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Design, synthesis and *in vitro* antitumor evaluation of novel pyrazole-benzimidazole derivatives

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ABSTRACT

A series of novel pyrazole-benzimidazole derivatives (**6**–**42**) have been designed, synthesized and evaluated for their *in vitro* antiproliferative activity against the HCT116, MCF-7 and Huh-7 cell lines. Among them, compounds **17**, **26** and **35** showed significant antiproliferative activity against HCT116 cell lines with the IC_{50} values of 4.33, 5.15 and 4.84 μM, respectively. Moreover, fluorescent staining studies showed compound **17** could induce cancer cells apoptosis. The flow cytometry assay revealed that compound **17** could induce cell cycle arrest at G0/G1 phase. All in all, these consequences suggest that pyrazole-benzimidazole derivatives could serve as promising compounds for further research to develop novel and highly potent cancer therapy agents.

Introduction

Cancer, described as the uncontrolled proliferation and spread of abnormal cells, is one of the most siginificant health problems today.[1] Chemotherapy drugs are widely used in the clinical treatment of cancer. However chemotherapeutic drugs such as cisplatin, thiotepa, paclitaxel and chlorambucil failed to control the growth of cancer cells completely. The traditional chemotherapy is facing the challenge of lack selectivity towards tumor cells.[2] Therefore, the development of the new antitumor agents has been getting more and more attention.[3]

Heterocyclic compounds are well-documented pharmaceutically active products, and have been found to play a significant role in the drug design and discovery. Hence, heterocyclic moieties are often used in the design and synthesis of efficient and pharmacological agents for the cure of various diseases, such as antioxidant, anticancer agent, antibacterial agent. Among the *N*-heterocyclic pharmacophores, benzimidazole[4–8] and pyrazole[9–12] moieties are of interest because of their wide range of clinical applications.

Benzimidazole nucleus is used as privileged structural motif in the development of a wide range of drugs with interest in numerous medicinal areas, such as anti-inflammation, anti-microbial, and anti-cancer.[13–15] The heterocycle has been applied into various marketed anticancer drugs such as bendamustine, veliparib, pracinostat and galeterone (Fig. 1).

1,3-Diphenylpyrazole moieties are highly important pharmacologically active organic compounds found in medicinally active substances. Recent years there have been reported an increasing number of molecules containing 1,3-diphenylpyrazole moieties showed various bioactivities such as antitumor, immunosuppressive, antiotubercular and antibacterial (some moleculars are showed in Fig. 2).[16–19]

Inspired by the combination principle of some molecules encompassing benzimidazole moiety and 1,3-diphenylpyrazole moiety for the synthesis of new bioactive compounds[20–23] (Fig. 3), we designed and synthesized a new series of heterocyclic antitumor molecules by the joining of two important pharmacophores: 1,3-diphenylpyrazole and benzimidazole. Different substituents on benzimidazole ring and benzene ring were introduced to investgate their anti-proliferative activity against cancer cells *in vitro*.

The synthesis of the target compounds was described in Scheme 1. 4-Substituted acetophenone (**1a** and **1b**) was condensed with phenylhydrazine (**2**) in reflux ethanol containing 5% glacial acetic acid.[24,25] The ethylidene hydrazine (**3a** and **3b**) was formed as precipitate at room temperature and filtered. The next step was Vilsmeier-Haack-Arnold ring closing formylation,[26] by treating the ethylidene hydrazine (**3a** and **3b**) with $POCl_3$/DMF.[27] The compounds (**5a** and **5b**) were obtained in good yields by heating aldehyde intermediates (**4a** and **4b**) with *O*-phenylenediamine in DMF and sodium metabisulfite.[28] Then respectively reacted with various alkyl bromide in the presence of cesium carbonate in acetonitrile at room temperature or in the presence of sodium hydride in tetrahydrofuran at 0 °C to yield the desired compounds pyrazol-benzimidazole derivatives (**6**–**42**). 37 novel derivatives were characterized by ^{1}H NMR, ^{13}C NMR, ^{19}F NMR and (HR)ESI-MS. The

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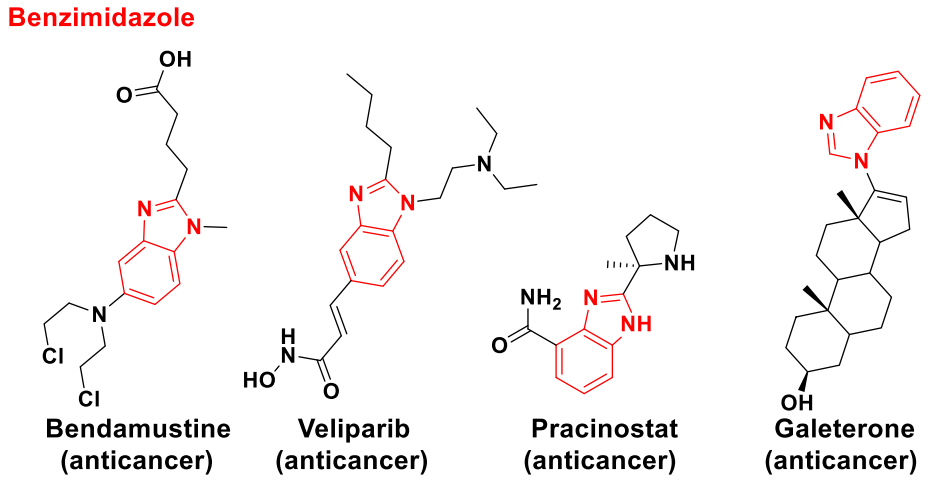


Fig. 1. Drugs possessing benzimidazole motifs.

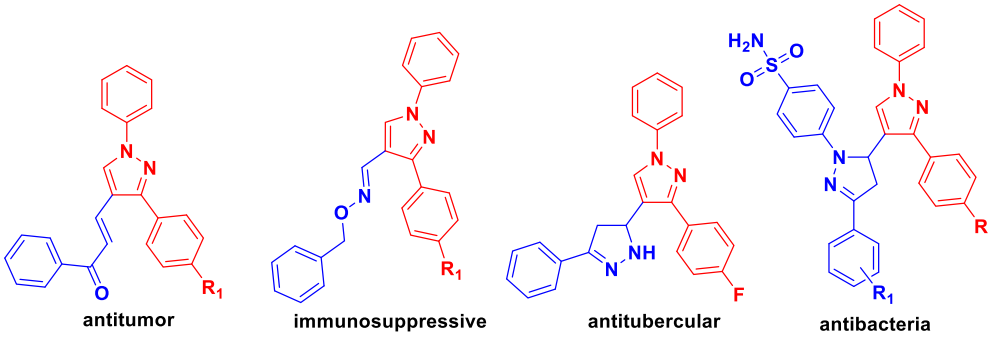


Fig. 2. Examples of molecules with 1,3-diphenylpyrazole.

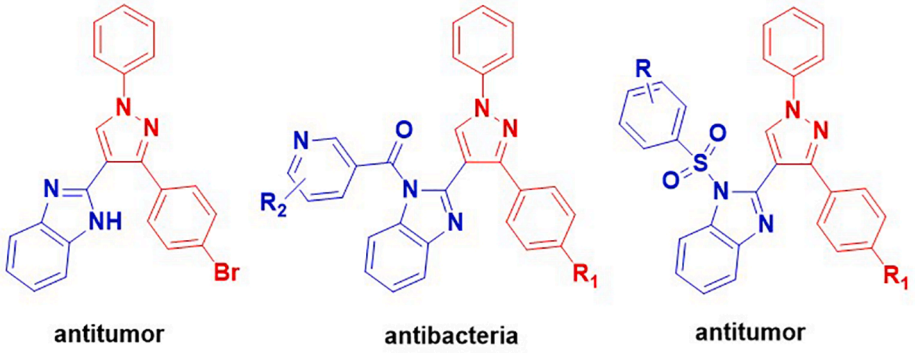


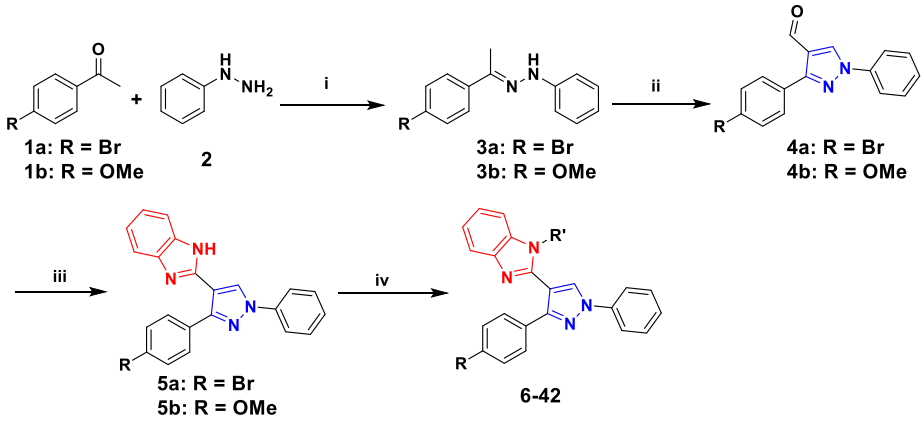
Fig. 3. Examples of pyrazole-benzimidazole derivatives.

purity of all derivatives was greater than 97% by HPLC detection.

All the synthesized benzimidazole-pyrazoles derivatives (**6**–**42**) were screened for their antiproliferative activity against three human tumor cell lines: HCT116, MCF-7 and Huh-7 cancer cells lines by using sulforhodamine B (SRB) growth inhibition assay.[29] The derivatives were dissolved in DMSO (less than 0.1%) and etoposide (VP-16) was used as positive control. The IC_{50} values of the derivatives are listed in Table 1.

The derivatives (except compound **6** and **38**), possessing different substitituted aryl or alkyl group on benzimidazole, showed higher antiproliferative activity (IC_{50} ranges from 4.33 to 10.0 μM) than positive control etoposide (VP-16, IC_{50} = 15.6 μM) against HCT116 cells. Furthermore, most derivatives showed moderate anti-prolifeative activities against MCF-7 cells with IC_{50} values ranging from 10 to 16.4 μM, which is better than VP-16 (IC_{50} = 18.9 μM). However, the derivatives showed poor activities (IC_{50} > 20 μM) against Huh-7 cells. In general, the compounds showed more selectively significant anti-proliferative activities against HTC116 than MCF-7 and Huh-7 cells.

In comparison with compound **6** and **7**–**25**, when R was substituted with Br, derivatives with single or multiple substitution on the benzyl group exhibited considerable antiproliferative activities against HCT116



R = Br
6: R' = Benzyl
7: R' = 2-Fluorobenzyl
8: R' = 3-Fluorobenzyl
9: R' = 4-Fluorobenzyl
10: R' = 2-Chlorobenzyl
11: R' = 4-Chlorobenzyl
12: R' = 2-Bromobenzyl
13: R' = 3-Bromobenzyl
14: R' = 2-Iodobenzyl
15: R' = 3-Iodobenzyl
16: R' = 2-(Trifluoromethyl)benzyl
17: R' = 4-(Trifluoromethyl)benzyl
18: R' = 3,5-Difluorobenzyl
19: R' = 2-Chloro-4-fluorobenzyl
20: R' = 2-Bromo-5-fluorobenzyl
21: R' = 2-Bromo-5-chlorobenzyl
22: R' = 2-Bromo-4-methoxybenzyl
23: R' = 4-Bromo-2-fluorobenzyl
24: R' = 3,5-Dimethylbenzyl
25: R' = 2,3,4,5,6-Pentafluorobenzyl
26: R' = Propyl
27: R' = Allyl
28: R' = Propargyl
29: R' = Ethoxycarbonylmethyl
30: R' = 1-Methylallyl
R = OMe
31: R' = 4-Fluorobenzyl
32: R' = 4-Chlorobenzyl
33: R' = 2-Bromobenzyl
34: R' = 3-Bromobenzyl
35: R' = 3-Iodobenzyl
36: R' = 4-Cyanobenzyl
37: R' = 3,5-Difluorobenzyl
38: R' = 2-Bromo-5-chlorobenzyl
39: R' = 2-Bromo-5-methoxybenzyl
40: R' = 3,5-Dimethylbenzyl
41: R' = Cinnamyl
42: R' = 1-Methylallyl

Scheme 1. Reagents and conditions: (i) HOAc, EtOH, reflux, yield: for **3a**: 95%; for **3b**: 90%; (ii) $POCl_3$, DMF; $NaHCO_3$, reflux, yield: for **4a**: 90%; for **4b**: 86% (iii) *o*-diaminobenzene, sodium metabisulfite, DMF, 110 °C, 4 h, reflux, yield: for **5a**: 80%; for **5b**: 74% (iv) alkyl bromide, Cs_2CO_3, MeCN, rt, yield: 60–85%; for compound **26**: propyl bromide, NaH, THF, 0 °C, yield: 70%.

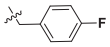
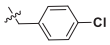
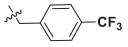
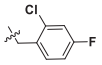
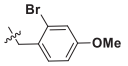
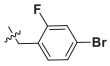
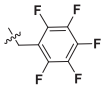
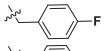
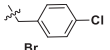
(colon cancer), but did not significantly affect the inhibitory activity against MCF-7 (breast cancer) and Huh-7 (liver cancer), which indicated that the introduction of substituted benzyl groups can increase the selectivity index of the derivative against HCT116 (breast cancer) when R was substituted with Br. Derivatives with same substituents in different positions will also exhibit different inhibitory activities. For instance, for derivatives **7**, **8** and **9**, the order of their antiproliferative activities against HCT116 cells is *meta* > *ortho* > *para*, and derivative **8** (with the substitution of 3-fluorobenzyl group) exhibited higher inhibitory activity against HCT116 cells (IC_{50} = 5.43 μM) and selectivity index (3.1). For derivatives **12**–**15,** the inhibitory activity and selectivity of *meta*-substituted derivatives are also higher than other positions. Derivative **17** bearing 4-(trifluoromethyl)benzyl group showed better activities than derivative **16** bearing 2-(trifluoromethyl)benzyl group, **17** also exhibited the highest antitumor activity against HCT116 with IC_{50} value of 4.33 μM and with high selectity index value of 3.4. Then we introduced the hydrocarbon group in the benzimidazole ring to investigate its performance. For derivatives **26**–**28**, increasing its hydrophobic properties can also increase the inhibitory activity against HCT116 and selectivity index. Interestingly, derivative **29** exhibited lower cytotoxic effect against two cell lines (HCT116, MCF-7) and selectivity index probably because of the bulkiness of the substituted alkyl group. Derivatives with R substituted with OMe showed higher activities against MCF-7 cells than R substituted with Br (for example, **31** vs **9**, **33** vs **12**, **34** vs **13**, **35** vs **15**), but it has weak or poor influence on the inhibitory activity against HCT116, this leads to the decrease of the selectity index.

The values of topological polar surface area (TPSA) and percent absorption (%ABS) could be used to predict absorption of the pyrazole-benzimidazole derivatives. TPSA is the surface belonging to polar atoms and is described as a value connected with passive molecular transport through membranes, and thus enables estimation of transport properties in the intestine and blood brain barrier.[30] TPSA values of more than half of derivatives were 35.65 Å, while the TPSA value of VP-16 is 160.83 Å. The absorption (%ABS) was determine using the equation %ABS = 109 − 0.345 × TPSA following the method of Zhao et al.[31] Compounds **6**–**29** derivatives showed %ABS values more than 95%, indicating potential for good oral bioavailability. To find out whether the calculated clogP (the logarithm of partition coefficient of a compound between *n*-octanol and water) and antiproliferative activity are related, the clogP values of these derivatives were calculated via Molinspiration and the results were showed in Table 2. It showed that there was no obvious connection between clogP values and their antiproliferative activities, while introducing substituents on benzimidazole core affected the hydrophobicity to a certain extent.

The morphological characteristics displayed by cells would exhibits some changes like cell shrinkage and chromatin accumulation when cell apoptosis occurs. In order to understand whether compound **17** can induce apoptosis, HCT116 cells were treated with different concentrations of compound **17** (1, 2.5 or 5 μM) for 48 h and then stained with Hoechst 33258. The morphological changes were observed by using fluorescence microscopy (Fig. 4). The control (DMSO) exhibited slight blue fluorescence, while HCT116 cells treated with different concentrations of compound **17** exhibited bright blue fluorescence due to staining of the condensed chromatin in apoptotic cells (Fig. 5). The results showed that compound **17** was significant in inducing apoptosis in HCT116 cells. We speculate that compound **17** might exert an effect on inhibitory effects on tubulin polymerization.

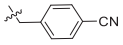
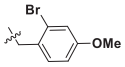
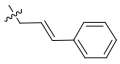
To determine whether the derivatives could induce alterations in the cell cycle progression, we evaluated the effect of the compound **17** on the cell cycle progression by using flow cytometric analysis in HCT116 cells after treatment with 1 or 5 μM of the compound for 24 h. As shown

Table 1
The *in vitro* anti-proliferative evaluation of target compounds **6**–**42** against selected cell lines.

No.	R	R′	IC_{50}, μM[a]			
			HCT116	MCF-7	Huh-7	SI[b]
6	Br		19.1 ± 1.1	15.5 ± 2.1	19.0 ± 0.3	0.8
7	Br		6.53 ± 0.14	15.9 ± 0.3	>20	2.4
8	Br		5.43 ± 0.36	16.7 ± 1.3	>20	3.1
9	Br		8.01 ± 0.09	15.2 ± 0.3	>20	1.9
10	Br		9.65 ± 0.07	14.2 ± 0.2	>20	1.5
11	Br		7.26 ± 0.04	15.5 ± 0.2	>20	2.1
12	Br		7.49 ± 0.27	15.2 ± 0.3	>20	2.0
13	Br		6.67 ± 0.36	17.1 ± 0.6	>20	2.6
14	Br		6.63 ± 0.36	15.4 ± 1.9	>20	2.3
15	Br		5.40 ± 0.15	19.5 ± 0.9	>20	3.6
16	Br		8.80 ± 0.08	16.4 ± 0.3	18.8 ± 0.3	1.9
17	Br		4.33 ± 0.26	14.7 ± 2.3	>20	3.4
18	Br		9.79 ± 0.13	13.4 ± 0.6	19.3 ± 1.0	1.4
19	Br		6.35 ± 0.15	14.0 ± 0.4	>20	2.2
20	Br		8.97 ± 0.44	14.7 ± 0.6	>20	1.6
21	Br		7.83 ± 0.03	19.4 ± 0.90	>20	2.5
22	Br		9.67 ± 0.18	16.0 ± 0.4	>20	1.7
23	Br		8.07 ± 0.50	14.8 ± 0.5	>20	1.8
24	Br		5.96 ± 0.15	15.2 ± 0.4	>20	2.6
25	Br		9.83 ± 0.14	12.7 ± 0.3	>20	1.3
26	Br		5.15 ± 0.10	16.4 ± 1.1	>20	3.2
27	Br		5.19 ± 0.38	16.3 ± 0.9	>20	3.1
28	Br		5.22 ± 0.09	14.7 ± 0.7	>20	2.8
29	Br		7.49 ± 0.35	16.8 ± 0.5	>20	2.2
30	Br		7.26 ± 0.22	15.3 ± 0.3	>20	2.1
31	Br		6.17 ± 0.24	12.3 ± 1.6	>20	2.0
32	OMe		6.72 ± 0.26	11.9 ± 1.5	>20	1.8
33	OMe		7.19 ± 0.73	12.5 ± 1.1	18.4 ± 0.5	1.7
34	OMe		5.78 ± 0.03	11.1 ± 0.9	>20	1.9

(*continued on next page*)

Table 1 (*continued*)

No.	R	R′	IC_{50}, μM[a]			
			HCT116	MCF-7	Huh-7	SI[b]
35	OMe		4.84 ± 0.14	12.8 ± 1.1	>20	2.6
36	OMe		5.58 ± 0.22	11.0 ± 1.4	>20	2.0
37	OMe		5.99 ± 0.14	11.3 ± 1.3	>20	1.9
38	OMe		16.5 ± 0.20	11.5 ± 0.4	>20	0.7
39	OMe		10.0 ± 0.51	12.5 ± 2.6	>20	1.3
40	OMe		6.27 ± 0.14	13.9 ± 1.1	>20	2.2
41	OMe		6.03 ± 0.24	10.6 ± 1.3	>20	1.8
42	OMe		7.98 ± 0.42	11.7 ± 1.6	>20	1.5
E[c]			15.6 ± 0.21	18.9 ± 0.8	17.3 ± 0.6	1.2

[a] Values are means from three independent experiments. Compounds were measured at different concentrations for 48 h.
[b] Selective Index (SI): [IC_{50} MCF-7]/[IC_{50}HCT116].
[c] Positive control etoposide (VP-16).

Table 2
clogP, TPSA and %ABS values of the derivatives **6**–**42**.[a]

Compound	clogP	TPSA	%ABS	Compound	clogP	TPSA	%ABS
6	7.69	35.65	96.70	**25**	8.62	35.65	96.70
7	7.83	35.65	96.70	**26**	7.23	35.65	96.70
8	7.83	35.65	96.70	**27**	6.95	35.65	96.70
9	7.83	35.65	96.70	**28**	6.27	35.65	96.70
10	7.83	35.65	96.70	**29**	7.26	35.65	96.70
11	8.40	35.65	96.70	**30**	6.47	61.96	87.62
12	8.55	35.65	96.70	**31**	6.93	44.89	93.51
13	8.55	35.65	96.70	**32**	7.49	44.89	93.51
14	8.51	35.65	96.70	**33**	7.64	44.89	93.51
15	8.81	35.65	96.70	**34**	7.64	44.89	93.51
16	8.57	35.65	96.70	**35**	7.90	44.89	93.51
17	8.57	35.65	96.70	**36**	6.21	68.68	85.31
18	7.98	35.65	96.70	**37**	7.07	44.89	93.51
19	8.55	35.65	96.70	**38**	8.36	44.89	93.51
20	8.70	35.65	96.70	**39**	7.70	44.89	93.51
21	9.27	35.65	96.70	**40**	7.78	44.89	93.51
22	8.61	44.89	93.51	**41**	7.97	44.89	93.51
23	8.70	35.65	96.70	**42**	6.35	44.89	93.51
24	8.96	35.65	96.70	**VP-16**	−0.11	160.83	53.51

[a]TPSA calculations were done by using Molinspiration. ClogP values were calculated by using Molinspiration. %ABS: Percentage of absorption. %ABS = 109 − 0.345 * TPSA31.

in Fig. 4, with the concentrations of compound **17** increased, the percentage of HCT116 cells in the G0/G1 phase increased from 44.1% to 67.1%, while the percentage of HCT116 cells in the S phase decreased from 45.0% to 22.7%. These results showed that compound **17** induced cell cycle arrest at the G0/G1 phase in a concentration-dependent manner. Its' mechanism is significantly different from the positive control VP-16.

In conclusion, we designed and synthesized 37 novel pyrazole-benzimidazole derivatives and evaluated their anti-proliferative activities against the tested cells. Moreover, a majority of derivatives exhibited potent antiproliferative activities against HCT116 with IC_{50} values ranging from 4.33 μM to 9.83 μM. Among them, compound **17** exhibited the most potent anti-proliferative activity against the HCT116 cells with IC_{50} value and selective index of 4.33 μM and 3.4, respectively. In addition, mechanism of action research shown that compound **17** could significantly induce cell cycle arrest and apoptosis in HCT116 cells. Furthermore, all the consequences showed that pyrazole-benzimidazole derivatives will serve as antiproliferative leads for further chemical optimization.

Declaration of Competing Interest

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

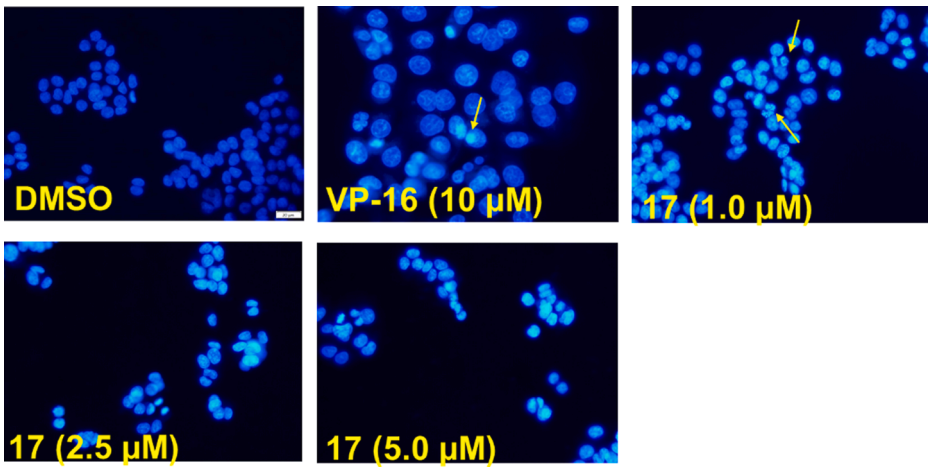


Fig. 4. Hoechst staining of compound **17** in HCT116 cells. After the treatment with compound **17** (1, 2.5 or 5 μM) for 48 h, HCT116 cells were stained by Hoechst 33258 solution, then detected by fluorescent microscope (20×). Error bar (white in DMSO): 20 μm.

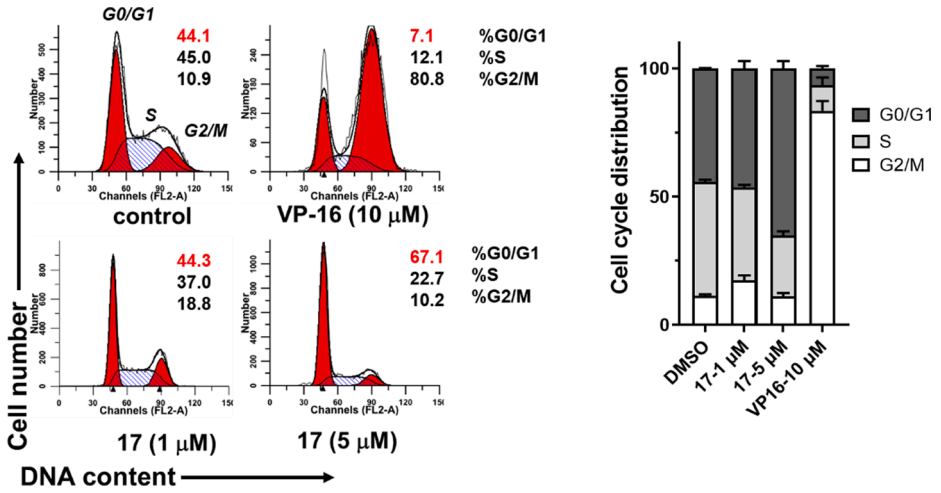


Fig. 5. Cell cycle analysis of different concentrations of compound **17** and VP-16 in HCT116 cells. HCT116 cells were treated with compound **17** (1 or 5 μM) and VP-16 (10 μM) for 24 h, then stained with PI. A flow cytometer was used to analyze.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data (experimental section and spectral data containing m.p., ^{1}H NMR, ^{13}C NMR, ^{19}F NMR, (HR)ESI-MS and HPLC) to this article can be found online at https://doi.org/10.1016/j.bmcl.2021.128097.

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