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Graphical Abstract

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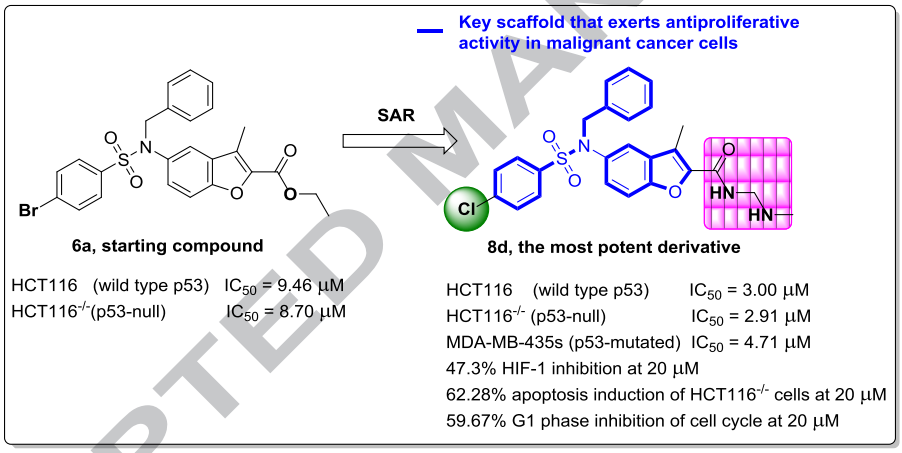

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ABSTRACT

p53-independent malignant cancer is still severe health problem of human beings. HIF-1 pathway is believed to play an important role in the survival and developing progress of such cancers. In the present study, with the aim to inhibit the proliferation of p53-independent malignant cells, we disclose the optimization of **6a**, the starting compound which is discovered in the screening of in-house compound collection. The structure-activity relationship (SAR) is summarized. The most potent derivative **8d**, inhibits the proliferation of both p53-null and p53-mutated cells through inhibition of HIF-1 pathway. Our findings here provide a new chemotype in designing potent anticancer agent especially against those p53-independent malignant tumors.

Cancer is a major public health problem that causes the death of human beings worldwide. In recent years, there are increasing pre-clinical and clinical achievements in the treatment of cancers. Among these discoveries, p53 signaling pathway attracts the attention of researchers all over the world. It induces cell cycle arrest and apoptosis in the presence of metabolic disorder or genetic damage. Activating the function of p53, which is mainly achieved by blocking the p53-MDM2 protein-protein interaction with small molecules such as **Nutlin-3a**,[1] is considered to be a promising therapeutic strategy. However, for those malignant cancer cell lines, especially the ones that are independent of p53 because of mutation or deletion of p53, there have been only minimal therapeutic improvements. Mutation or deletion of p53 occurs in approximately 50 % of cancers. That will cause the loss of tumor suppressor function of p53, and what's worse, will lead to new oncogenic gain-of-function properties in the cancer cells including tumorigenesis, invasion, and metastasis[2]. As a result, obtaining efficient inhibitors against p53-independent cells is still an emergent task for medicinal chemists.

A unique feature of malignant cancers is that the rapid growth of the cells often resulted in hypoxia microenvironment, leading to reduced oxygen availability of the cells due to the formation of inadequate or aberrant vasculature.[3] The hypoxic condition can reduce the sensibility of cancer cells to radiotherapy[4] and conventional chemotherapy[5], thus leading to poor therapeutic outcome.[6] At the molecular level, the transcription factor hypoxia inducible factor-1 (HIF-1) has been recognized as the key modulator of the response to hypoxia in malignant cells. It enhances the transactivation of many genes that are involved in cell survival, metabolism, angiogenesis and invasion.[7] Therefore, inhibition of HIF-1 function is considered as an attractive strategy for the malignant cancer therapy.[8-10]

As a key functional moiety, benzofuran appears in the structures of many natural products and approved drugs. Recently, benzofuran derivatives have been verified to exert antiproliferative activity through multiple mechanisms, such as tubulin polymerization inhibition,[11,12] multi-drug resistance (MDR) reversal activity[13] and HIF-1 inhibition.[14] For instance, moracin family are bioactive benzofuran derivatives isolated from species of the *moraceae* family. **Moracin O** and **Moracin P** were reported for their ability to inhibit HIF-1 activation, with IC_{50} values of 0.14 and 0.65 μM, respectively.[14,15] Another benzofuran derivative **BNC-105P**, a tubulin polymerization inhibitor, is being developed in early clinical development at Bionomics for the treatment of progressive or metastatic clear cell renal cell carcinoma (Phase II),[16] asbestos-related mesothelioma (Phase II)[17] and ovarian cancer (Phase I/II).[18]

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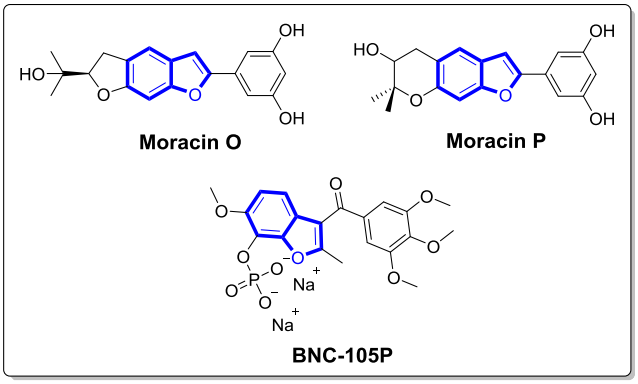


Figure 1. Representation of benzofuran derivatives

Our research groups focused on the discovery and optimization of new therapeutic agents against malignant cancers for over a decade.[19-22] Previously, we performed cell-based screenings of in-house compound collections with the aim to identify new compounds against p53-independent malignant cancer cell lines. During screening, compound **6a** showed moderate inhibition of two human colorectal carcinoma cell lines, HCT116 (with wild-type p53) and HCT116$^{-/-}$(with p53 deleted), with IC_{50} value of 9.46 ± 1.01 and 8.70 ± 1.76 μM, respectively. Considering that the compound showed better inhibitory activity on p53-independent cell HCT116$^{-/-}$ than traditional p53-MDM2 inhibitor **Nutlin-3a**, we infer that **6a** may be more sensitive to p53-independent cells. Therefore, the compound was selected as the starting compound for further optimization.

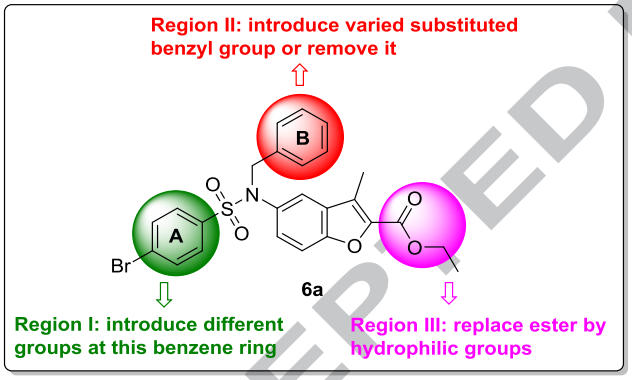
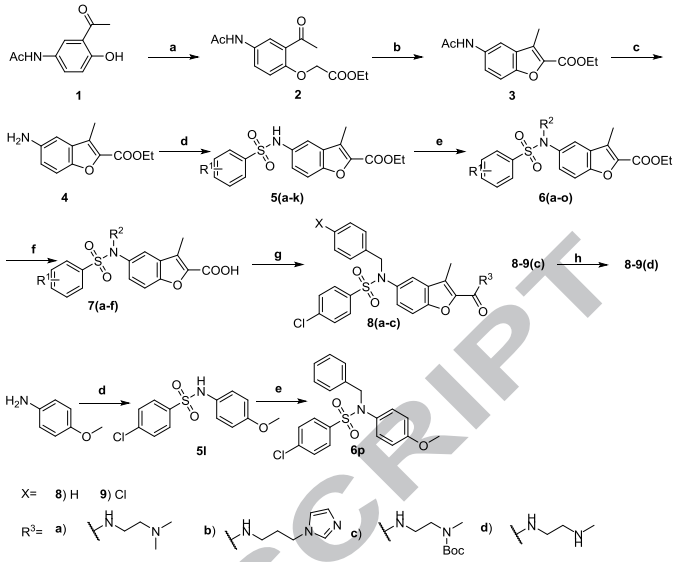


Figure 2. Structural optimization strategy of the starting compound **6a**.

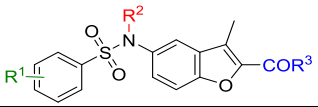
In order to identify the key component of compound **6a**, the scaffold of this compound was firstly analyzed. Two series of analogues **6a~p** and **7a~g** were synthesized (**Scheme 1**) in order to evaluate the importance of the two aromatic ring (ring A and B, respectively). Additionally, we observed that **6a** contained several hydrophobic rings, which can lead to the poor solubility of the compound (cLogP of **6a** was 6.14 calculated by Discovery Studio 4.0). To solve this problem, the ester group (Region III) was replaced by other polar groups to explore their effects on antitumor activities as well as the physicochemical properties.

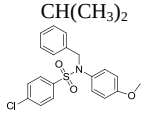
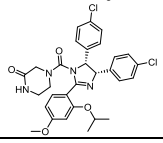
Compound **1** was prepared as described in literature[23] with p-anisidine as the starting material. In the presence of potassium carbonate, **1** was alkylated with ethyl bromoacetate to give **2**. Cyclization of **2** in the presence of potassium carbonate under microwave irradiation afforded benzofuran **3**.[24] Hydrolysis of **3** with HCl gave **4**. Sulfonylation of **4** with substituted benzenesulfonyl chloride and pyridine generated analogues **5**.[25] N-alkylation of **5** led to analogues **6**. Hydrolysis of carboxylic ester **6** with sodium hydroxide led to **7**. **8a~c** and **9a~c** were obtained by amidation of **7** and substituted amine. Deprotection of Boc group in **8c** and **9c** gave **8d** and **9d**.



Scheme 1. Reagents and conditions: (a) $BrCH_2COOEt$, KI, K_2CO_3, acetone, reflux, 2h, 91%; (b) K_2CO_3, DMF, microwave (350W, 145 °C, 7 min) 59%; (c) 3M HCl, EtOH, reflux, 6-8 h, 63%; (d) pyridine, substituted benzenesulfonyl chloride, DCM, r.t., 6h, 41-82%; (e) R^2Br or R^2Cl, K_2CO_3, KI, acetone, reflux, 2-8 h, 41-90%; (f) NaOH/EtOH, reflux, 2 h, 69-85%; (g) EDCI, HoBt, DIPEA, RNH_2, r.t., overnight, 43-88%; (h) CF_3COOH, 30 °C, overnight, 98%.

Table 1. Antiproliferative activities of the synthesized analogs of **6a** against human colorectal carcinoma cells

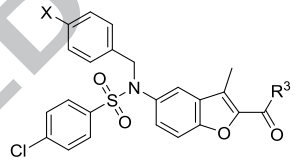


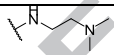
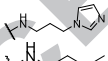
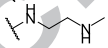
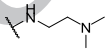
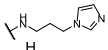
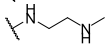
Compd.	R^1	R^2	R^3	IC_{50}(μM)	
				HCT116	HCT116$^{-/-}$
6a	4-Br	Benzyl	OEt	9.46 ± 1.01	8.70 ± 1.76
6b	3-Br	Benzyl	OEt	>40	>40
6c	2-Br	Benzyl	OEt	>40	>40
6d	4-CH_3	Benzyl	OEt	>40	>40
6e	4-OMe	Benzyl	OEt	>40	>40
6f	4-NO_2	Benzyl	OEt	>40	>40
6g	4-CF_3	Benzyl	OEt	26.7 ±1.8	12.7 ± 2.6
6h	4-Cl	Benzyl	OEt	4.86 ±0.56	23.2 ± 1.1
6i	3-Cl	Benzyl	OEt	>40	>40
6j	4-F	Benzyl	OEt	5.82 ± 0.73	33.1 ± 3.6
6k	H	Benzyl	OEt	>40	>40
6l	4-Br	4-Br-benzyl	OEt	10.9 ± 1.7	5.22 ± 1.41
6m	4-Cl	4-Cl-benzyl	OEt	9.48 ± 0.84	9.64 ± 1.43
6n	4-Cl		OEt	14.1 ±1.5	>40
6o	4-Cl	$CH(CH_3)_2$	OEt	7.83 ± 1.71	>40
6p				18.3 ± 2.5	>40
5a	4-Br	H	OEt	9.31 ± 0.75	>40
5g	4-CF_3	H	OEt	5.03 ± 0.50	24.2 ± 1.1
5h	4-Cl	H	OEt	24.3 ± 1.08	>40
5j	4-F	H	OEt	10.5 ± 1.0	>40
7a	4-Br	Benzyl	OH	7.09 ± 0.48	20.0 ± 2.3
7b	4-Br	4-Br-benzyl	OH	8.58 ± 0.82	>40
7c	4-Cl	Benzyl	OH	9.13 ± 0.93	33.4 ± 2.0
7d	4-Cl	4-Cl-benzyl	OH	3.67 ± 0.20	27.2 ± 2.7
7e	4-F	Benzyl	OH	>40	>40
7f	4-CF_3	Benzyl	OH	4.13 ± 0.94	21.1 ± 1.4
Nutlin-3a				5.21 ± 0.39	21.5 ± 3.7

All the compounds were assessed *in vitro* against HCT116 and HCT116$^{-/-}$ (p53-null) cell lines. **Nutlin-3a** was used as the positive control. For the structure-activity relationship (SAR) of these compounds, we firstly observed that the substituted position of ring A played critical role for the compounds. Changes from 4-bromine to 3- or 2-bromine (**6b** or **6c**) led to the completely loss of antitumor activity. Similar manner was observed in -Cl substitution (**6h** and **6i**). The results indicated that *para* substitution of ring A was one of the determinants for the antiproliferative activity. As a result, we then introduced other groups at the *para* position of ring A. The bromine atom was replaced by different kinds of substituent, including electron-donating groups (**6d**, **6e**) and electron-withdrawing groups (**6f**, **6g**, **6j**). We found electron-donating groups were not preferred, as **6d** and **6e** completely loss their antiproliferative activity. While electron-withdrawing groups slightly reduced the activity, with an exception of **6f**, this can be explained by its poor solubility during the assay. When we removed the substituent of ring A (**6k**), the activity was completely lost, indicating appropriate group at *para* position of ring A was necessary. When halogen atom was introduced (**6l**, **6m**), the activity was slightly improved. Additionally, we found the **6l** showed improved potency on HCT116$^{-/-}$ cells, indicating that bromine at *para* position of ring B was preferred in designing potent compounds against p53-independent cells. When ring B was replaced by cyclohexylmethyl (**6n**) or isopropyl (**6o**), the activity on wild type HCT116 cells was retained, however, they completely loss the activities on HCT116$^{-/-}$ cells, indicating the benzene ring was important in designing potent compounds targeting p53-independent cells. Similar manner was observed when the benzene ring was removed (**5a**, **5g**, **5h**, **5j**). The results further supported the conclusion mentioned above. To further evaluate the importance of benzofuran core, we replaced it by benzene (**6p**). The compound also lost the activity on HCT116$^{-/-}$ cells, indicating the benzofuran core need to be retained.

We then optimized region III by replacing the ester with carboxyl group (**7a**~**7f**) to improve the aqueous solubility of the compounds. The activities of compounds **7a**~**7f** on HCT116 cells were retained, however, the HCT116$^{-/-}$ inhibition remarkably decreased. We inferred that the carboxyl group may be too polar to permeate the cell membrane of HCT116$^{-/-}$ cells. Compounds with both high cell permeability and good aqueous solubility were more likely to show good inhibitory activities. As a result, we then replaced the carboxyl group with N containing alkyl chains (**8a**, **8b**, **8d**, **9a**, **9b**, **and 9d**). We observed that all these six compounds exhibited obviously improved antiproliferative activity on HCT116$^{-/-}$ cells compared to carboxyl substituted compounds. To further verify that compounds were active in a p53-independent manner, we evaluated the antiproliferative activities of the compounds on two breast cancer cell lines. MCF7 was p53 wild type while MDA-MB-435s was more malignant with p53 mutation. We observed that all the compounds showed good antiproliferative activities on MDA-MB-435s cells, while the positive control, **Nutlin-3a**, exhibited about 5-fold decrease in inhibiting MDA-MB-435s cells compared to MCF7 cells. The most potent compound **8d** and **9d**, showed IC_{50} against the four cell lines in a low micromolar range, indicating that they can serve as the starting compounds for further optimization.

Table 2. Antiproliferative activities against five cancer cell lines and physicochemical properties of derivatives of **6h** and **6m**



Compd.	X	R^3	Antiproliferative activity, IC_{50} (μM)				HIF-1 IR[a] at 20 μM (%)
			HCT116	HCT116$^{-/-}$	MCF7	MDA-MB-435s	
8a	**H**		7.06 ± 0.51	6.31 ± 0.54	6.72 ± 1.69	9.24 ± 2.74	<10
8b	**H**		17.1 ± 4.9	5.00 ± 0.08	31.8 ± 5.24	5.60 ± 0.65	32.3 ± 6.0
8d	**H**		3.00 ± 0.86	2.91 ± 0.73	4.56 ± 0.27	4.71 ± 0.26	47.3 ± 5.7
9a	**Cl**		5.38 ± 0.49	3.87 ± 0.33	5.84 ± 0.89	10.8 ± 0.5	12.7 ± 4.4
9b	**Cl**		2.43 ± 0.61	1.89 ± 0.26	>40	7.25 ± 0.80	27.0 ± 9.1
9d	**Cl**		1.81 ± 0.33	4.03 ± 0.85	5.67 ± 0.75	1.63 ± 0.06	22.2 ± 3.8 (10 μM)[b]
Nutlin-3a	-	-	5.21 ± 0.39	21.5 ± 3.7	5.01 ± 0.05	29.6 ± 5.3	<10

[a] inhibitory rate; [b] **9d** caused completely cell death at a concentration of 20 μM.

Considering HIF-1 signaling pathway played an important role in malignant cancers, we then evaluated whether the compounds exerted their p53-independent antiproliferative effects through HIF-1 pathway. We initially assayed the compounds by a cell-based luciferase reporter assay on the basis of evaluating the hypoxia response elements (HREs). **8d** showed 47.3 ± 5.7 % inhibition of HRE at the concentration of 20 μM, while **9d** showed 22.2 ± 3.8 % inhibition at 10 μM. These data suggested that the compounds, at least partially, exerted their antiproliferative activities through HIF-1 inhibition. To further support the conclusion, we next selected **8d** to determine whether it could inhibit the gene level of endothelial growth factor (*VEGF*), a well-known downstream target gene of HIF-1. **8d** reduced the expression of *VEGF* gene in a dose dependent manner based on real-time PCR (rt-PCR, **Fig. 3**). The data were consistent with HRE assay, confirming that **8d** inhibited HIF-1 pathway, which would enhance the antitumor activity of the compound under hypoxia condition.

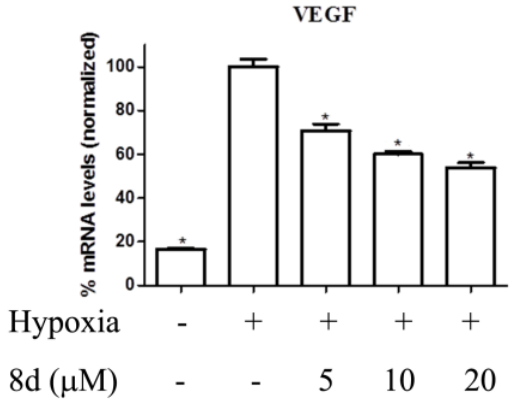


Figure 3. VEGF mRNA was measured by real-time PCR analyses in MCF7 cell line after **8d** (0, 5, 10, 20 μM) treated for 24 h under hypoxic condition. GAPDH expression was used as control. All error bars are SD. *Represents a significant difference compared to hypoxia control, $p < 0.05$.

We next evaluated whether **8d** could induce cell apoptosis through inhibiting HIF-1 pathway. After 48 h treatment of **8d** in HCT116$^{-/-}$ cells, a significantly morphologic change was observed, indicating that **8d** strongly induced the death of HCT116$^{-/-}$ cells (**Fig. 4A** and **4B)**. To characterize the mode of cell death induced by **8d**, biparametric flow cytometric analysis with annexin-V/propidium iodide (PI) was performed on HCT116$^{-/-}$ cells. After treatment with 5, 10 or 20 μM of **8d** for 48 h, the early apoptotic rates in control and treated HCT116$^{-/-}$ cells were 6.96, 15.42, 55.86 and 62.28%, respectively, and the late apoptotic rates were 3.11, 6.74, 14.99, and 34.63% respectively (**Fig. 4C**). These results demonstrated that **8d** treatment induced both early and late apoptosis in a concentration-dependent manner. To determine whether the suppression of HCT116$^{-/-}$ cell by compound **8d** was caused by a regulation of the cell cycle progress, a cell-cycle cytotoxicity assay was performed by treating HCT116$^{-/-}$ cells at different concentrations of compound **8d** (0, 5, 10, 20 μM) for 12 h . Cells in the G1 phase increased from 27.78% in control to 36.24%, 52.82% and 59.67%. Therefore, compound **8d** induced cell cycle arrest at the G1 phase in a concentration manner **(Fig. 4D)**.

Finally, we evaluated the expression of apoptosis related proteins procaspase-3 and procaspase-9 after the treatment of **8d** in HCT116$^{-/-}$ cells by western blot analysis (**Fig. 5**). The expression levels of the two proteins were remarkably decreased, indicating an activation of apoptosis. While p53 wild-type dependent compound **Nutlin-3a** did not show such effect. Taken together, compound **8d** induced cell apoptosis in a p53-independent manner.

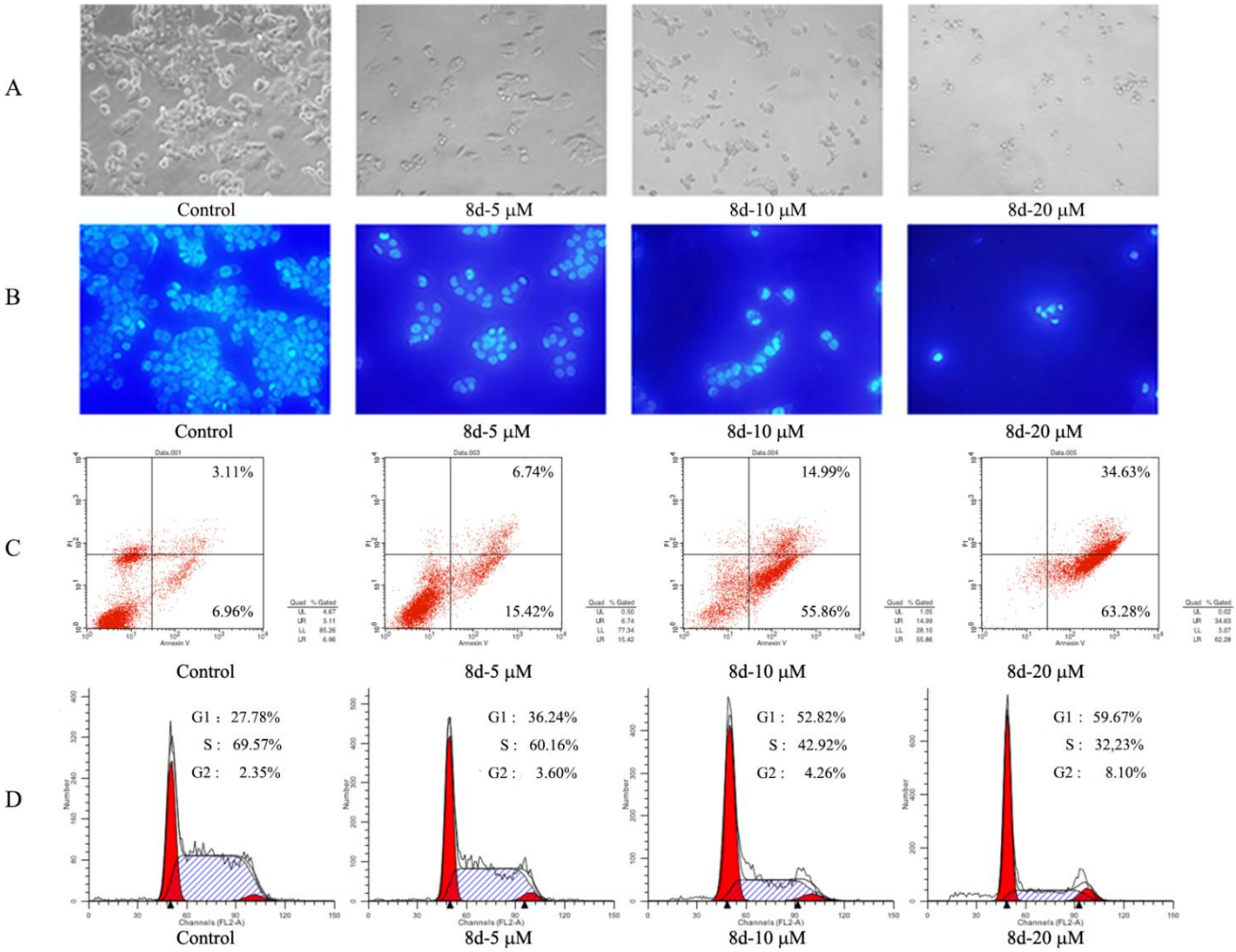


Figure 4. (A) Morphologic changes and (**B**) the nuclei of HCT116$^{-/-}$ (p53-null) cells were treated with 0, 5, 10 and 20 μM compound **8d** for 48h. (**C**) Apoptosis analysis of HCT116$^{-/-}$ (p53-null) cells was performed by flow cytometer after **8d** treatment for 48 h. (**D**) Cell cycle analysis of HCT116$^{-/-}$ (p53-null) cells was performed by flow cytometer after **8d** treatment for 12 h.

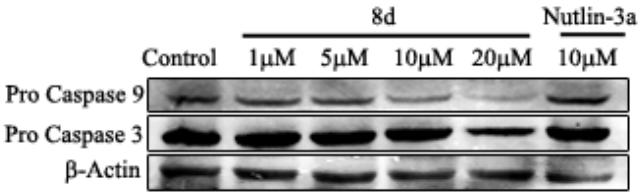


Figure 5. Cellular activity of **8d** for the caspase cascade pathway activation detected by western blotting (HCT116$^{-/-}$, 24 h treatment).

In conclusion, a new series of benzofuran derivatives were synthesized and evaluated against several cancer cell lines. Preliminary SAR found that 4-halogen phenylsulfonyl (Region I), benzyl (Region II) and benzofuran contributed significantly to the antiproliferative activities against cancer cell lines, especially those p53-independent cells. Although substitutions at region II do not affect the activity remarkably, it deserves to be mentioned that proper groups at place can improve the sensitivity of the compound to p53 independent cells. Further optimization can be performed guided by this information. Replacing the ester group (Region III) by N containing alkyl chains can remarkably enhance the activity, which may be attributed to the improved aqueous solubility. The SAR was summarized in **Fig. 6**.

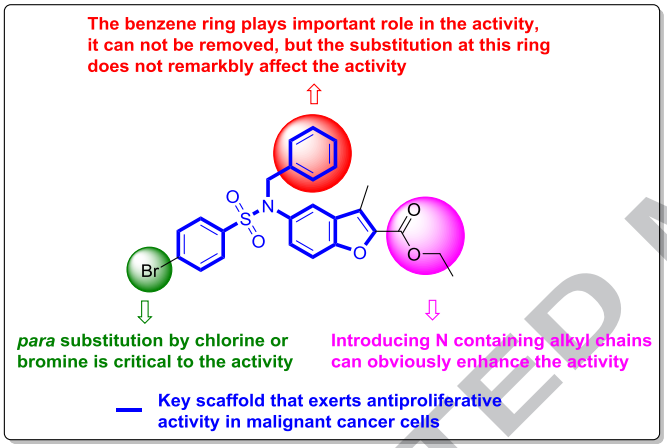


Figure 6. The SAR summary of the benzofuran derivatives.

Biological study showed that **8d**, the most potent derivative inhibited the growth of both p53-null and p53-mutated cells, inhibited the function of HIF-1 through HRE assay. The downstream of HIF-1 signaling was also suppressed. Further study confirmed that **8d** induced cell cycle arrest in G1 phase and apoptosis in a concentration-dependent manner. Taken together, our findings provide a new chemotype in designing potent anticancer agent especially against those p53-independent malignant tumors.

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Supplementary data

The structure determination and the methods for biological evaluation are supplied in supplementary data.

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