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Development of novel chromeno[4,3-*c*]pyrazol-4(2*H*)-one derivates containing piperazine as inhibitors of PI3Kα

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ABSTRACT

PI3K pathway has been heavily studied and is one of the most potential targets for various cancer treatment. Herein, we designed and synthesized a series of novel chromeno[4,3-*c*]pyrazol-4(2*H*)-one derivates contained piperazine based on our previous research. They were evaluated for their PI3Kα wild-type and H1047R mutant inhibitory activities and anticancer effects in vitro. Most of these compounds displayed the potential antiproliferative activities against four cancer cell lines (HCT-116, A549, Huh7 and HL60). Among them, Compound **4p** revealed the remarkable antiproliferative activity and was selected for further biological evaluation. Compound **4p** displayed the potent activity against both PI3Kα wild-type and H1047R mutant, and a certain degree of selectivity for PI3Kα over PI3Kβ, γ and δ, and meanwhile it can remarkable down-regulate the phosphorylation of Akt. In addition, compound **4p** was found to induce cell apoptosis *via* upregulation of Bax and cleaved-caspase 3/9, and downregulation of Bcl-2. The above results suggested that compound **4p** could be considered as a promising PI3Kα inhibitor.

1. Introduction

As we all known, phosphatidylinositol 3-kinases (PI3Ks) play an extremely important role in many cellular functions such as cell motility, differentiation, proliferation, growth and intracellular trafficking [1–3]. These physiological roles are regulated by phosphatidylinositol 3,4,5-triphosphate (PIP3), a second messenger who converted from phosphatidylinositol(4,5)diphosphate (PIP2) catalyzed by PI3Ks [4–7]. Meanwhile, PIP3 levels are strictly controlled by phosphatase and tensin homologue (PTEN), which converts PIP3 into PIP2 [8,9]. Based on structural features and substrate specificity, the PI3K family is categorized into three classes (classes I, II, and III) [10–12]. Class I PI3Ks comprise four different isoforms (PI3Kα, β, γ and δ) and consist of heterodimers between a p110 catalytic subunit and a p85 regulatory subunit, they are studied most extensively and have been an attractive approach for cancer therapy over the past decades [13–16]. In addition, PI3Kα and PI3Kβ are expressed commonly, nevertheless PI3Kδ and PI3Kγ are present in the central nervous system, epithelial cells and the hematopoietic system [17–21]. As one of the most frequently dysregulated signaling pathways in oncogenesis and other human pathologies, PI3K pathway have been heavily studied and is one of the most potential approaches for various cancer treatment.

Nowadays several PI3K inhibitors have been identified and developed, and they are in preclinical studies or early clinical trials, such as Wortmannin, LY294002, BEZ235, GDC-0941, GDC-0980, GSK2269557, PI-3065 and so on [22–27]. In our previous research, chromeno[4,3-*c*]pyrazol-4(2*H*)-one has been explored according to the structural feature of Wortmannin, LY294002, BEZ235 and GDC-0941. Subsequently, chromeno[4,3-*c*]pyrazol-4(2*H*)-one derivates have been identified as PI3Kα inhibitors [28,29]. However, the selectivity was unsatisfactory. Piperazine fragment is commonly found in many active molecules and used to design drug [30]. In addition, it contributes to improve the flexibility and solubility of molecule, and it is observed in the structure of GDC-0980, GSK2269557 and PI-3065 (Fig. 1). On the basis of our

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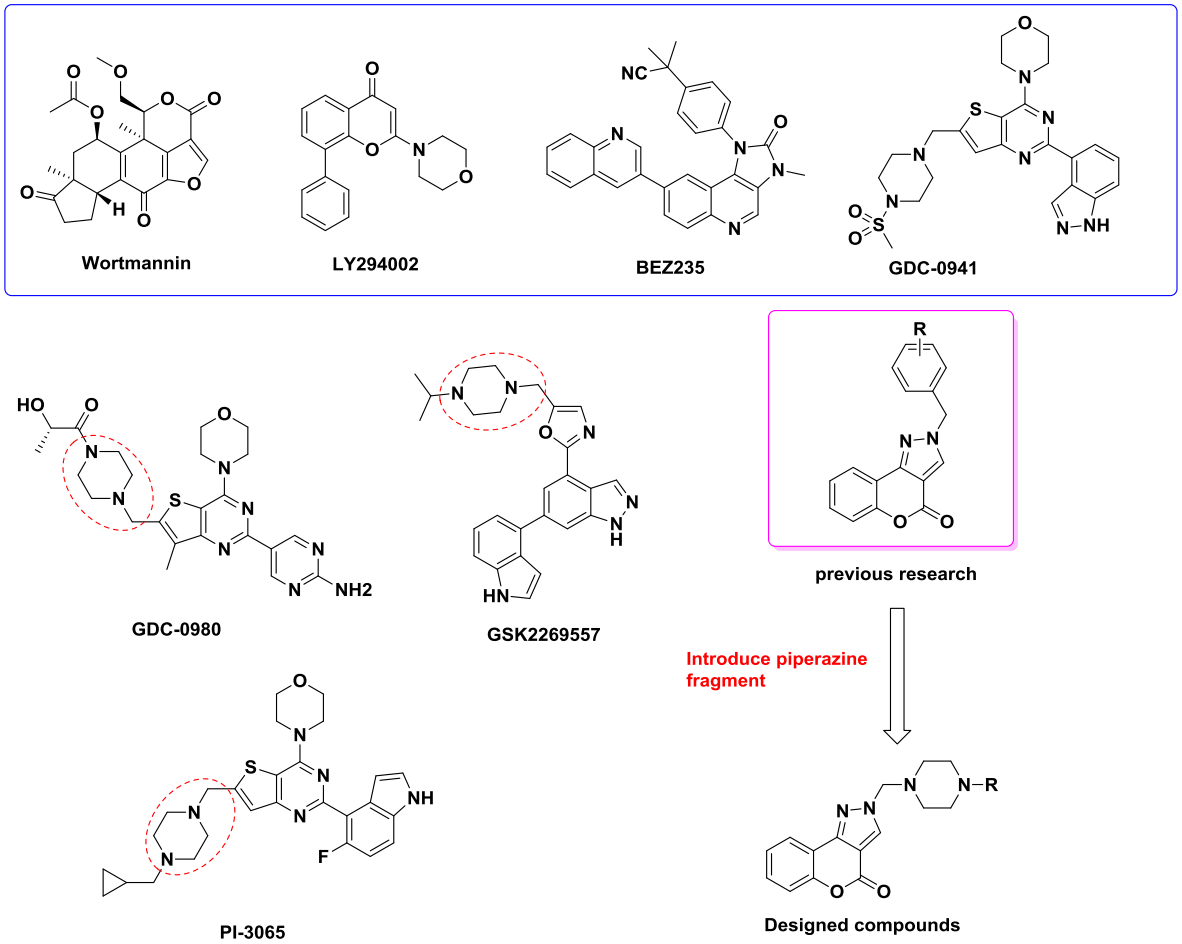


Fig. 1. Various PI3K inhibitors and the design compounds.

previous work and the function of piperazine, a series of chromeno[4,3-*c*]pyrazol-4(2*H*)-one derivates containing piperazine fragment was designed and synthesized, and some other nitrogen heterocycles were also introduced to chromeno[4,3-*c*]pyrazol-4(2*H*)-one to testify the importance of fragment.

In this research, we designed and synthesized a series of chromeno [4,3-*c*]pyrazol-4(2*H*)-one derivates containing piperazine, and they were evaluated for their PI3K inhibitory activities and anticancer effects in vitro.

2. Results and discussion

2.1. Chemistry

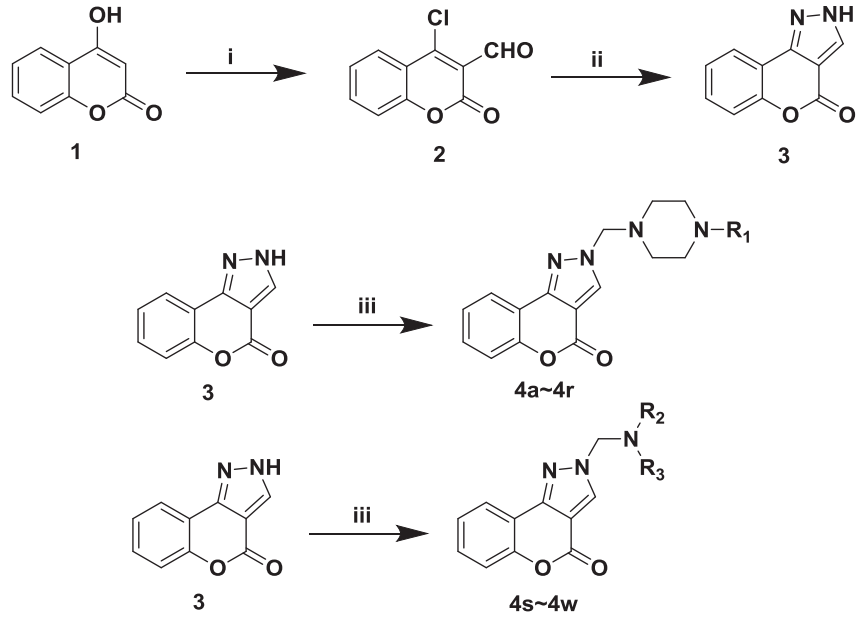
The synthetic routes for the desired compounds followed the general pathway outlined in Scheme 1. Firstly, synthesis of the so-called core region described as our previous research [28]. Briefly, Chromeno [4,3-*c*]pyrazol-4(2*H*)-one (**3**) was produced *via* a Vilsmeier-Haack reaction of 4-hydroxycoumarin (**1**) in the presence of dimethylformamide and phosphoryl chloride followed by cyclization with 85% hydrazine hydrate, and the yield was up to 85%. It was purified by crystallization to afford the satisfactory product. And then, chromeno[4,3-*c*]pyrazol-4(2H)-one (**3**) and various amines were reacted under 40% formaldehyde condition to get the designed compounds (**4a**-**4w**).

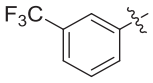
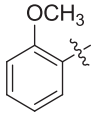
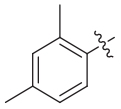
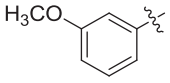
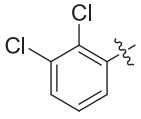
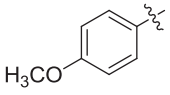
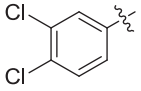
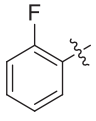
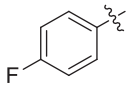
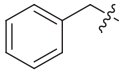
2.2. Antiproliferative assays in vitro

All the designed compounds were evaluated for their antiproliferative activities against four cancer cell lines: HL60, Huh7, A549 and HCT-116, and the results were shown in Table 1. Most of the designed compounds showed the good antiproliferative activities against four cancer cell lines. Compared to the positive drug LY294002, a great improvement of their activities was performed. Out of them, compounds **4p**, **4q** and **4r** exhibited the potent and top-3 antiproliferative activities, particularly, compound **4p** revealed the potent antiproliferative activity against four cancer cell lines with the IC_{50} value of 0.08 μM, 0.08 μM, and 0.07 μM, 0.05 μM for Huh7, A549, HL60 and HCT-116, respectively.

As can be seen in Table 1, an interesting point was observed that the compounds containing the piperazine fragment (**4a**-**4r**, **4v**) exhibited the better antiproliferative activities than that of other compounds without piperazine fragment (**4s**, **4t** and **4w**). What' more, the compounds containing the phenylpiperazine or benzylpiperazine moiety revealed the potent antiproliferative activities against cancer cell lines. It was indicated that phenylpiperazine or benzylpiperazine moiety play a crucial role for the designed compounds in the effect of antiproliferative activities.

Obviously, the compounds bearing diphenylmethylpiperazine fragment exhibited the better antiproliferative activities than that of compounds containing phenylpiperazine and other compounds, such as compounds **4p**, **4q** and **4r**, and it was observed that the more substituents on the benzene ring, the poorer antiproliferative activities. Among the compounds **4a**-**4m** which contained benzylpiperazine moiety, compounds with disubstituted groups on the benzene ring showed antiproliferative activities were superior to that of mono-substituted, *e.g.* compound **4k**, **4l** and **4m**. For the compounds which contained disubstituted groups on the benzene ring, different substituents bring the different effect on compounds, and they exhibited



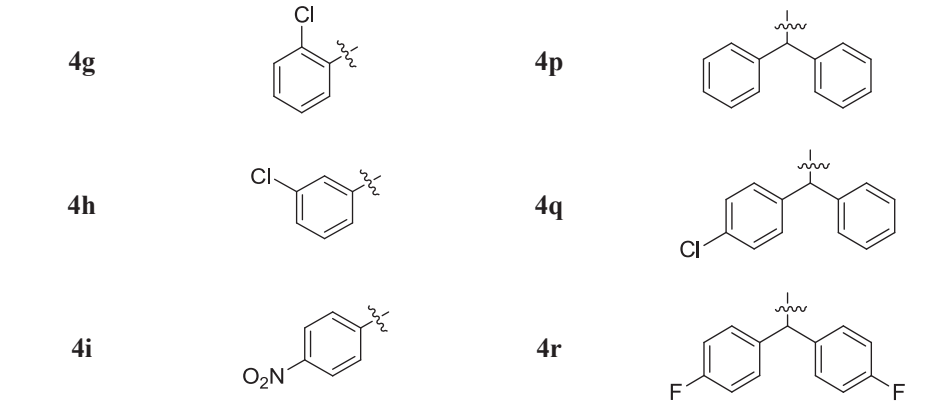
Compounds	R_1	Compounds	R_1
4a		**4j**	
4b		**4k**	
4c		**4l**	
4d		**4m**	
4e		**4n**	
4f		**4o**	

Scheme 1. Synthesis of compounds **4a-4w**. Reagents and conditions: (i) DMF, $POCl_3$, 60 °C, 6 h. (ii) Ethanol, Et_3N, 85% hydrazine hydrate, r.t., 14 h. (iii) formaldehyde/ various *N*-heterocyclic compounds, EtOH, r.t., 4–6 h.

antiproliferative activities with the trend of order was OCH_3 > NO_2 > F > CF_3 > Cl. Meanwhile, it was observed that the substituted position on the benzene ring displayed the antiproliferative activities with the trend of *ortho* > *para* > *meta*, such as compounds **4b**, **4c** and **4d**. Besides, for other compounds, hydrophilic group played a positive role for the activity of these compounds, *e.g.* compound **4k**-**4w**. In brief, the aboved results indicated that *N*-substituted piperazine group is an essential fragment for the designed compounds to displayed the potent antiproliferative activities, and especially diphenylmethylpiperazine group, exerting the remarkable efficacy.

2.3. PI3K enzymatic activity

To validate whether the antiproliferative effect of compounds was exerted by targeting PI3K protein, the twelve compounds which revealed the highest activities were selected to test against PI3K enzymatic activity, and the results are shown in Table 2. Among these compounds, most compounds displayed the inhibition activities against



Compounds	R_1	Compounds	R_2R_3
4s		**4u**	
4t		**4v**	
		4w	

Scheme 1. (*continued*)

Table 1
Antiproliferative activities in vitro of targeted compounds **4a-4w** against four cancer cell lines.

Compound	IC_{50} ± SD[a] (μM)			
	HCT-116	A549	Huh7	HL60
4a	22.03 ± 1.92	28.47 ± 2.18	27.08 ± 2.23	27.10 ± 2.23
4b	0.76 ± 0.06	1.04 ± 0.12	0.79 ± 0.07	0.92 ± 0.07
4c	3.40 ± 0.24	6.15 ± 0.59	7.81 ± 0.79	3.91 ± 0.25
4d	1.30 ± 0.12	1.14 ± 0.13	1.37 ± 0.14	1.85 ± 0.14
4e	2.84 ± 0.24	4.68 ± 0.45	2.97 ± 0.21	3.12 ± 0.21
4f	3.27 ± 0.31	7.04 ± 0.68	5.31 ± 0.48	4.09 ± 0.37
4g	9.36 ± 0.86	14.67 ± 1.22	16.41 ± 1.39	14.44 ± 1.26
4h	7.18 ± 0.62	10.81 ± 0.94	11.68 ± 1.14	12.61 ± 1.18
4i	1.95 ± 0.16	2.49 ± 0.23	3.15 ± 0.28	3.65 ± 0.39
4j	5.79 ± 0.57	8.66 ± 0.71	12.51 ± 1.14	8.74 ± 0.81
4k	0.26 ± 0.02	0.38 ± 0.03	0.67 ± 0.06	0.51 ± 0.04
4l	0.33 ± 0.02	0.61 ± 0.04	0.82 ± 0.07	0.49 ± 0.04
4m	0.17 ± 0.01	0.43 ± 0.03	0.31 ± 0.02	0.32 ± 0.02
4n	11.39 ± 1.27	27.10 ± 3.22	21.31 ± 2.27	19.73 ± 1.44
4o	14.63 ± 1.45	19.56 ± 2.03	32.60 ± 2.94	22.71 ± 2.08
4p	0.05 ± 0.004	0.08 ± 0.006	0.08 ± 0.005	0.07 ± 0.006
4q	0.08 ± 0.006	0.11 ± 0.01	0.12 ± 0.01	0.11 ± 0.01
4r	0.10 ± 0.007	0.12 ± 0.01	0.17 ± 0.01	0.14 ± 0.01
4s	62.80 ± 6.43	> 100	89.04 ± 9.12	> 100
4t	47.57 ± 4.36	81.04 ± 8.37	67.46 ± 6.33	84.93 ± 8.74
4u	> 100	> 100	92.80 ± 9.31	89.04 ± 8.21
4v	74.30 ± 6.92	78.64 ± 7.62	79.13 ± 9.28	56.84 ± 5.25
4w	> 100	58.33 ± 5.44	64.31 ± 5.81	68.74 ± 7.02
LY294002	51.82 ± 4.58	82.32 ± 7.26	67.18 ± 5.64	18.43 ± 2.03

[a] Values are average of three independent experimental measurements and expressed as Mean ± SD.

PI3Kα, β, γ and δ to a certain degree. Particularly, compounds **4p**-**4r** showed greatly improved inhibition activities against PI3Ks comparing to LY294002. Additionally, compound **4p** exhibited the prominent activity against PI3Kα with IC_{50} values of 0.012 μM, 0.12 μM, 0.068 μM and 0.19 μM for PI3Kα, PI3Kβ, PI3Kγ and PI3Kδ, respectively.

Table 2
Enzymatic activities of selected compounds against PI3K.

Compounds	IC_{50} ± SD[a] (μM)			
	PI3Kα	PI3Kβ	PI3Kγ	PI3Kδ
4b	2.45 ± 0.18	5.82 ± 0.67	4.13 ± 0.37	8.17 ± 0.74
4c	> 10	9.43 ± 0.84	> 10	> 10 ±
4d	5.84 ± 0.32	5.09 ± 0.37	3.97 ± 0.24	7.52 ± 0.66
4e	7.61 ± 0.31	> 10	7.82 ± 0.28	> 10
4f	9.37 ± 0.28	8.01 ± 0.12	> 10	9.74 ± 0.78
4i	4.37 ± 0.38	7.74 ± 0.37	8.17 ± 0.56	7.52 ± 0.54
4k	0.84 ± 0.02	0.42 ± 0.41	0.73 ± 0.037	1.58 ± 0.12
4l	1.74 ± 0.11	1.12 ± 0.22	0.40 ± 0.021	1.44 ± 0.11
4m	0.32 ± 0.02	0.65 ± 0.04	0.46 ± 0.023	0.66 ± 0.051
4p	0.012 ± 0.001	0.12 ± 0.003	0.068 ± 0.004	0.19 ± 0.009
4q	0.058 ± 0.002	0.20 ± 0.006	0.13 ± 0.008	0.75 ± 0.042
4r	0.10 ± 0.002	0.19 ± 0.006	0.11 ± 0.012	0.16 ± 0.011
LY294002	0.48 ± 0.07	0.98 ± 0.012	0.95 ± 0.082	1.36 ± 0.13

[a] Values are average of three independent experimental measurements and expressed as Mean ± SD.

Compared to other three PI3K isoforms, compound **4p** selectively inhibited PI3Kα, and it was better than that of LY294002.

What's more, compounds **4k-4m** and **4p-4r** were selected to test for their inhibitory activity against PI3Kα H1047R mutant, and the results were showed in Table 3. it was observed that all the selected compounds exhibit the compared inhibitory activities against PI3Kα wild-type and H1047R mutant. Interestingly, compound **4p** also exhibited the potent inhibitory activity against PI3Kα H1047R mutant (IC_{50} = 0.01 μM). All the results indicated that compound **4p** may be a new PI3Kα inhibitor.

2.4. Western blot assay

Once the PI3Kα activity was suppressed, downstream signal factor of PI3K which mediates cell growth and blocks cell apoptosis might also

Table 3
Inhibitory activity against PI3Kα wild type and H1047R mutant.

Compounds	IC_{50} ± SD[a] (μM)	
	PI3Kα (wild-type)	PI3Kα (H1047R mutant)
4k	0.84 ± 0.02	0.76 ± 0.08
4l	1.74 ± 0.11	1.46 ± 0.11
4m	0.32 ± 0.02	0.21 ± 0.03
4p	0.012 ± 0.001	0.010 ± 0.002
4q	0.058 ± 0.002	0.076 ± 0.004
4r	0.10 ± 0.002	0.088 ± 0.005

[a] Values are average of three independent experimental measurements and expressed as Mean ± SD.

be inhibited, and as we known, activation of PI3K leads to the Akt phosphorylated. Based on these, western blot was performed to assess whether effect on the expression levels of Akt and p-Akt in HCT-116 cell line after treated with compound **4p** at the concentration of 0.25 μM, 0.5 μM and 1 μM. As shown in Fig. 2, compound **4p** caused a decrease in p-Akt (S473) level obviously in a dose-dependent manner, indicating that **4p** might be a potential PI3K inhibitor.

2.5. Cell apoptosis assay

To identify whether compound **4p** could induce cell apoptosis, compound **4p** was used to induce cell apoptosis in HCT-116 cell line using Annexin V-FITC/PI FACS assay. After treated with **4p** at different concentration (0.25, 0.5 and 1 μM) in HCT-116 cell line for 24 h, the percentages of late apoptotic population from 17.6 to 30.1%, and the percentages of early apoptotic population from 18.4 to 27.0% (Fig. 3), which was remarkable influence in a dose-dependent manner by compound **4p**.

To further investigate the apoptosis mechanism by compound **4p** in HCT-116 cell line, apoptotic related proteins were examined in HCT-116 after treatment with **4p** at concentrations of 0.25, 0.5 and 1 μM for 24 h. As shown in Fig. 3, the proapoptotic protein Bax level was remarkably increased in a dose-dependent manner, in contrast, Bcl-2 level was decreased. What's more, the cleaved caspase-3 and cleaved caspase-9 levels which are recognized as bio-markers for cell apoptosis, were increased in a dose-dependent manner. These results indicated that compound **4p** induce cell apoptosis of HCT-116 cells in a dose-dependent manner.

2.6. Molecular docking study

To study a possible interaction mode between compound **4p** and PI3Kα H1047R mutant, molecular docking was carried out to fit compound **4p** into the active pocket of the PI3Kα H1047R mutant protein (PDB ID: 3HHM) using Discovery Studio 3.5. As shown in Fig. 4, the compound **4p** was well filled in the active pocket *via* one hydrogen bond, three π-cation interactions and one π-Sigma interaction. the carboxyl oxygen atoms of compound **4p** formed one hydrogen bond (distance:1.79 Å) with the amino of Lys802; Two π-cation interactions was formed by Lys776 and the coumarin ring, while another π-cation interaction was formed between Tyr836 and nitrogen atom of piperazine; The benzene ring of compound **4p** formed one π-sigma interaction with Met922. Interestingly, the four amino acid residues play a vital role in the active pocket of PI3Kα, and they are the key binding site of most PI3Kα inhibitors. These interactions resulting in compound **4p** binding well to the active pocket of PI3Kα protein, indicating that compound **4p** is a promising PI3Kα inhibitor.

3. Conclusion

In summary, twenty-three chromeno[4,3-*c*]pyrazol-4(2H)-one derivates containing piperazine fragment as PI3K inhibitors have been designed and synthesized, and their antiproliferative activities against HCT-116, Huh7, A549 and HL60 four cancer cell lines were evaluated, and most desired compounds showed better antiproliferative activities. Among of them, compound **4p** exhibited the remarkable antiproliferative activity (IC_{50} ranging from 0.05 to 0.08 μM), which was superior to that of reference drug LY294002. In addition, compound **4p** revealed the potent activity against both PI3Kα wild-type and H1047R mutant with IC50 values of 0.012, and 0.01 μM, respectively, comparing to another three PI3K isoforms (PI3Kβ, PI3Kγ and PI3Kδ), and an about 40-fold increase in comparison with LY294002. Western blotting assay showed that **4p** decrease the p-AKT phosphorylation level, indicating compound **4p** could inhibit PI3Kα activity. What's more, compound **4p** remarkably induced apoptosis in HCT-116 cell in a dose-dependent manner, up-regulated Bax, cleaved caspase-3 and cleaved caspase-9 expressions, and reduced the anti-apoptotic protein Bcl-2 level. Molecular docking indicating compound **4p** was docked well into the active pocket of PI3Kα and strong bind to the protein. These results suggested that compound **4p** could be a promising PI3Kα inhibitor for cancer therapy.

4. Experimental section

All chemicals and reagents were of analytical grade and purchased from commercial company. Melting points were determined by a melting point apparatus (Taike Corp., China). All reactions were monitored by thin layer chromatography (TLC; silica GF_{254} plates) which purchased from Merck. ^{1}H NMR spectra were recorded using Bruker DPX400 spectrometer and ^{13}C NMR spectra were determined by Bruker Ascend 600 spectrometer, and TMS as an internal standard. Chemical shifts are described in ppm (δ). ESI mass spectra were determined on a

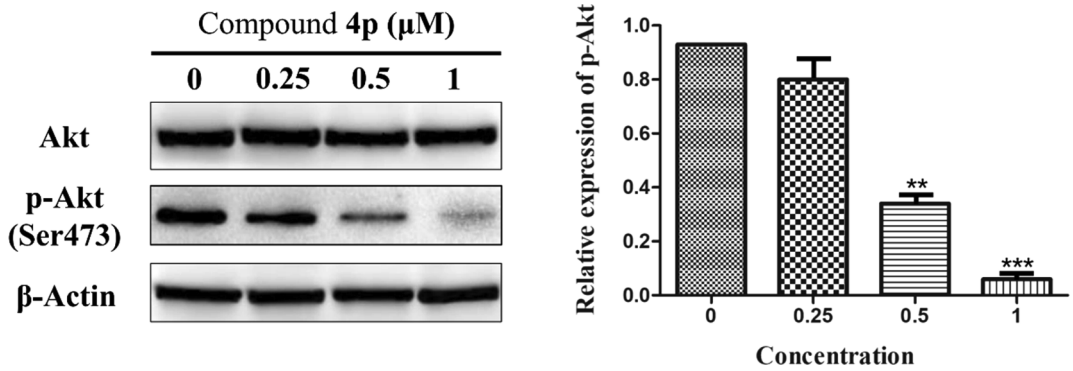


Fig. 2. Effects of compound **4p** on the AKT phosphorylation. HCT-116 cells were treated with compound **4p** for 24 h, total protein was extracted and subjected to Western blot analysis, β-actin was used as an internal control. Bar graphs represent the expression level of AKT and p-AKT proteins. Data were expressed as Mean ± SD (n = 3). *p < 0.05, **p < 0.01 and ***p < 0.005 *vs*. the control.

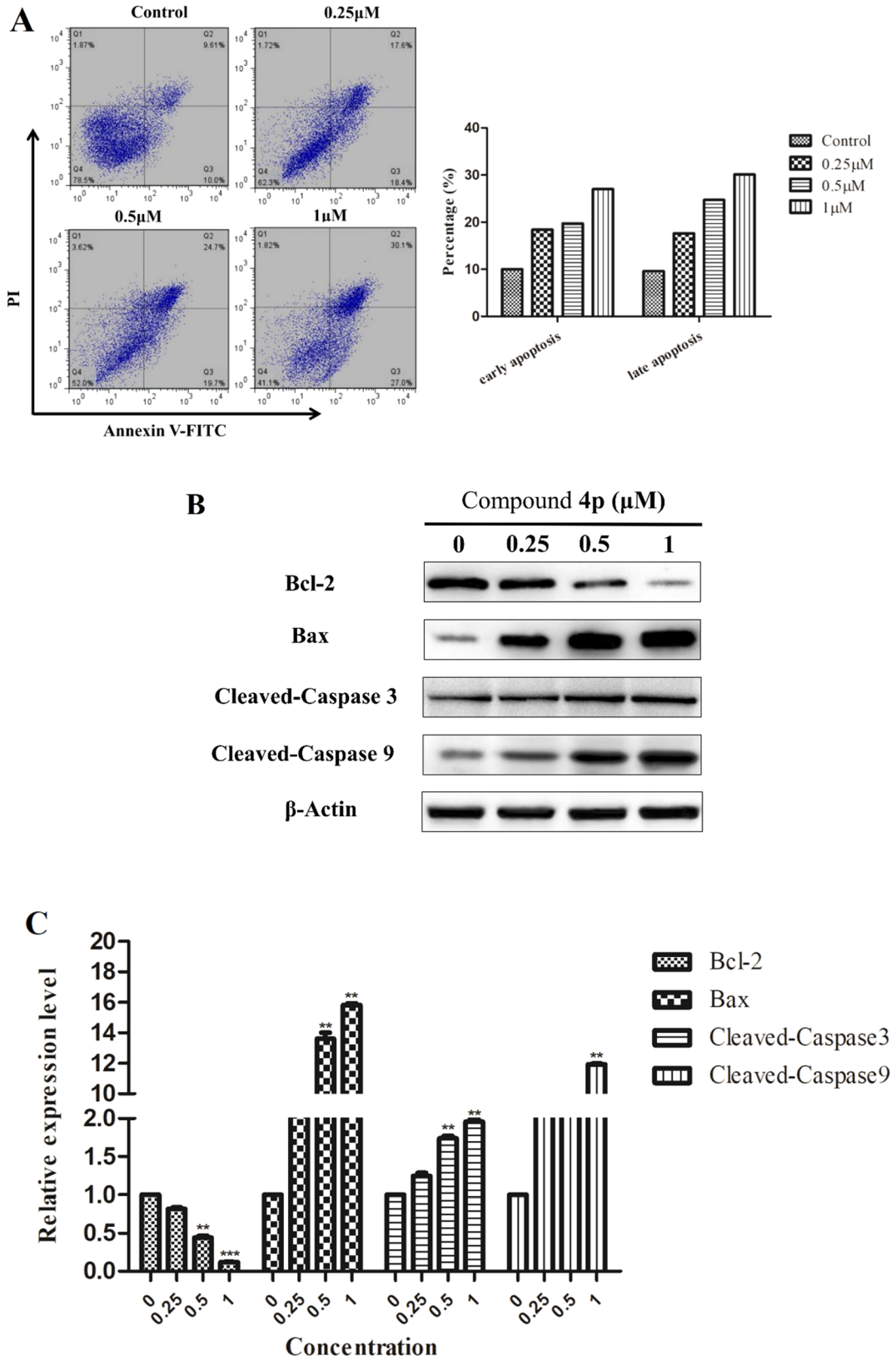


Fig. 3. Induced apoptotic effect of compound **4p** on HCT-116 cell. (A) Apoptotic assay by flow cytometry. HCT-116 cells were treated with compound **4p** at 0, 0.25, 0.5, and 1 μM for 24 h. The cells were stained with Annexin V-FITC/PI, then detected by a flow cytometer. (B and C) Effects of compound **4p** on Bcl-2, Bax, cleaved-caspase-3 and cleaved-caspase-9 expression. Cells were treated with compound **4p** for 24 h, then total protein was extracted and subjected to Western blot analysis, β-actin was used as an internal control. Bar graphs represent the expression level of Caspase proteins. Data were expressed as Means ± SD (n = 3). *p < 0.05, **p < 0.01 and ***p < 0.005 *vs*. the control.

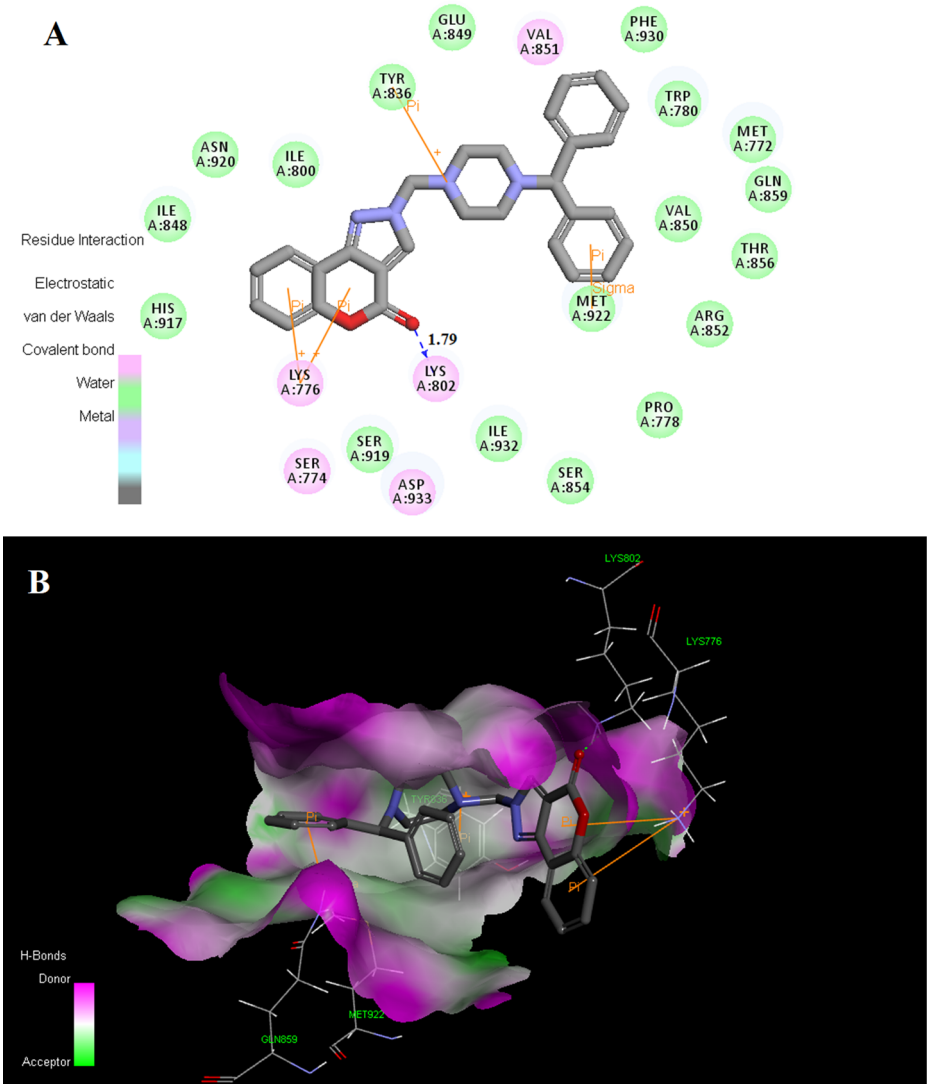


Fig. 4. (A) 2D molecular docking model of compound **4p** with 3HHM (PI3Kα H1047R mutant). (B) 3D conformation position of the compound **4p** in the binding pocket.

Mariner System 5304 mass spectrometer.

4.1. General procedure for the preparation of compound *3*

Chromeno[4,3-*c*]pyrazol-4(2*H*)-one (**3**) was synthesized described in the previous research [28]. Briefly, $POCl_3$ (0.15 mol) was added partly to a mixture of 4-hydroxycoumarin (0.05 mol) in anhydrous DMF (30 ml) under the ice-bath. The mixture was stirred for 0.5–1 h, and then heated to 65 °C and reacted for 6 h. Subsequently, ice water (200 ml) was poured into the reaction mixture and stirred vigorously, the yellow solid was precipitated out and then filtered, washed successively with aqueous Na_2CO_3 (5%) and water, crystallized with acetone to obtain the compound **2**.

85% Hydrazine hydrate (3 mmol) and TEA (6 mmol) were mixed in 65–70% ethanol (15 ml), the mixture was slowly added dropwise to the solution of compound **2** (3 mmol) in ethanol (15 ml) under the temperature did not exceed 25 °C. The mixture was stirred for 12 h at room temperature, and then removed ethanol *in vacuo*. The residue was purified by recrystallization to afford Chromeno[4,3-*c*]pyrazol-4(2*H*)-one (**3**).

4.2. General procedure for the preparation of compounds *4a-4w*

40% formaldehyde (300 μL) was added slowly into the ethanol solution of **3** (2 mmol) and various heterocyclic amine (2 mmol), the mixtures were stirred at room temperature for 5–7 h. A large quantity of solid was precipitated and then filtered, washed with cold ethanol, and crystallized with ethanol to get the designed compounds **4a-4w**.

4.2.1. 2-((4-Phenylpiperazin-1-yl)methyl)chromeno[4,3-c]pyrazol-4(2H)-one (*4a*)

White power, yield: 48%. Mp: 212–214 °C. ^{1}H NMR (400 MHz, $CDCl_3$): 8.29 (s, 1H, ArH), 8.09(m, 1H, ArH), 7.49–7.45(m, 1H, ArH), 7.38(t, J = 4.10 Hz, 1H, ArH), 7.32(m, 1H, ArH), 7.24(m, 2H, ArH), 6.89–6.84(m, 3H, ArH), 5.18(s, 2H, $-CH_2$), 3.21(t, J = 4.96, 4H, $-CH_2CH_2-$), 2.83(t, J = 4.94 Hz, 4H, $-CH_2CH_2-$). ^{13}C NMR (125 MHz, $CDCl_3$): 158.71, 152.96, 151.11, 149.21, 132.43, 130.42, 129.17(2), 124.54, 122.80, 120.25, 117.63, 116.52(2), 114.94, 107.98, 74.30, 50.11(2), 49.33(2). MS(ESI) *m/z*: 361.38 $[M+H]^+$ (Calcd for 361.42, $C_{21}H_{21}N_4O_2$).

4.2.2. 2-((4-(2-Methoxyphenyl)piperazin-1-yl)methyl)chromeno[4,3-c]pyrazol-4(2H)-one (*4b*)

White power, yield: 42%. Mp: 190–193 °C. 1H NMR (400 MHz,

CDCl3): 8.27(s, 1H, ArH), 8.08(d, J = 7.70 Hz, 1H, ArH), 7.49–7.45(m, 1H, ArH), 7.39(d, J = 8.40 Hz, 1H, ArH), 7.32(m, 1H, ArH), 7.00(m, 1H, ArH), 6.91(m, 2H, ArH), 6.83(d, J = 7.68 Hz, 1H, ArH), 5.19(s, 2H, $-CH_2$), 3.80(s, 3H, $-OCH_3$), 3.10(s, 4H, $-CH_2CH_2-$), 2.87(t, J = 4.76 Hz, 4H, $-CH_2CH_2-$). ^{13}C NMR (125 MHz, $CDCl_3$): 158.23, 152.95, 152.24, 149.20, 140.91, 132.39, 130.36, 124.49, 123.27, 122.80, 120.97, 118.30, 117.64, 114.98, 111.18, 107.98, 74.46, 55.35, 50.51(2), 50.28(2). MS(ESI) *m/z*: 391.27 $[M+H]^+$ (Calcd for 391.44, $C_{22}H_{23}N_4O_3$).

4.2.3. 2-((4-(3-Methoxyphenyl)piperazin-1-yl)methyl)chromeno[4,3-c] pyrazol-4(2H)-one (**4c**)

White power, yield: 49%. Mp: 168–169 °C. ^1H NMR (400 MHz, $CDCl_3$): 8.28(s, 1H, ArH), 8.08(d, J = 7.71 Hz, 1H, ArH), 7.47(t, J = 7.71 Hz, 1H, ArH), 7.39–7.30(m, 2H, ArH), 714(t, J = 8.49 Hz Hz, 1H, ArH), 6.49(d, J = 9.0 Hz, 1H, ArH), 6.41(d, J = 6.27 Hz, 2H, ArH), 5.17(s, 2H, $-CH_2$), 3.76(s, 3H, $-OCH_3$), 3.21(t, J = 4.77 Hz, 4H, $-CH_2CH_2-$), 2.82(t, J = 4.77 Hz, 4H, $-CH_2CH_2-$). ^{13}C NMR (125 MHz, $CDCl_3$): 160.59, 158.17, 152.96, 152.49, 149.23, 132.39, 130.43, 129.85, 124.54, 122.79, 117.64, 114.93, 109.25, 108.00, 104.93, 103.00, 74.30, 55.21, 50.07(2), 49.24(2). MS(ESI) *m/z*: 391.27 $[M+H]^+$ (Calcd for 391.44, $C_{22}H_{23}N_4O_3$).

4.2.4. 2-((4-(4-Methoxyphenyl)piperazin-1-yl)methyl)chromeno[4,3-c] pyrazol-4(2H)-one (**4d**)

White power, yield: 43%. Mp: 193–194 °C. ^1H NMR (400 MHz, $CDCl_3$): 8.28(s, 1H, ArH), 8.09(m, 1H, ArH), 7.47(m, 1H, ArH), 7.38(d, J = 8.08 Hz, 1H, ArH), 7.32(m, 1H, ArH), 6.86(d, J = 8.88 Hz, 2H, ArH), 6.81(d, J = 9.08 Hz, 2H, ArH), 5.17(s, 2H, $-CH_2$), 3.75(s, 3H, $-OCH_3$), 3.10(s, 4H, $-CH_2CH_2-$), 2.84(s, 4H, $-CH_2CH_2-$). ^{13}C NMR (125 MHz, $CDCl_3$): 158.18, 152.96, 149.23, 145.45, 132.37, 130.42, 124.53, 122.79, 118.71, 117.65, 114.94, 114.49(2), 107.98, 74.32, 55.58, 50.79(2), 50.21(2). MS(ESI) *m/z*: 391.27 $[M+H]^+$ (Calcd for 391.44, $C_{22}H_{23}N_4O_3$).

4.2.5. 2-((4-(2-Fluorophenyl)piperazin-1-yl)methyl)chromeno[4,3-c] pyrazol-4(2H)-one (**4e**)

Light yellow power, yield: 39%. Mp: 197–199 °C. ^1H NMR (400 MHz, $CDCl_3$): 8.29(s, 1H, ArH), 8.09(d, J = 6.20 Hz, 1H, ArH), 7.47(m, 1H, ArH), 7.38(d, J = 7.64 Hz, 1H, ArH), 7.32(m, 1H, ArH), 7.04(m, 1H, ArH), 7.01(m, 1H, ArH), 6.99–6.91(m, 2H, ArH), 5.18(s, 1H, $-CH_2$), 3.14(d, J = 3.24 Hz, 4H, $-CH_2CH_2-$), 2.86(t, J = 3.60 Hz, 4H, $-CH_2CH_2-$). ^{13}C NMR (125 MHz, $CDCl_3$): 158.19, 155.74(d, J = 294 Hz), 152.97, 149.25, 139.78(d, J = 9 Hz), 132.36, 130.41, 124.53, 124.50(d, J = 4.5 Hz), 122.88, 122.82, 119.11(d, J = 4.5 Hz), 117.64, 116.21(d, J = 25.5 Hz), 114.95, 108.01, 74.38, 50.41(2), 50.19(2). MS(ESI) *m/z*: 379.33 $[M+H]^+$ (Calcd for 379.41, $C_{21}H_{20}FN_4O_2$).

4.2.6. 2-((4-(4-Fluorophenyl)piperazin-1-yl)methyl)chromeno[4,3-c] pyrazol-4(2H)-one (**4f**)

Light yellow power, yield: 42%. Mp: 197–200 °C. ^1H NMR (400 MHz, $CDCl_3$): 8.28(s, 1H, ArH), 8.08(m, 1H, ArH), 7.48(m, 1H, ArH), 7.38(t, J = 4.14 Hz, 1H, ArH), 7.31(m, 1H, ArH), 6.93(m, 2H, ArH), 6.83(m, 2H, ArH), 5.17(s, 2H, $-CH_2$), 3.12(t, J = 4.92 Hz, 4H, $-CH_2CH_2-$), 2.83(t, J = 4.94 Hz, 4H, $-CH_2CH_2-$). ^{13}C NMR (125 MHz, $CDCl_3$): 158.61, 157.46(d, J = 285 Hz), 152.96, 149.23, 147.76, 132.41, 130.44, 124.54, 122.78, 118.35(d, J = 9 Hz)(2), 117.64, 115.61(d, J = 25.5 Hz)(2), 114.92, 107.99, 74.25, 50.32(2), 50.09(2). MS(ESI) *m/z*: 379.33 $[M+H]^+$ (Calcd for 379.41, $C_{21}H_{20}FN_4O_2$).

4.2.7. 2-((4-(2-Chlorophenyl)piperazin-1-yl)methyl)chromeno[4,3-c] pyrazol-4(2H)-one (**4g**)

Light yellow power, yield: 45%. Mp: 211–213 °C. ^1H NMR (400 MHz, $CDCl_3$): 8.29(s, 1H, ArH), 8.09(m, 1H, ArH), 7.47(m, 1H, ArH), 7.38(d, J = 8.28 Hz, 1H, ArH), 7.32(m, 2H, ArH), 7.21(m, 1H, ArH), 7.02(m, 1H, ArH), 6.96(m, 1H, ArH), 5.18(s, 2H, $-CH_2$), 3.09(d, J = 4.32 Hz, 4H, $-CH_2CH_2-$), 2.86(t, J = 4.58 Hz, 4H, $-CH_2CH_2-$). ^{13}C NMR (125 MHz, $CDCl_3$): 158.23, 152.98, 149.23, 148.90, 132.32, 130.72, 130.39, 128.85, 127.61, 124.52, 124.00, 122.83, 120.43, 117.64, 114.99, 108.05, 74.43, 51.08(2), 50.33(2). MS(ESI) *m/z*: 395.71 $[M+H]^+$ (Calcd for 395.86, $C_{21}H_{20}ClN_4O_2$).

4.2.8. 2-((4-(3-Chlorophenyl)piperazin-1-yl)methyl)chromeno[4,3-c] pyrazol-4(2H)-one (**4h**)

Light yellow power, yield: 51%. Mp: 193–195 °C. ^1H NMR (400 MHz, $CDCl_3$): 8.28(s, 1H, ArH), 8.08(m, 1H, ArH), 7.47(m, 1H, ArH), 7.38(d, J = 4.15 Hz, 1H, ArH), 7.31(m, 1H, ArH), 7.13(t, J = 8.05 Hz, 1H, ArH), 6.82(m, 2H, ArH), 6.74(m, 1H, ArH), 5.17(s, 2H, $-CH_2$), 3.21(t, J = 4.98 Hz, 4H, $-CH_2CH_2-$), 2.81(t, J = 4.98 Hz, 4H, $-CH_2CH_2-$). ^{13}C NMR (125 MHz, $CDCl_3$): 158.16, 152.96, 152.11, 149.26, 134.99, 132.42, 130.47, 130.09, 124.56, 122.80, 119.83, 117.64, 116.18, 114.90, 114.36, 108.02, 74.21, 49.90(2), 48.83(2). MS (ESI) *m/z*: 395.71 $[M+H]^+$ (Calcd for 395.86, $C_{21}H_{20}ClN_4O_2$).

4.2.9. 2-((4-(4-Nitrophenyl)piperazin-1-yl)methyl)chromeno[4,3-c] pyrazol-4(2H)-one (**4i**)

Yellow power, yield: 40%. Mp: 221–224 °C. ^1H NMR (400 MHz, $CDCl_3$): 8.27(s, 1H, ArH), 8.07(m, 3H, ArH), 7.47(m, 1H, ArH), 7.37(d, J = 8.24 Hz, 1H.ArH), 7.30(m, 1H, ArH), 6.78(d, J = 9.44 Hz, 2H, ArH), 5.19(s, 2H, $-CH_2-CH_2$), 3.45(t, J = 5.04 Hz, 4H, $-CH_2CH_2-$), 2.82(t, J = 5.02 Hz, 4H, $-CH_2CH_2-$). ^{13}C NMR (125 MHz, $CDCl_3$): 158.06, 154.59, 152.97, 149.38, 138.91, 132.42, 130.57, 125.94(2), 124.59, 122.77, 117.67, 114.81, 113.07(2), 108.11, 74.03, 49.58(2), 47.11(2). MS(ESI) *m/z*: 406.28 $[M+H]^+$ (Calcd for 406.41, $C_{21}H_{20}N_5O_4$).

4.2.10. 2-((4-(3-(Trifluoromethyl)phenyl)piperazin-1-yl)methyl)chromeno [4,3-c]pyrazol-4(2H)-one (**4j**)

Yellow power, yield: 47%. Mp: 174–176 °C. ^1H NMR (400 MHz, $CDCl_3$): 8.28(s, 1H, ArH), 8.08(m, 1H, ArH), 7.48(m, 1H, ArH), 7.38(d, J = 8.24 Hz, 1H.ArH), 7.32(m, 2H, ArH), 7.08(d, J = 5.96 Hz, 2H.ArH), 7.02(m, 1H, ArH), 5.19(s, 2H, $-CH_2$), 3.26(t, J = 4.97 Hz, 4H, $-CH_2CH_2-$), 2.84(m, J = 4.97 Hz, 4H, $-CH_2CH_2-$). ^{13}C NMR (125 MHz, $CDCl_3$): 158.13, 152.98, 151.19, 149.30, 132.39, 130.51(d, J = 264 Hz), 130.48, 124.56, 122.79, 119.22, 117.66, 116.42(d, J = 4.5 Hz), 114.89, 112.60(d, J = 9 Hz), 112.59, 108.05, 74.20, 49.92(2), 48.85(2), 29.73. MS(ESI) *m/z*: 429.31 $[M+H]^+$ (Calcd for 429.42, $C_{22}H_{20}F_3N_4O_2$).

4.2.11. 2-((4-(2,4-Dimethylphenyl)piperazin-1-yl)methyl)chromeno[4,3-c]pyrazol-4(2H)-one (**4k**)

White power, yield: 43%. Mp: 226–228 °C. ^1H NMR (400 MHz, $CDCl_3$): 8.28(s, 1H, ArH), 8.11(m, 1H, ArH), 7.48(m, 1H, ArH), 7.39(d, J = 7.52 Hz, 1H, ArH), 7.32(m, 1H, ArH), 6.96(d, J = 6.32 Hz, 2H, ArH), 6.91(d, J = 8.72, 1H, ArH), 5.19(s, 2H, $-CH_2$), 2.92(t, J = 4.64 Hz, 4H, $-CH_2CH_2-$), 2.82(d, J = 4.44 Hz, 4H, $-CH_2CH_2-$), 2.25(s, 3H, $-CH_3$), 2.18(s, 3H, $-CH_3$). ^{13}C NMR (125 MHz, $CDCl_3$): 158.26, 152.99, 149.16, 148.69, 132.96, 132.66, 132.30, 131.87, 130.38, 127.05, 124.52, 122.84, 119.02, 117.64, 115.04, 108.01, 74.54, 51.79(2), 50.65(2), 20.70, 17.67. MS(ESI) *m/z*: 389.32 $[M+H]^+$ (Calcd for 389.47, $C_{23}H_{25}N_4O_2$).

4.2.12. 2-((4-(2,3-Dichlorophenyl)piperazin-1-yl)methyl)chromeno[4,3-c] pyrazol-4(2H)-one (**4l**)

Light yellow power, yield: 56%. Mp: 220–222 °C. ^1H NMR (400 MHz, $CDCl_3$): 8.30(s, 1H, ArH), 8.08(m, 1H, ArH), 7.47(m, 1H, ArH), 7.38(d, J = 8.12 Hz, 1H, ArH), 7.31(t, J = 7.48 Hz, 1H, ArH), 7.14(m, 2H, ArH), 6.92(m, 1H, ArH), 5.18(s, 2H, $-CH_2$), 3.07(s, 4H, $-CH_2CH_2-$), 2.86(t, J = 4.38 Hz, 4H, $-CH_2CH_2-$). ^{13}C NMR (125 MHz, $CDCl_3$): 158.21, 152.97, 150.87, 149.24, 134.13, 132.34,

130.42, 127.62, 127.48, 124.93, 124.54, 122.84, 118.67, 117.63, 114.98, 108.07, 74.36, 51.18(2), 50.26(2). MS(ESI) *m/z*: 430.18 $[M+H]^+$ (Calcd for 430.30, $C_{21}H_{19}Cl_2N_4O_2$).

*4.2.13. 2-((4-(3,4-Dichlorophenyl)piperazin-1-yl)methyl)chromeno[4,3-c]pyrazol-4(2H)-one (**4m**)*

Light yellow power, yield: 53%. Mp: 216–219 °C. ^{1}H NMR (400 MHz, $CDCl_3$): 8.28(s, 1H, ArH), 8.07(m, 1H, ArH), 7.47(m, 1H, ArH), 7.37(d, J = 8.20 Hz, 1H, ArH), 7.31(m, 1H, ArH), 7.24(d, J = 9.00 Hz, 1H, ArH), 6.90(d, J = 2.80 Hz, 1H, ArH), 6.67 (m, 1H, ArH), 5.17(s, 1H, $-CH_2$), 3.17(t, J = 4.96 Hz, 4H, $-CH_2CH_2-$), 2.80(t, J = 4.96 Hz, 4H, $-CH_2CH_2-$). ^{13}C NMR (125 MHz, $CDCl_3$): 158.13, 152.95, 150.43, 149.27, 132.84, 132.43, 130.51, 130.49, 124.57, 122.79, 122.71, 117.64, 117.62, 115.75, 114.88, 108.02, 74.14, 49.76(2), 48.80(2). MS(ESI) *m/z*: 430.18 $[M+H]^+$ (Calcd for 430.30, $C_{21}H_{19}Cl_2N_4O_2$).

*4.2.14. 2-((4-(Pyridin-2-yl)piperazin-1-yl)methyl)chromeno[4,3-c]pyrazol-4(2H)-one (**4n**)*

Light yellow power, yield: 49%. Mp: 192–194 °C. ^{1}H NMR (400 MHz, $CDCl_3$): 8.28(s, 1H, ArH), 8.13(m, 1H, ArH), 8.06 (t, J = 6.20 Hz, 1H, ArH), 7.45(m, 2H, ArH), 7.37–7.28(m, 2H, ArH), 6.60(t, J = 6.20 Hz, 2H, ArH), 5.17(s, 2H, $-CH_2$), 3.58(t, J = 5.02 Hz, 4H, $-CH_2CH_2-$), 2.77(t, J = 5.05 Hz, 4H, $-CH_2CH_2-$). ^{13}C NMR (125 MHz, $CDCl_3$): 159.11, 158.15, 152.94, 149.20, 147.93, 137.58, 132.39, 130.41, 124.52, 122.79, 117.61, 114.91, 113.62, 107.97, 107.21, 74.39, 49.92(2), 45.11(2). MS(ESI) *m/z*: 362.18 $[M+H]^+$ (Calcd for 361.41, $C_{20}H_{20}N_5O_2$).

*4.2.15. 2-((4-Benzylpiperazin-1-yl)methyl)chromeno[4,3-c]pyrazol-4(2H)-one (**4o**)*

White power, yield: 41%. Mp: 169–172 °C. ^{1}H NMR (400 MHz, DMSO): 8.23(s, 1H, ArH), 8.05(d, J = 7.70 Hz, 1H, ArH), 7.46(m, 1H, ArH), 7.38(d, J = 8.12 Hz, 1H, ArH), 7.30(m, 1H, ArH), 7.28–7.21(m, 5H, ArH), 5.11(s, 2H, $-CH_2$), 3.48(s, 2H, $-CH_2$), 2.69(s, 4H, $-CH_2CH_2-$), 2.49(s, 4H, $-CH_2CH_2-$). ^{13}C NMR (125 MHz, $CDCl_3$): 158.20, 152.94(2), 149.11, 132.29, 130.35, 129.13, 128.29(2), 127.20, 124.50, 122.78(2), 117.62, 114.98, 107.89, 74.35, 62.88, 52.77(2), 49.99(2). MS(ESI) *m/z*: 375.33 $[M+H]^+$ (Calcd for 375.44, $C_{22}H_{23}N_4O_2$).

*4.2.16. 2-((4-Benzhydrylpiperazin-1-yl)methyl)chromeno[4,3-c]pyrazol-4(2H)-one (**4p**)*

White power, yield: 36%. Mp: 191–194 °C. ^{1}H NMR (400 MHz, $CDCl_3$): 8.23(s, 1H, ArH), 8.09(t, J = 7.62 Hz, 1H, ArH), 7.48(t, J = 7.71 Hz, 1H, ArH), 7.39(d, J = 8.40 Hz, 1H, ArH), 7.34(m, 5H, ArH), 7.23(m, 4H, ArH), 7.15(m, 2H, ArH), 5.12(s, 2H, $-CH_2$), 4.21(s, 1H, $-CH$), 2.68(s, 4H, $-CH_2CH_2-$), 2.44(s, 4H, $-CH_2CH_2-$). ^{13}C NMR (125 MHz, $CDCl_3$): 158.27, 152.98, 149.06, 142.50(2), 132.38, 130.37, 128.56(4), 127.86(2), 127.05(2), 124.53, 122.84, 117.64, 115.08, 107.90, 76.06, 74.26, 51.64(2), 50.20(2). MS(ESI) *m/z*: 451.21 $[M+H]^+$ (Calcd for 451.54, $C_{28}H_{27}N_4O_2$).

*4.2.17. 2-((4-((4-Chlorophenyl)(phenyl)methyl)piperazin-1-yl)methyl)chromeno[4,3-c]pyrazol-4(2H)-one (**4q**)*

Light yellow power, yield: 39%. Mp: 228–230 °C. ^{1}H NMR (400 MHz, $CDCl_3$): 8.22(s, 1H, ArH), 8.09(d, J = 7.65 Hz, 1H, ArH), 7.48(m, 1H, ArH), 7.39(d, J = 7.77 Hz, 1H, ArH), 7.33 (m, 5H, ArH), 7.25–7.15(m, 5H, ArH), 5.12(s, 2H.$-CH_2$), 4.19(s, 1H, $-CH$), 2.67(s, 4H, $-CH_2CH_2-$), 2.42(s, 4H, $-CH_2CH_2-$). ^{13}C NMR (125 MHz, $CDCl_3$): 158.25, 152.98, 149.09, 141.90, 141.08, 132.69, 132.31, 130.40, 129.11(2), 128.74(2), 128.68(2), 127.76(2), 127.29, 124.53, 122.82, 117.65, 115.04, 107.94, 75.28, 74.22, 51.54(2), 50.14(2). MS(ESI) *m/z*: 485.78 $[M+H]^+$ (Calcd for 485.98, $C_{28}H_{26}ClN_4O_2$).

*4.2.18. 2-((4-(Bis(4-fluorophenyl)methyl)piperazin-1-yl)methyl)chromeno[4,3-c]pyrazol-4(2H)-one (**4r**)*

Light yellow power, yield: 34%. Mp: 201–203 °C. ^{1}H NMR (400 MHz, $CDCl_3$): 8.22(s, 1H, ArH), 8.09(m, 1H, ArH), 7.49(m, 1H, ArH), 7.39(d, J = 2.70 Hz, 1H, ArH),7.31(m, 1H, ArH), 7.27(m, 4H, ArH),6.93(m, 4H, ArH), 5.13(s, 2H, $-CH_2$), 4.20(s, 1H, $-CH$), 2.67(s, 4H, $-CH_2CH_2-$), 2.40(s, 4H, $-CH_2CH_2-$). ^{13}C NMR (125 MHz, $CDCl_3$): 161.87(J = 292.5 Hz)(2), 158.24, 152.98, 149.10, 137.96(J = 3 Hz)(2), 132.32, 130.42, 129.18(J = 9 Hz)(4), 124.54, 122.82, 117.66, 115.49(J = 25.5 Hz)(4), 115.03, 107.94, 74.31, 74.19, 51.46(2), 50.13(2). MS(ESI) *m/z*: 487.26 $[M+H]^+$ (Calcd for 487.52, $C_{28}H_{25}F_2N_4O_2$).

*4.2.19. 2-((4-Ethylpiperazin-1-yl)methyl)chromeno[4,3-c]pyrazol-4(2H)-one (**4s**)*

White power, yield: 44%. Mp: 185–189 °C. ^{1}H NMR (400 MHz, $CDCl_3$): 8.24(s, 1H, ArH), 8.06(d, J = 7.79 Hz, 1H, ArH), 7.45 (m, 1H, ArH), 7.36(d, J = 7.88 Hz, 1H, ArH), 7.29 (m, 1H, ArH), 5.10(s, 2H, $-CH_2$), 2.71(s, 4H, $-CH_2CH_2-$), 2.49(s, 4H, $-CH_2CH_2-$), 2.39(m, 2H, $-CH_2$), 1.03(s, 3H, $-CH_3$). ^{13}C NMR (125 MHz, $CDCl_3$): 158.18, 152.91, 149.13, 132.36, 130.34, 124.49, 122.76, 117.59, 114.92, 107.84, 74.28, 52.49, 52.19(2), 49.94(2), 11.94. MS (ESI): 313.17 $[M+H]^+$ (Calcd for 313.37, $C_{17}H_{21}N_4O_2$).

*4.2.20. 2-((4-(2-Hydroxyethyl)piperazin-1-yl)methyl)chromeno[4,3-c]pyrazol-4(2H)-one (**4t**)*

White power, yield: 36%. Mp: 200–202 °C. ^{1}H NMR (400 MHz, $CDCl_3$): 8.25(s, 1H, ArH), 8.07(m, 1H, ArH), 7.47(m, 1H, ArH), 7.36(d, J = 8.20 Hz, 1H, ArH), 7.31(m, 1H, ArH), 5.11(s, 2H, $-CH_2$), 3.57(t, J = 5.30 Hz, 2H, $-CH_2$), 2.71(s, 4H, $-CH_2CH_2-$), 2.56(s, 4H, $-CH_2CH_2-$), 2.53(t, J = 5.35 Hz, 2H, $-CH_2$). ^{13}C NMR (125 MHz, $CDCl_3$): 158.18, 152.96, 149.19, 132.33, 130.41, 124.53, 122.79, 117.62, 114.94, 107.93, 74.24, 59.19, 57.78, 52.62(2), 50.01(2). MS (ESI): 329.24 ($C_{17}H_{21}N_4O_3$, $[M+H]^+$ (Calcd for 329.37, $C_{17}H_{21}N_4O_3$).

*4.2.21. 2-(Piperidin-1-ylmethyl)chromeno[4,3-c]pyrazol-4(2H)-one (**4u**)*

White power, yield: 49%. Mp: 187–190 °C. ^{1}H NMR (400 MHz, $CDCl_3$): 8.23(s, 1H, ArH), 8.09(d, J = 7.64 Hz, 1H, ArH), 7.47(t, J = 5.21 Hz, 1H, ArH), 7.38(d, J = 8.36 Hz, 1H, ArH), 7.31(m, 1H, ArH), 5.10(s, 2H, $-CH_2$), 2.60(s, 4H, $-CH_2CH_2-$), 1.60(d, J = 3.80 Hz, 4H, $-CH_2CH_2-$), 1.38(d, J = 3.92 Hz, 2H, $-CH_2$). ^{13}C NMR (125 MHz, $CDCl_3$): 158.17, 152.94, 149.22, 132.18, 130.28, 124.48, 122.77, 117.61, 114.79, 107.94, 75.42, 51.40(2), 25.87(2), 23.60. MS (ESI): 285.14 ($C_{16}H_{17}N_4O_2$, $[M+H]^+$ (Calcd for 285.32, $C_{16}H_{17}N_4O_2$).

*4.2.22. 2-(Piperazin-1-ylmethyl)chromeno[4,3-c]pyrazol-4(2H)-one (**4v**)*

White power, yield: 52%. Mp: 265–268 °C. ^{1}H NMR (400 MHz, DMSO): 8.82(s, 1H, ArH), 8.00(d, J = 6.30 Hz, 1H, ArH), 7.52–7.58(m, 1H, ArH), 7.41–7.44(d, J = 8.28 Hz, 1H, ArH), 7.34–7.39(t, J = 7.50 Hz, 1H, Ar), 5.15(s, 2H, $-CH_2$), 3.31(s, 4H, $-CH_2CH_2-$), 2.61(s, 4H, $-CH_2CH_2-$), 1.03–1.07(t, J = 6.99 Hz, 1H, $-NH$). ^{13}C NMR (125 MHz, $CDCl_3$): 158.13, 152.94, 149.11, 132.29, 130.33, 124.46, 122.79, 117.64, 114.94, 107.88, 74.32, 51.14(2), 50.91(2). MS(ESI) *m/z*: 284.23 $[M+H]^+$ (Calcd for 284.33, $C_{16}H_{18}N_3O_2$).

*4.2.23. 2-(Morpholinomethyl)chromeno[4,3-c]pyrazol-4(2H)-one (**4w**)*

White power, yield: 58%. Mp: 135–138 °C. 1H NMR (400 MHz, CDCl3): 8.26(s, 1H, ArH), 8.08(m, 1H, ArH), 7.48(m, 1H, ArH), 7.38(d, J = 7.76 Hz, 1H, ArH), 7.32(m, 1H, ArH), 5.09(s, 2H, $-CH_2$), 3.73(t, J = 4.66 Hz, 4H, $-CH_2CH_2-$), 2.67(t, J = 4.66 Hz, 4H, $-CH_2CH_2-$). ^{13}C NMR (125 MHz, $CDCl_3$): 158.14, 152.98, 149.26, 132.33, 130.46, 124.55, 122.78, 117.66, 114.90, 108.04, 74.51, 66.71(2), 50.32(2). MS (ESI) *m/z*: 286.18 $[M+H]^+$ (Calcd for 286.30, $C_{15}H_{16}N_3O_3$).

4.3. Cell proliferation assay

Four human cell lines HCT-116, A549, HL60 and Huh7 were cultured growth medium (RPMI-1640 medium with 100 mg/mL streptomycin, 100 U/mL penicillin and 10% FBS, and incubated at 37 °C under the atmosphere of 5% CO_2 and 20% O_2.

The antiproliferative activity in vitro was measured using the MTT assay. The tested compounds were dissolved in DMSO and diluted to the different concentrations. Cells in logarithmic phase were harvested and divided into 96-well plates (0.5×10^4 each well), culture medium containing the test compounds at different concentrations were added to each well and incubated for 48 h. Viable cells were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay kit (MTT, Sigma) according to the manufacturer's instructions. Triplicate wells were used for each concentration and each assay was carried out at least three times. The cytotoxic activity was expressed as the IC_{50} values.

4.4. PI3K enzyme assay

The inhibition of PI3K activity was determined by competitive fluorescence polarization kinase activity assay as our previous research described [28]. Briefly, 10 μL PI3K (Perkin-Elmer) reactions were performed in pH 7, 2.5 mM $MgCl_2$, 5 mM HEPES, 50 mM ATP and 10 mM DTT using diC_8-PI(4,5)P_2 (Echelon Biosciences) as the substrate. Firstly, tested compounds (ranging from 3.2 nM to 1 mM) were added into per 10 μL reaction volume (prepared with 50 ng of enzyme and 10 mM of substrate). After incubating for 3 h at room temperature, a chelator was added to quench the reactions. And then a mixture of phosphoinositide binding protein was added and mixed, subsequently, a fluorophor-elabeled phosphoinositide tracer was added. Samples were incubated in the dark for 1 h to equilibrate after mixing in 384-well black Corning nonbinding plates. Finally, polarization values were recorded using red fluorophores with appropriate filters to determine the extent of enzyme activity in the reaction.

4.5. Apoptosis analysis

1×10^5 HCT-116 cells in exponential growth was seeded into each well of 6-well plate, and treated with compound **4p** at different concentrations for 24 h, the cells were collected, centrifuged and re-suspended in 500 ml AnnexinV binding buffer, and incubated for 15 min on the ice in the darkness. Samples were analyzed using a FACS Calibur flow cytometer (Bectone Dickinson, San Jose, CA, USA).

4.6. Western blotting

HCT-116 cells were incubated in the presence of **4p** for 24 h. Subsequently, trypsinized the cells and collected, the prepared 1 × RIPA lysis buffer (1% NP-40,50 mM Tris-HCl, 150 mM NaCl, pH 7.4, 0.25% deoxycholic acid, 1 mM EDTA containing protease inhibitors PMSF) (Amresco, Solon, USA) was added to extract the total proteins. The proteins was separated by sodium dodecyl sulfate (8% or 10%) polyacrylamide gel electrophoresis (SDS-PAGE, BioRad Laboratories, Hercules, CA), and transferred from the gel onto to PVDF membrane (BioRad Laboratories, Hercules, CA), blotted with primary antibodies, probed with secondary isotype specific antibodies tagged with horseradish peroxidase (Cell Signaling Technology). Bound immunocomplexes were detected using a ChemiDOC™ XRS + system (BioRad Laboratories, Hercules, CA).

4.7. Molecular docking

Docking study was performed by Discovery Studio 3.5 and the PI3Kα H1047R mutant (PDB:3HHM) was downloaded from RCSB Protein Date Bank (www.rcsb.org). The protein and all ligands were prepared by minimization with CHARMM force field. Homology Modeling in Discovery Studio was used to treat with the missing residues in protein, which mainly based on MODELER program. Molecular docking was carried out using DS-CDOCKER protocol without constraint, all bound water and ligands were eliminated from the protein and the polar hydrogen was added to the proteins.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.103238.

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