (m, 2H, Ph), 7.52–7.44 (m, 2H, Ph), 7.44–7.34 (m, 2H, Ph), 6.94 (d, J = 9.7 Hz, 1H, Ph), 6.74 (s, 1H, CH), 6.43 (s, 1H, 3-H of indole), 4.58 (s, 2H, CH_2Ph), 2.45 (s, 3H, CH_3). ^{13}C NMR (100 MHz, CD_3OD) δ 174.03, 164.97, 158.42, 155.51, 140.36, 138.87, 133.81, 130.05, 129.92, 129.65, 128.72, 128.10, 125.82, 122.14, 122.01, 117.59, 105.81, 45.14, 14.47. m.p.: 58–60 °C. HRMS calcd for $C_{21}H_{19}N_5O$ $[M+Na]^+$ 380.1481, found 380.1485.

*4.2.15. N-(1-(4-(benzylamino)-7,8-dihydro-5H-pyrano[4,3-d]pyrimidin-2-yl)-2- methyl-1H-indol-4-yl)acetamide **(26)***

Compound **26** was synthesized from **5c** and **13a** according to the procedure for preparing **16**. 69% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.72 (d, J = 8.3 Hz, 1H, Ph), 7.56 (d, J = 7.8 Hz, 1H, Ph), 7.28–7.17 (m, 5H, Ph), 6.95 (t, J = 8.0 Hz, 1H, Ph, 6.18 (s, 1H, 3-H of indole), 4.62 (d, J = 5.4 Hz, 2H, OCH_2), 4.46 (s, 2H, CH_2Ph), 3.96 (t, J = 5.6 Hz, 2H, $OC\underline{H}_2CH_2$), 2.79 (t, J = 5.8 Hz, 2H, $OCH_2C\underline{H}_2$), 2.14 (s, 6H, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.54, 159.98, 158.78, 155.70, 138.45, 137.57, 128.92, 127.61, 122.41, 113.84, 111.03, 107.28, 101.04, 64.99, 62.77, 45.13, 31.36, 24.66, 14.30. m.p.: 87–89 °C. HRMS calcd for $C_{25}H_{25}N_5O_2$ $[M+Na]^+$ 450.1900, found 450.1902.

*4.2.16. N-(1-(4-(benzylamino)-7,8-dihydro-5H-pyrano[4,3-d]pyrimidin-2-yl)-2- methyl-1H-indol-4-yl)-2-chloroacetamide **(27)***

Compound **27** was synthesized from **5c** and **13b** according to the procedure for preparing **16**. 73% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.53 (s, 1H, Ph), 7.51 (s, 1H, Ph), 7.37–7.21 (m, 5H, Ph), 6.88 (t, J = 8.1 Hz, 1H, Ph), 6.55 (s, 1H, 3-H of indole), 4.65 (d, J = 5.9 Hz, 2H, OCH_2), 4.59 (s, 2H, CH_2Ph), 4.37 (s, 2H, $COCH_2$), 3.98 (t, J = 5.6 Hz, 2H, $OC\underline{H}_2CH_2$), 2.75 (t, J = 5.7 Hz, 2H, $OCH_2C\underline{H}_2$), 2.46 (s, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6) δ 164.80, 158.97, 158.76, 154.70, 139.68, 136.97, 136.39, 128.66, 128.42, 126.74, 126.59, 120.95, 113.20, 110.48, 108.21, 102.05, 63.97, 62.75, 43.62, 43.52, 30.66, 15.75. m.p.: 64–66 °C. HRMS calcd for $C_{25}H_{24}ClN_5O_2$ $[M+H]^+$ 462.1691, found 462.1734.

*4.2.17. N-(1-(4-(benzylamino)-7,8-dihydro-5H-pyrano[4,3-d]pyrimidin-2-yl)-2- methyl-1H-indol-4-yl)acrylamide **(28)***

Compound **28** was synthesized from **5c** and **13c** according to the procedure for preparing **16**. 64% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.74 (s, 1H), 7.64 (d, J = 2.4 Hz, 1H, Ph), 7.52 (d, J = 8.3 Hz, 1H, Ph), 7.38–7.21 (m, 5H, Ph), 6.87 (t, J = 8.1 Hz, 1H, Ph), 6.72–6.62 (m, 1H, $COC\underline{H}CH_2$), 6.60 (s, 1H, 3-H of indole), 6.26 (dd, J_1 = 2.1 Hz, J_2 = 17.0 Hz, 1H $COCHC\underline{H}_2$), 5.74 (dd, J_1 = 2.1 Hz, J_2 = 10.2 Hz, 1H $COCHC\underline{H}_2$), 4.65 (d, J = 5.8 Hz, 2H, OCH_2), 4.60 (s, 2H, CH_2Ph), 3.98 (t, J = 5.6 Hz, 2H, $OC\underline{H}_2CH_2$), 2.75 (t, J = 5.7 Hz, 2H, $OCH_2C\underline{H}_2$), 2.46 (s, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6) δ 163.24, 159.03, 158.73, 154.80, 139.70, 136.94, 136.13, 132.03, 129.22, 128.39, 126.70, 126.58, 121.57, 113.08, 110.11, 108.12, 102.22, 66.37, 63.97, 43.59, 30.70, 15.77. m.p.: 36–38 °C. HRMS calcd for C26$H_{25}N_5O_2$ $[M+H]^+$ 440.2081, found 440.2084.

*4.2.18. N-(1-(4-(benzylamino)-7,8-dihydro-5H-pyrano[4,3-d]pyrimidin-2-yl)-2-methyl −1H-indol-4-yl)cinnamamide **(29)***

Compound **29** was synthesized from **5c** and **13d** according to the procedure for preparing **16**. 75% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.76 (s, 1H, NH), 7.68–7.64 (m, 1H, Ph), 7.63 (s, 1H, Ph), 7.53 (d, J = 8.3 Hz, 1H, CH), 7.50–7.22 (m, 10*H*, Ph), 7.13 (d, J = 15.7 Hz, 1H, CH), 6.89 (t, J = 8.1 Hz, 1H, Ph), 6.67 (s, 1H, 3-H of indole), 4.66 (d, J = 5.9 Hz, 2H, OCH_2), 4.60 (s, 2H, CH_2Ph), 3.98 (t, J = 5.7 Hz, 2H, $OC\underline{H}_2CH_2$), 2.76 (t, J = 5.7 Hz, 2H, $OCH_2C\underline{H}_2$), 2.49–2.45 (s, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6) δ 159.05, 158.75, 154.83, 139.91, 139.72, 136.95, 136.13, 135.00, 129.09, 128.41, 127.75, 126.73, 126.59, 122.63, 121.69, 120.46, 108.14, 102.12, 63.99, 62.76, 43.60, 31.21, 15.83. m.p.: 89–91 °C. HRMS calcd for $C_{32}H_{29}N_5O_2$ $[M+Na]^+$ 538.2213, found 538.2216.

*4.2.19. (E)-N-(1-(4-(benzylamino)-7,8-dihydro-5H-pyrano[4,3-d]pyrimidin-2-yl)-2- methyl-1H-indol-4-yl)-4-(dimethylamino)but-2-enamide **(30)***

Compound **30** was synthesized from **5c** and **13e** according to the procedure for preparing **16**. 60% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.67 (s, 1H, NH), 7.72–7.60 (m, 1H, Ph), 7.52 (d, J = 8.3 Hz, 1H, Ph), 7.43–7.28 (m, 5H, Ph), 6.96–6.83 (m, 1H, Ph), 6.79–6.68 (m, 1H, CH), 6.62 (s, 1H, CH), 6.60–6.50 (m, 1H, 3-H of indole), 4.65 (d, J = 5.9 Hz, 2H, OCH_2), 4.59 (s, 2H, CH_2Ph), 3.98 (t, J = 5.6 Hz, 2H, $OC\underline{H}_2CH_2$), 3.17 (d, J = 6.0 Hz, 2H, CH_2), 2.75 (t, J = 5.6 Hz, 2H, $OCH_2C\underline{H}_2$), 2.46 (s, 3H, CH_3), 2.26 (s, 6H, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6) δ 174.66, 163.21, 159.05, 158.76, 154.85, 139.74, 136.97, 136.07, 128.43, 126.74, 126.61, 121.61, 112.93, 110.55, 110.02, 108.14, 102.30, 64.01, 62.80, 59.50, 48.68, 43.62, 31.40, 13.51. m.p.: 60–62 °C. HRMS calcd for $C_{29}H_{32}N_6O_2$ $[M+Na]^+$ 519.2478, found 519.2483.

*4.2.20. N-(1-(4-(benzylamino)-7,8-dihydro-5H-pyrano[4,3-d]pyrimidin-2-yl)-2-methyl-1H-indol-4-yl)-2-(pyrrolidin-1-yl)acetamide **(31)***

Compound **31** was synthesized from **5c** and **13f** according to the procedure for preparing **16**. 72% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.56 (d, J = 7.7 Hz, 1H, Ph), 7.51 (d, J = 8.3 Hz, 1H, Ph), 7.37–7.20 (m, 5H, Ph), 6.87 (t, J = 8.0 Hz, 1H, Ph), 6.34 (m, 1H, 3-H of indole), 4.64 (d, J = 5.9 Hz, 2H, OCH_2), 4.59 (s, 2H, CH_2Ph), 3.98 (t, J = 5.6 Hz, 2H, $OC\underline{H}_2CH_2$), 3.31 (s, 2H, $COCH_2$), 2.75 (t, J = 5.6 Hz, 2H, $OCH_2C\underline{H}_2$), 2.65 (m, 4H, $NC\underline{H}_2CH_2$), 2.45 (s, 3H, CH_3), 1.78 (m, 4H, $NCH_2C\underline{H}_2$). ^{13}C NMR (100 MHz, DMSO-d_6) δ 168.87, 159.46, 159.14, 155.20, 140.12, 137.29, 136.94, 129.23, 128.82, 127.13, 127.00, 122.08, 120.91, 112.89, 110.39, 108.58, 101.56, 64.39, 63.16, 59.51, 54.23, 44.03, 31.12, 23.99, 16.17. m.p.: 59–61 °C. HRMS calcd for $C_{29}H_{32}N_6O_2$ $[M+H]^+$ 497.2659, found 497.2697.

*4.2.21. N-(2-methyl-1-(4-((pyridin-4-ylmethyl)amino)-7,8-dihydro-5H-pyrano[4,3-d] pyrimidin-2-yl)-1H-indol-4-yl)acrylamide **(32)***

Compound **32** was synthesized from **5d** and **13c** according to the procedure for preparing **16**. 78% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.73 (s, 1H, NH), 8.51 (d, J = 4.8 Hz, 2H, Py), 7.77–7.58 (m, 2H, Py), 7.41 (d, J = 8.4 Hz, 1H, Ph), 7.32 (d, J = 5.0 Hz, 2H, Ph), 6.83 (t, J = 8.1 Hz, 1H, 3-H of indole), 6.58 (s, 1H, COC<u>H</u>CH$_2$), 6.26 (d, J = 16.9 Hz, 1H, COCHC<u>H$_2$</u>), 5.74 (d, J = 10.3 Hz, 1H, COCHC<u>H$_2$</u>), 4.65 (s, 1H, CH), 4.63 (s, 2H, CH$_2$Ph), 4.62–4.42 (m, 2H, OCH$_2$), 3.99 (t, J = 5.8 Hz, 2H, OC<u>H$_2$</u>CH$_2$), 2.76 (s, 2H, OCH$_2$C<u>H$_2$</u>), 2.39 (s, 3H, CH$_3$). ^{13}C NMR (100 MHz, DMSO-d_6) δ 163.26, 158.60, 151.99, 146.17, 136.86, 136.55, 136.04, 131.99, 129.30, 126.62, 122.81, 121.48, 120.79, 113.15, 102.35, 66.40, 64.00, 43.14, 32.95, 15.65. m.p.: 112–114 °C. HRMS calcd for $C_{26}H_{25}N_5O_2$ [M + Na]$^+$ 463.1900, found 463.1928.

*4.2.22. N-(1-(4-((cyclopropylmethyl)amino)-7,8-dihydro-5H-pyrano[4,3-d]pyrimidin- 2-yl)-2-methyl-1H-indol-4-yl)acrylamide **(33)***

Compound **33** was synthesized from **5f** and **13c** according to the procedure for preparing **16**. 68% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.77 (s, 1H, NH), 7.86 (d, J = 8.3 Hz, 1H, Ph), 7.71 (d, J = 7.7 Hz, 1H, Ph), 7.39 (d, J = 8.7 Hz, 1H, Ph), 7.13–6.96 (m, 1H, COCH), 6.76–6.60 (m, 1H, 3-H of indole), 6.27 (dd, J_1 = 2.1 Hz, J_2 = 17.0 Hz, 1H, COCHC<u>H$_2$</u>), 5.80–5.71 (m, 1H, COCHC<u>H$_2$</u>), 4.49 (s, 2H, OCH$_2$), 3.95 (t, J = 5.7 Hz, 2H, OC<u>H$_2$</u>CH$_2$), 3.29 (d, J = 6.1 Hz, 2H, CH$_2$), 2.76–2.69 (m, 2H, OCH$_2$C<u>H$_2$</u>), 2.63 (dd, J_1 = 1.0 Hz, J_2 = 4.5 Hz, 3H, CH$_3$), 1.14–1.06 (m, 1H, CH), 0.45–0.39 (m, 2H, CH$_2$), 0.25–0.19 (m, 2H, CH$_2$). ^{13}C NMR (100 MHz, DMSO-d_6) δ 163.29, 158.82, 158.73, 154.87, 137.07, 136.14, 132.06, 129.38, 126.63, 121.65, 120.83, 113.10, 110.18, 107.83, 102.25, 63.97, 62.80, 55.00, 31.21, 16.11, 10.93, 3.49. m.p.: 68–70 °C. HRMS calcd for $C_{23}H_{25}N_5O_2$ [M + Na]$^+$ 426.1900, found 426.1916.

*4.2.23. N-(2-methyl-1-(4-((naphthalen-1-ylmethyl)amino)-7,8-dihydro-5H-pyrano [4,3-d]pyrimidin-2-yl)-1H-indol-4-yl)acrylamide **(34)**.*

Compound **34** was synthesized from **5g** and **13c** according to the procedure for preparing **16**. 73% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.67 (s, 1H, NH), 8.17–8.10 (m, 1H, Ph), 8.02–7.96 (m, 1H, Ph), 7.85 (d, J = 7.9 Hz, 1H, Ph), 7.76–7.68 (m, 1H, Ph), 7.58–7.53 (m, 2H, Ph), 7.50–7.34 (m, 4H, Ph), 6.52 (d, J = 4.0 Hz, 1H, COC<u>H</u>CH$_2$), 6.51–6.45 (m, 1H, 3-H of indole), 6.27–6.19 (m, 1H, COCHC<u>H$_2$</u>), 5.74–5.69 (m, 1H, COCHC<u>H$_2$</u>), 5.12 (d, J = 5.5 Hz, 2H, CH$_2$Ph), 4.66 (s, 2H, OCH$_2$), 4.03–3.96 (m, 2H, OC<u>H$_2$</u>CH$_2$), 2.77 (t, J = 5.7 Hz, 2H, OCH$_2$C<u>H$_2$</u>), 2.39–2.34 (m, 3H, CH$_3$). ^{13}C NMR (100 MHz, DMSO-d_6) δ 163.20, 159.12, 158.83, 154.88, 136.85, 134.55, 133.36, 132.00, 130.75, 128.64, 127.24, 126.23, 125.86, 125.55, 123.38, 123.15, 121.26, 120.69, 108.25, 102.29, 64.02, 62.84, 55.00, 31.21, 14.05. m.p.: 82–84 °C. HRMS calcd for $C_{30}H_{27}N_5O_2$ [M + H]$^+$ 490.2237, found 490.2242.

*4.2.24. N-(1-(4-(benzylamino)-7,8-dihydro-5H-pyrano[4,3-d]pyrimidin-2-yl)-2-methyl − 1H-indol-4-yl)methanesulfonamide **(35)***

Compound **35** was synthesized from **5c** and **13g** according to the procedure for preparing **16** in 76% yield. ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.37–7.33 (m, 5H, Ph), 7.20 (d, J = 7.6 Hz, 1H, Ph), 7.08 (t, J = 8.0 Hz, 1H, Ph), 6.44 (s, 1H, 3-H of indole), 6.36 (s, 1H, Ph), 4.76 (d, J = 5.3 Hz, 2H, OCH$_2$), 4.58 (s, 2H, CH$_2$Ph), 4.08 (t, J = 5.6 Hz, 2H, OC<u>H$_2$</u>CH$_2$), 2.95 (s, 3H, CH$_3$), 2.92 (t, J = 5.7 Hz, 2H, OCH$_2$C<u>H$_2$</u>), 2.62 (s, 3H, CH$_3$). ^{13}C NMR (100 MHz, DMSO-d_6) δ 159.06, 158.75, 154.75, 139.70, 137.19, 136.83, 128.42, 128.20, 126.72, 126.54, 123.35, 121.79, 115.41, 111.17, 108.23, 102.38, 63.98, 62.75, 43.60, 30.71, 15.82. m.p.: 111–113 °C. HRMS calcd for $C_{24}H_{25}N_5O_3S$ [M + H]$^+$ 464.1750, found 464.1720.

*4.2.25. N-(1-(4-(benzylamino)-7,8-dihydro-5H-pyrano[4,3-d]pyrimidin-2-yl)-2-methyl − 1H-indol-4-yl)benzenesulfonamide **(36)***

Compound **36** was synthesized from **5c** and **13h** according to the procedure for preparing **16**. 74% yield. ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.68–7.44 (m, 5H, Ph), 7.41–7.34 (m, 5H, Ph), 6.99–6.94 (m, 2H, Ph), 6.60 (s, 1H, 3-H of indole), 6.14 (t, J = 1.0 Hz, 1H, Ph), 4.72 (d, J = 5.3 Hz, 2H, OCH$_2$), 4.56 (d, J = 5.4 Hz, 2H, CH$_2$Ph), 4.06–4.00 (m, 2H, OC<u>H$_2$</u>CH$_2$), 2.89–2.83 (m, 2H, OCH$_2$C<u>H$_2$</u>), 2.52 (s, 3H, CH$_3$). ^{13}C NMR (100 MHz, DMSO-d_6) δ 158.99, 158.71, 154.66, 140.20, 139.66, 138.84, 137.04, 134.65, 132.69, 129.51, 129.11, 128.41, 128.23, 127.63, 126.72, 126.53, 121.57, 114.64, 110.99, 108.17, 102.24, 63.95, 62.73, 43.56, 31.21, 15.75. m.p.: 84–86 °C. HRMS calcd for $C_{29}H_{27}N_5O_3S$ [M + Na]$^+$ 548.1726, found 548.1731.

*4.2.26. N-(1-(4-(benzylamino)-7,8-dihydro-5H-pyrano[4,3-d]pyrimidin-2-yl)-2-methyl-1H-indol-4-yl)cyclopropanesulfonamide **(37)***

Compound **37** was synthesized from **5c** and **13i** according to the procedure for preparing **16**. 64% yield. ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.38–7.31 (m, 5H, Ph), 7.22 (d, J = 7.7 Hz, 1H, Ph), 7.06 (t, J = 8.0 Hz, 1H, Ph), 6.42 (s, 1H, 3-H of indole), 6.41 (d, J = 1.1 Hz, 1H, Ph), 4.76 (d, J = 5.4 Hz, 2H, OCH$_2$), 4.58 (d, J = 1.4 Hz, 2H, CH$_2$Ph), 4.07 (t, J = 5.6 Hz, 2H, OC<u>H$_2$</u>CH$_2$), 2.94–2.87 (m, 2H, OCH$_2$C<u>H$_2$</u>), 2.62 (d, J = 1.0 Hz, 3H, CH$_3$), 2.43 (m, 1H, CH), 1.15–1.12 (m, 2H, CH$_2$), 0.89–0.85 (m, 2H, CH$_2$). ^{13}C NMR (100 MHz, DMSO-d_6) δ 159.04, 158.75, 154.78, 139.69, 137.13, 136.68, 128.41, 128.22, 126.71, 126.54, 123.69, 121.68, 115.80, 111.11, 108.17, 102.54, 63.98, 62.76, 43.59, 30.71, 15.86, 5.15. m.p.: 83–85 °C. HRMS calcd for $C_{26}H_{27}N_5O_3S$ [M + Na]$^+$ 512.1726, found 512.1727.

*4.2.27. N-(1-(4-(benzylamino)-7,8-dihydro-5H-pyrano[4,3-d]pyrimidin-2-yl)-2- methyl-1H-indol-4-yl)-1,1,1-trifluoromethanesulfonamide **(38)***

Compound **38** was synthesized from **5c** and **13j** according to the procedure for preparing **16**. 66% yield. ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.36–7.27 (m, 5H, Ph), 7.09–7.02 (m, 2H, Ph), 6.92 (t, J = 8.0 Hz, 1H, Ph), 6.38 (s, 1H, 3-H of indole), 4.66 (s, 2H, OCH$_2$), 4.53 (s, 2H, CH$_2$Ph), 4.00 (t, J = 5.6 Hz, 2H, OC<u>H$_2$</u>CH$_2$), 2.81 (t, J = 5.7 Hz, 2H, OCH$_2$C<u>H$_2$</u>), 2.46 (s, 3H, CH$_3$). ^{13}C NMR (100 MHz, DMSO-d_6) δ 163.26, 159.35, 153.71, 139.51, 136.53, 136.29, 128.32, 128.21, 126.59, 126.42, 122.35, 120.59, 117.37, 111.16, 108.25, 103.89, 63.45, 62.80, 43.25, 31.41, 14.75. m.p.: 50–52 °C. HRMS calcd for $C_{24}H_{22}F_3N_5O_3S$ [M + Na]$^+$ 540.1287, found 540.1290.

4.3. Biological assay

4.3.1. Kinase inhibition assay

The ATPase assay was performed according to the following protocol: compounds were diluted in DMSO with a 3-fold 10-point serial dilution starting at 10 μM. The tenth concentration point was solvent control group (no drug). The assay was done in a 384-well plate with each row as a single dilution series with duplicate of each compound concentration point. In 4 μL total volume, 60 μg/mL p97 hexameric enzyme and 100 μM ATP were added to start the reaction. The plate was sealed and incubated at 25 °C for 60 min after mixing thoroughly in an orbital shaker. ADP Glo reagents 1 and 2 were added according to the manufacturer's protocol (Promega, Madison, WI). The luminescence was measured by CLARIO Star Plate Reader as the end point of the reaction. The program Graphpad Prism 6 was used to fit nonlinear curve and calculate IC_{50} of each compounds.[31]

4.3.2. Cell culture and inhibition of cell proliferation

A549 cell lines were cultured according to ATCC guidelines. Cells were plated (2000 cells/well) in a volume of 90 μL/well of complete media in 96-well cell culture plates and cultured at 37 °C with 5% CO_2 for 24 h. Inhibitors were dissolved in DMSO (less than 0.1%) and tested in duplicate utilizing 3-fold serial dilutions with the highest concentration at 10 μM. Inhibitors were incubated with cells at 37 °C with

5% CO_2. After 72 h treatment, Cell Counting Kit-8 (CCK-8) was added to the plates to measure cell viabilities. Absorbance at 450 nm was measured and using GraphPad software to fit sigmoidal curve to determine IC_{50} value.

4.3.3. In vitro liver microsome stabilities

The liver microsomal incubations consisted of PBS (pH 7.4) containing 1 μM compounds **35** and **CB-5083**, 40 mM NADP, 80 mM G6P, 100 U/mL G6PDH, 120 mM $MgCl_2$ and 0.5 mg/mL liver microsomes (mouse, rat, dog, monkey and human, respectively). The reaction mixtures were pre-incubated for 3 min at 37 °C before the addition of corresponding liver microsomes, then terminated at 0, 5, 10, 20, 30, 60 min by adding equal volume of ice-cold acetonitrile. The final concentration of organic solvents was < 0.1% in all incubations. The samples were centrifuged at 4000 rpm for 20 min at 4 °C and then analyzed by liquid chromatography-mass spectrometry (ABSciex API4000).

4.3.4. In vivo pharmacokinetics

Male Sprague-Dawley rats (200 g) were administrated with the test compounds intravenously (iv) at 5 mg/kg or orally (ig) at 30 mg/kg. Compound **35** was dissolved in mixture of 40% PEG400, 10% Castor oil, 5% EtOH and 45% buffered saline for tail-vein or oral administration. Blood samples (0.1 mL) were then obtained via orbital sinus puncture at 2 min, 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 8 h and 24 h time points and collected into heparinized tubes. Heparinized blood samples collected for PK analyses were centrifuged at 4000 rpm for 10 min at 4 °C. LC/MS/MS analysis of compound **35** was performed under optimized conditions to obtain the best sensitivity and selectivity of the analyte in selected reaction monitoring mode (SRM). Plasma concentration-time data were analyzed by a non-compartment model using the software Kinetica 5.1.

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