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Graphical abstract

Inhibition of colony formation

G2/M phase arrest

Morphological Changes

Loss of MMP

apoptosis

Compound 11E
IC$_{50}$ on MGC-803 cells:
7.59±0.28μM

Bax, Cleaved-Caspase 3/9,
Cleaved PARP and DR4

Bcl-xl and Bcl-2
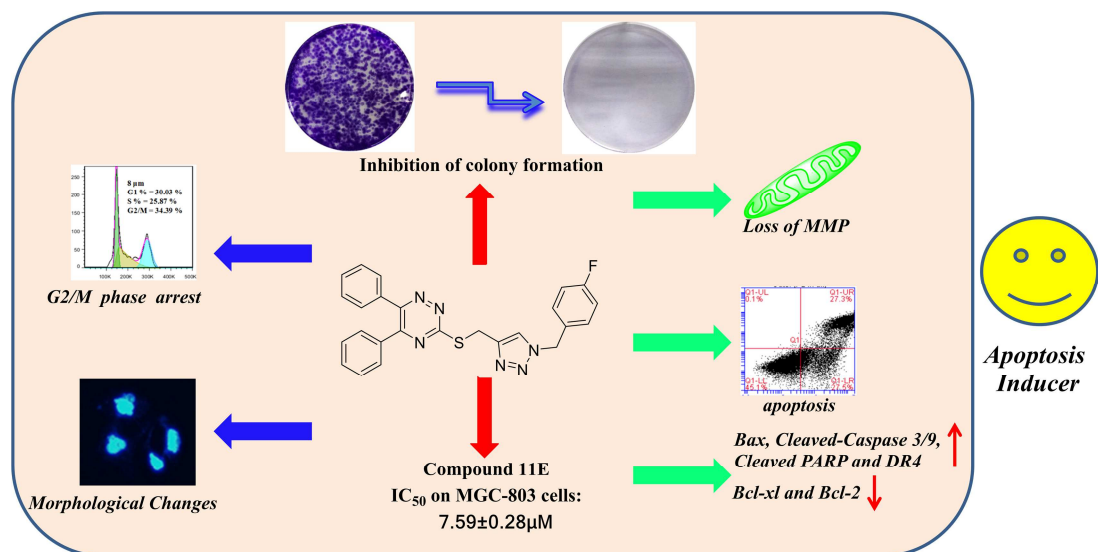
*Apoptosis
Inducer*

Discovery of 5,6-diaryl-1,2,4-triazines hybrids as potential apoptosis inducers

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Abstract: A series of 5,6-diaryl-1,2,4-triazines hybrids bearing a 1,2,3-triazole linker were synthesized by molecular hybridization strategy and evaluated for antiproliferative activity against three selected cancer cell lines (MGC-803, EC-109 and PC-3). The first structure-activity relationship (SAR) for these 5,6-diaryl-1,2,4-triazines is explored in this report with evaluation of 15 variants of the structural class. Among these chemical derivatives, 3-(((1-(4-fluorobenzyl)-1H-1,2,3-triazol-4-yl)methyl)thio)-5,6-diphenyl-1,2,4-triazine (**11E**) showed the more potent inhibitory effect against three cell lines than 5-Fu. Cellular mechanism studies in MGC-803 cells elucidated **11E** inhibited colony formation and arrested cell cycle at G2/M phase. Furthermore, compound **11E** caused morphological changes, decreased mitochondrial membrane potential, and induced apoptosis through the apoptosis-related proteins in MGC-803 cells. It was the first time, to our knowledge, that 5,6-diaryl-1,2,4-triazines bearing a 1,2,3-triazole linker were used as potential apoptosis inducers.

Keywords: 5,6-diaryl-1,2,4-triazines; molecular hybridization strategy; morphological changes; apoptosis; apoptosis-related proteins.

1. Introduction

5,6-Diaryl-1,2,4-triazine, an important heterocyclic skeleton, has been reported to exhibit a variety of biological activities [1-7]. 2-((5,6-diphenyl-1,2,4-triazin-3-yl)thio)-N-arylacetamide derivative **1** with strong electron-withdrawing nitro group on the arylacetamide moiety exhibited potent α-glucosidase inhibitory activity (IC$_{50}$ = 12.46 ± 0.13 μM) [8]. 5,6-diaryl-1,2,4-triazine bearing 3-morpholinoethylamine moiety **2** displayed a promising antithrombotic profile *in vivo*, which demonstrated less ulcerogenicity in rats as compared to aspirin [9]. 1,2,4-triazine derivative **3** was synthesized and screened for inhibition of cyclooxygenases (COX-

1 and COX-2) with anti-oxidant activity based on a cellular assay using human whole blood and lipoxygenase [10]. However, few studies investigated 5,6-diaryl-1,2,4-triazine moiety as a promising anticancer scaffold (**Fig. 1**).
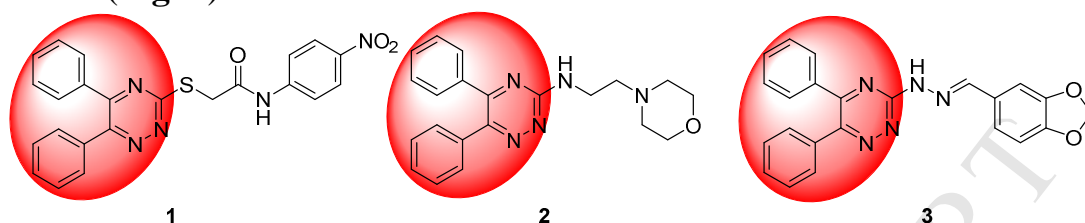

Fig. 1. Bioactive 5,6-diaryl-1,2,4-triazine derivatives.

1,2,3-triazole, a privileged scaffold in drug discovery, displayed a wide array of biological activities as antibacterial, anti-fungal, anti-HIV, anti-allergic, anti-tubercular and anti-inflammatory agents [11-14]. Recently, our group have reported four series of 1,2,3-triazole derivatives as potential antitumor agents (**Fig. 2**): 1,2,3-triazole-chalcone hybrid **4** inhibited the proliferation of SK-N-SH cancer cells by inducing apoptosis and arresting the cell cycle at the G1 phase [15]; the novel 1,2,3-triazole-chalcone **5** bearing a 4,5-dihydrothiazole showed the potent activity with an IC_{50} value of 8.16 μM against neuroendocrine cancer cells [16]; 1,2,3-triazole-dithiocarbamate based selective lysine specific demethylase 1 inactivator **6** inhibited gastric cancer cell apoptosis, invasion, and migration [17]; the steroidal hybrid **7** arrested cell cycle at G2/M phase, induced apoptosis accompanied with decrease of mitochondrial membrane potential [18].
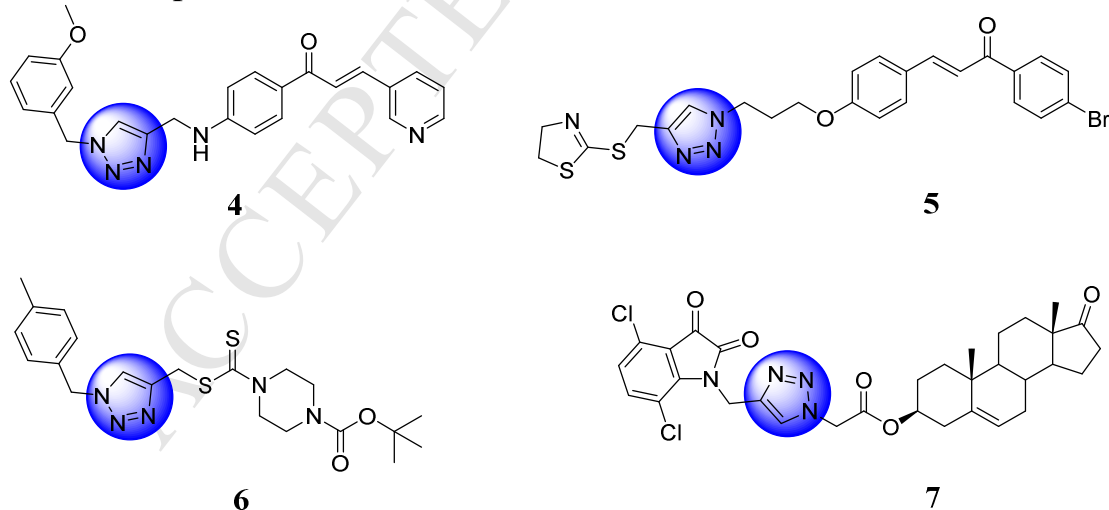

Fig. 2. 1,2,3-triazole derivatives as antiproliferative agents.

Molecular hybridization strategy is a useful concept in drug design and development based on the combination of pharmacophoric moieties of different bioactive substances to produce a new hybrid with improved affinity and efficacy, when compared to the parent drugs [19, 20]. These

above interesting findings and our continuous quest to identify more potent anticancer agents led to the molecular hybridization of 5,6-diaryl-1,2,4-triazine and bioactive scaffolds by a 1,2,3-triazole linker to integrate them in one molecular platform to generate a new hybrid with a potential antiproliferative activity.

As shown in **Fig. 3**, a molecular hybridization strategy based on the structures of a 5,6-diaryl-1,2,4-triazine and a 1,2,3-triazole yielded a scaffold which has three parts: (i) a 5,6-diaryl-1,2,4-triazine scaffold as a central core, (ii) a 1,2,3-triazole as a linker to combine different antiproliferative active skeletons, and (iii) a variety of reported antiproliferative skeletons (benzyl moiety, chalcone, formononetin, coumarin, sulfanilamide) [21-23]. To the best of our knowledge, there have been no literature reports regarding 5,6-diaryl-1,2,4-triazine hybrids bearing a 1,2,3-triazole linker as antiproliferative agents so far. These findings also encouraged us to investigate the potential synergistic effect of 1,2,3-triazole and 5,6-diaryl-1,2,4-triazine scaffolds.
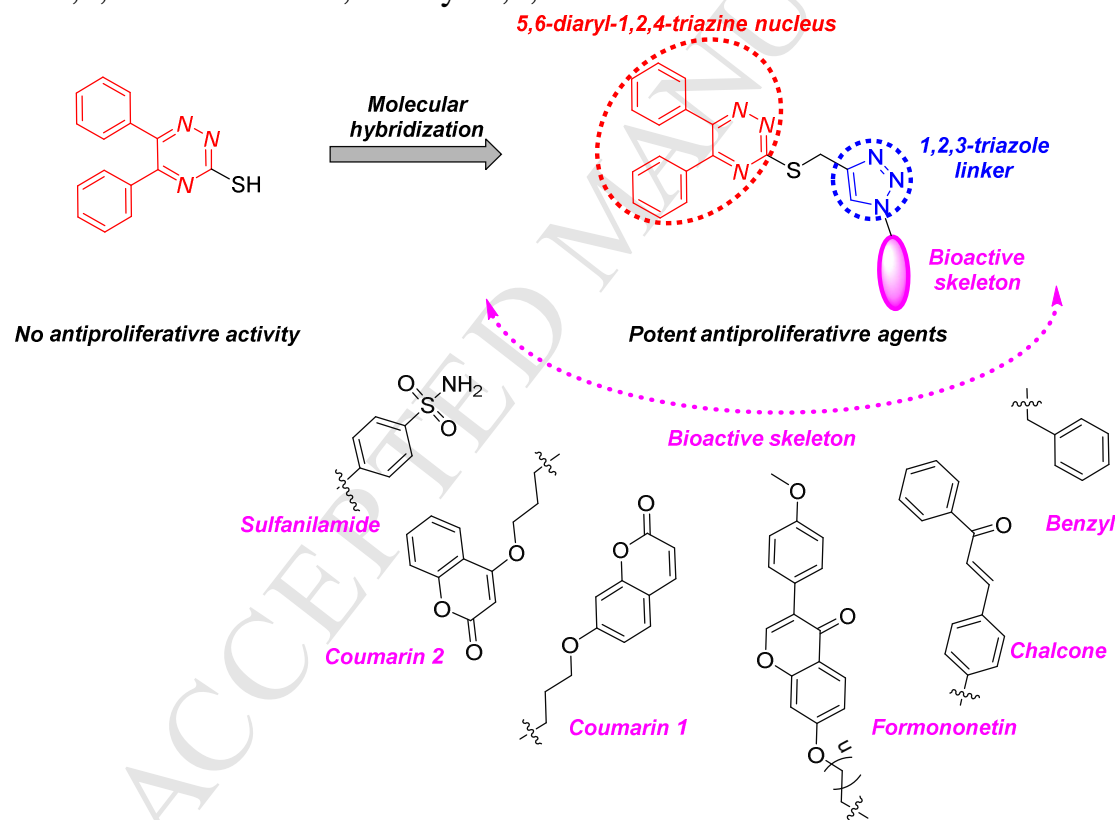

Fig. 3. Rational molecular hybridization design.

## 2. Results and discussion

2.1. Chemistry

To explore the potential synergistic effect of 1,2,3-triazole and 5,6-diaryl-1,2,4-triazine scaffolds, 5,6-diaryl-1,2,4-triazine derivatives without triazole moiety **10A-10C** were synthesized in **Scheme 1**.

Condensation of commercially available compound **8** with thiosemicarbazide in acetic acid at 120 ^{o}C for 3.5 h provided the 5,6-diphenyl-1,2,4-triazine-3-thiol **9** [24], which was then reacted with different bromides (1-bromopropan-2-one, 3-bromoprop-1-yne, and 3-bromopropanoic acid) in the presence of triethylamine as base to obtain compound **10A-10C**.
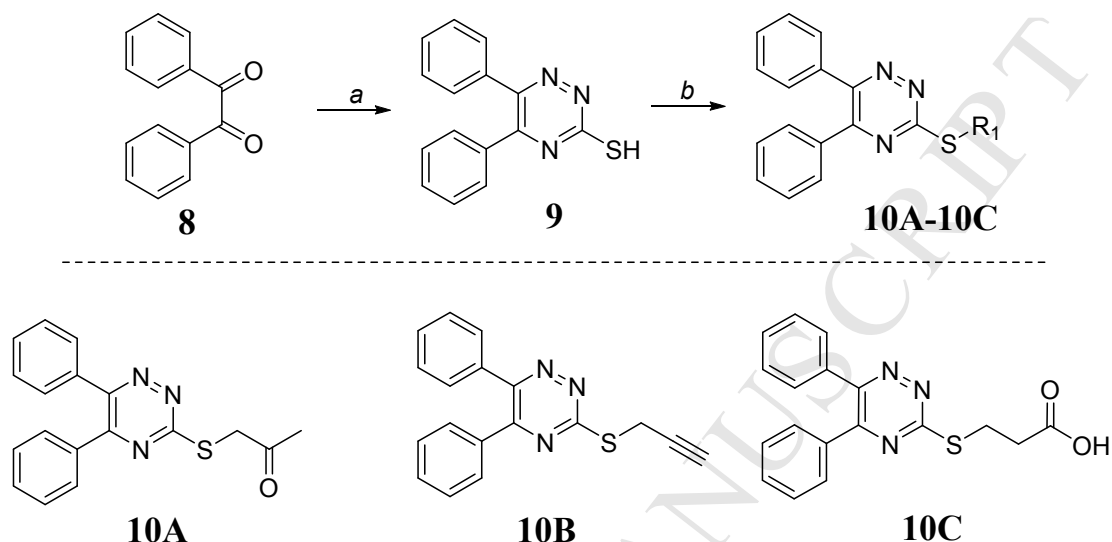

Scheme 1. Reagents and conditions: (a) AcOH, thiosemicarbazide, reflux, 3.5 h; (b) Et$_3$N, bromides (1-bromopropan-2-one or 3-bromoprop-1-yne, or 3-bromopropanoic acid), Acetone, reflux.

In order to obtain the target 5,6-diaryl-1,2,4-triazines hybrids bearing a 1,2,3-triazole linker, various azide derivatives **11a-11l** were used to synthesize the target compounds by click reaction. The structures of azide derivatives **11a-11l** in this work were shown in **Scheme 2**. The synthetic methods and structure confirmation of azide derivatives **11a-11l** were described in the Supporting Information.

11a **11b** **11c** **11d**

11e **11f** **11g**

11h **11i** **11j**

11k **11l**
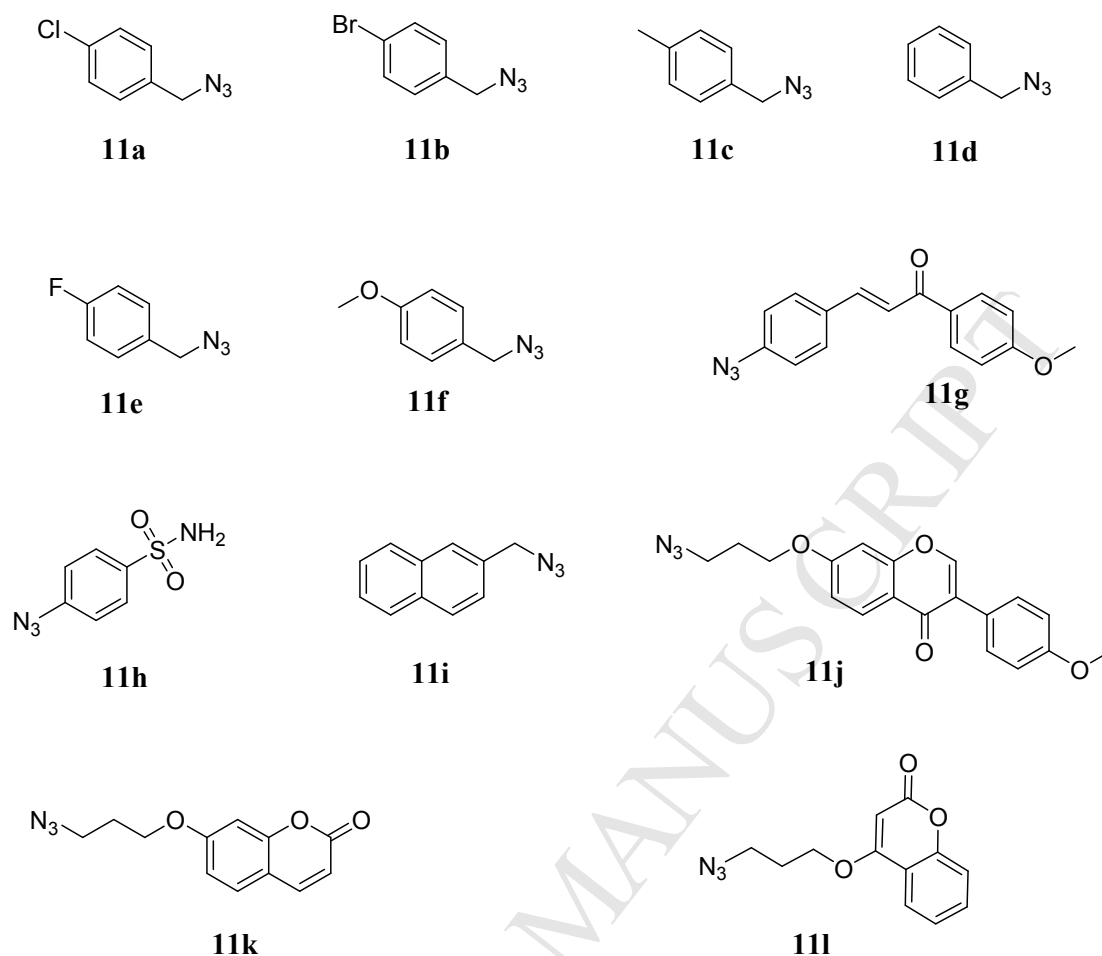
Scheme 2. Azide derivatives **11a-11l** in this work.

As shown in **Scheme 3**, 5,6-diaryl-1,2,4-triazine-triazole derivatives **11A-11L** were achieved by reaction of compound **10B** with various azides [25, 26] using sodium ascorbate and $CuSO_4 \cdot 5H_2O$ in THF at room temperature. The structures of all the new synthesized compounds **10A-10C** and **11A-11L** were characterized by ^{1}H NMR、^{13}C NMR and HRMS.
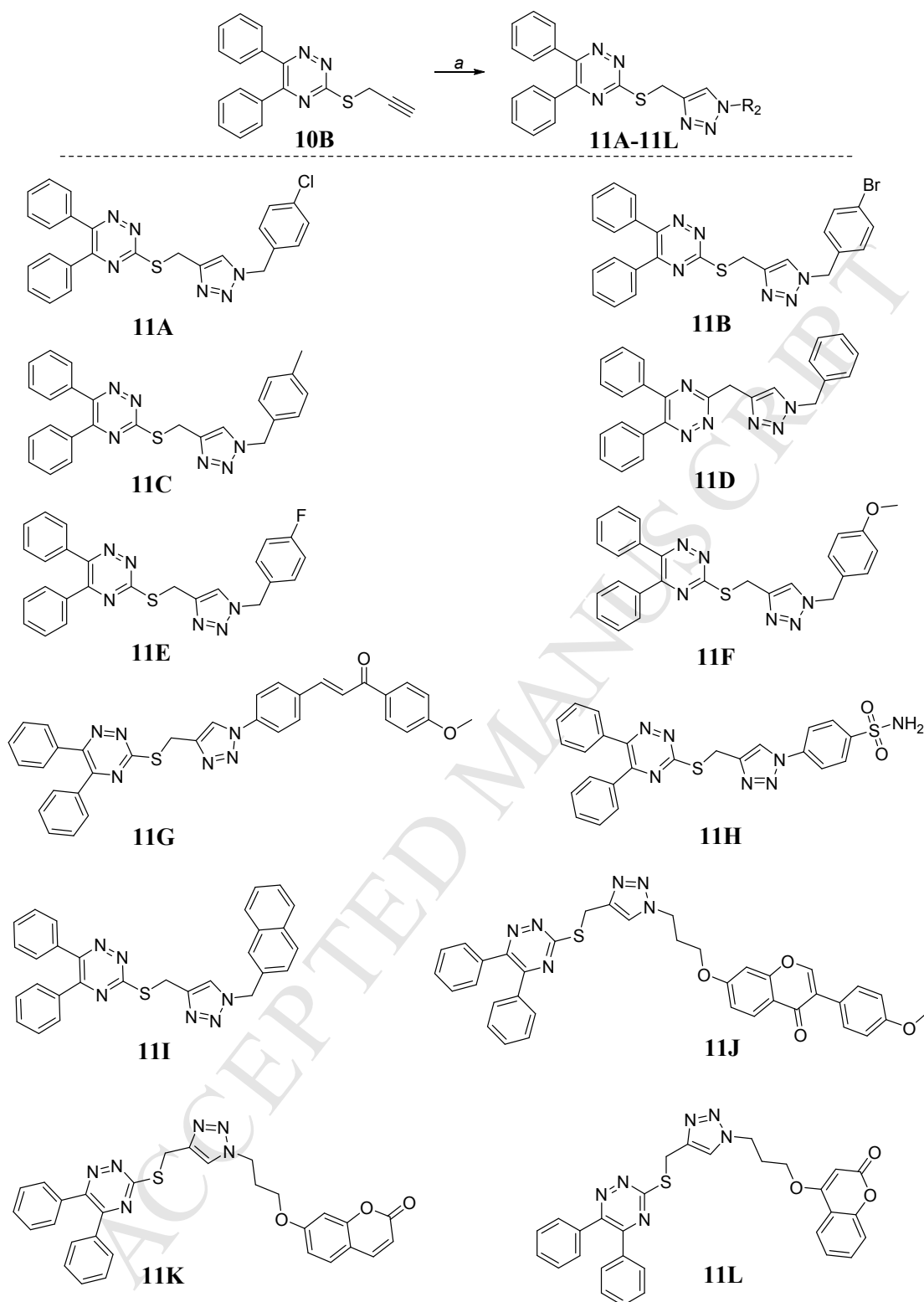
Scheme 3. Reagents and conditions: (a) Sodium ascorbate, $CuSO_4 \cdot 5H_2O$, azides **11a-11l**, THF/H_2O, r.t.

2.2. Antiproliferative activity

In continuation with our efforts toward the identification of novel derivatives with anticancer potential, we evaluated the antiproliferative activity of 5,6-diaryl-1,2,4-triazine derivatives **10A-10C** and 5,6-diaryl-

1,2,4-triazine-triazole hybrids **11A-11L** using the MTT assay. 1,2,3-triazole derivatives have reported as potent antiproliferative agents against PC-3 cells, MGC-803 cells and EC-109 cells [27]. All the target compounds in this work contained a 1,2,3-triazole moiety. Therefore, these three cancer cell lines (MGC-803, EC-109, PC-3) were selected to evaluate their antiproliferative activity. When some 1,2,3-triazole derivatives were reported as potent antiproliferative agents, 5-fluorouracil (5-FU) was selected as the control [27]. Therefore, 5-Fu was also used as the reference drug in this MTT assay.

The antiproliferative activity results against all three cancer cells for the candidate compounds were shown in **Table 1**. In order to investigate the effect of the inhibitory activity of 1,2,3-triazole, non-1,2,3-triazole-5,6-diphenyl-1,2,4-triazines **10A-10C** and 1,2,3-triazole-5,6-diphenyl-1,2,4-triazines **11A-11L** were examined for their antiproliferative activity. Removal of the 1,2,3-triazole was clearly detrimental for the inhibitory activity against the three cancer cell lines, as shown by compounds **10A-10C**. The introduction of 1,2,3-triazole scaffold resulted in an improvement of inhibitory activity. Especially, compound **11E** showed the more potent inhibitory effect against three cell lines than 5-Fu. This result suggested that 1,2,3-triazole moiety may play a synergistic role in determining activity.

In order to complete the structure activity relationship study, a series of 5,6-diphenyl-1,2,4-triazine-1,2,3-triazole hybrids were prepared and evaluated for their antiproliferative activity. With the exception of compounds **11A-11F**, all the compounds bearing a 1,2,3-triazole moiety exhibited potential antiproliferative activity. To determine whether the substituents on the phenyl ring have an effect on the inhibitory activity, compounds with various groups on the phenyl ring were compared with activity. We found that the substitution on the phenyl ring was important for the activity showing an over 4-fold activity loss, when the fluoro group was replaced with methyl group (compounds **11E** *vs*. **11C**). In addition, the size of halogen group on the phenyl ring may affected the antiproliferative activity. Replacement of the bromine atom of compound **11B** and chlorine atom of compound **11A** with a fluorine atom **11E** led to an increase of the activity.

Furthermore, compounds with a chalcone scaffold (**11G**), a sulfanilamide scaffold (**11H**), a naphthyl scaffold (**11I**), a formononetin scaffold (**11J**) and coumarin scaffolds (**11K-11L**) were synthesized to explore the effect of bioactive scaffolds for their antiproliferative activity. Compound bearing a chalcone scaffold **11G** displayed antiproliferative activity against MGC-803 cells with an IC_{50} value of 36.67 μM. However, compounds possessing a sulfanilamide group **11H** or a naphthyl group

11I were unactive against three cancer cell lines. We also found that the type of coumarin ring affected the activity comparing compound **11K** with compound **11L**. In addition, there is an interesting discovery that 5,6-diphenyl-1,2,4-triazine-1,2,3-triazole hybrid bearing a formononetin moiety **11J** exhibited more potent cytotoxicity against PC-3 cancer cells than 5-Fu. However, compound **11J** displayed no significant antiproliferative activity against MGC-803 cells and EC-109 cells indicating that compound **11J** had good selectivity among different cancer cells.

Compound **11E** displayed the potent antiproliferative activity against MGC-803 cells with an IC_{50} value of 7.59 μM. Compound **11E** was further examined for possible cytotoxicity against GES-1 (normal human gastric epithelial cell line). We found that compound **11E** exhibited weak cytotoxicity against GES-1 (31.71 ± 1.24 μM). The results indicated that compound **11E** had good selectivity between the selected cancer cell line (MGC-803) and a normal cell line (GES-1).

Table 1. Antiproliferative activity of target derivatives.

Compound	IC_{50} (μM)[a]		
	MGC-803	EC-109	PC-3
10A	>100	>100	>100
10B	>100	>100	>100
10C	>100	>100	>100
11A	13.23±0.99	24.24±1.16	14.66±0.09
11B	>100	>100	22.00±3.39
11C	34.75±0.51	12.42±0.04	22.63±2.02
11D	11.35±0.97	11.05±1.56	15.60±1.09
11E	7.59±0.28	19.12±1.11	10.63±0.28
11F	22.71±2.86	28.41±0.11	27.51±0.39
11G	36.67±4.69	>100	>100
11H	>100	>100	>100
11I	>100	>100	>100
11J	>100	>100	10.23±0.77
11K	>100	>100	92.34±8.27
11L	>100	>100	57.77±4.36
5-FU	9.79±0.17	56.20±5.48	12.87±1.20

[a] Antiproliferative activity was assayed by exposure for 48 h to substances and expressed as concentration required to inhibit tumor cell proliferation by 50% (IC_{50}). Data are presented as the means±SDs from the dose-response curves of three independent experiments.

2.3. Clone assays

Based on the most excellent activity against MGC-803 cells,

compound **11E** was chosen to perform colony formation to investigate whether **11E** could inhibit MGC-803 cells proliferation (**Fig. 4**). The assay measures the ability of MGC-803 cells to grow and form foci, which represents an indirect estimation of neoplastic transformation [28]. As shown in Fig. 4, MGC-803 cells treated with **11E** exhibited fewer and smaller colonies compared to the control, which indicated that compound **11E** could significantly inhibit the proliferation of MGC-803 cells in a concentration-dependent manner.
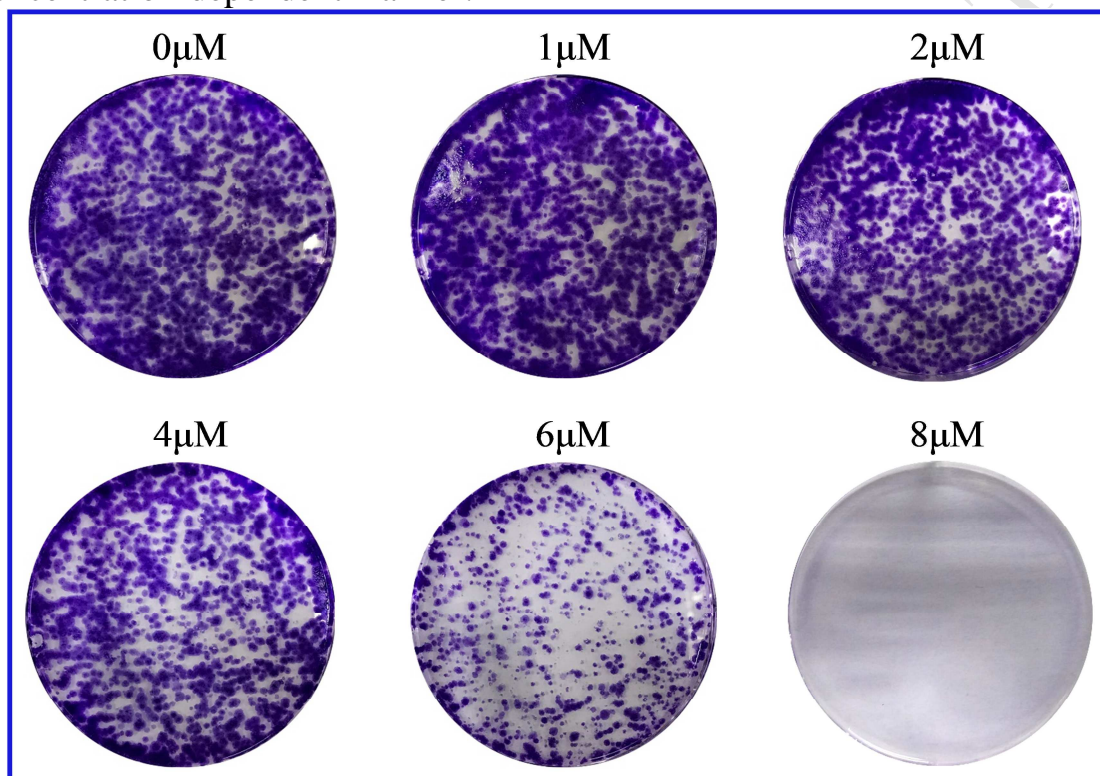

Fig. 4. Representative images of MGC-803 cells colonies after treatment **11E** with various concentrations for a week.

2.4. 11E induces G2/M phase arrest

Targeting the cell cycle of tumor cells has been recognized as a promising strategy for cancer therapy [29]. In this study, compound **11E** was chosen to investigate the effect of our designed derivatives on the cell cycle of MGC-803 cells. After treating MGC-803 cells with compound **11E** at different concentrations (0 μM, 6 μM, 8 μM) for 48 h, cells were then fixed and stained with PI for flow cytometry analysis. As shown in **Fig. 5**, compound **11E** concentration dependently arrested cell cycle at G2/M phase.
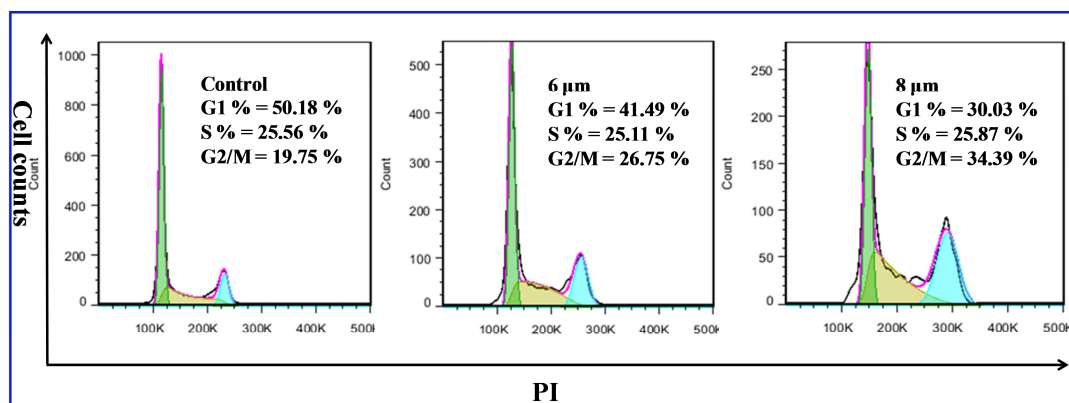
Fig. 5. Effects of **11E** on MGC-803 cell cycle progress for 48 h.

*2.5. Morphological changes of MGC-803 cells induced by compound **11E***

Morphological changes of cancer cells are always associated with the growth inhibition induced by cytotoxic agents [30]. Inspired by the good inhibition of compound **11E** against MGC-803 cells, we then investigated whether compound **11E** was able to induce morphological changes. After being incubated with **11E** for 48 h at different concentrations (0 μM, 6 μM, 8μM, 10 μM), the morphological changes of MGC-803 cells were recorded using an inverted microscope. As shown in **Fig. 6A**, significant changes of cell morphology such as rounding up and cell debris were observed, especially at high concentrations. After staining with Hoechst 33258, remarkable nuclear changes including the chromatin condensation, nuclear fragmentation and condensation were also observed (**Fig. 6B**).
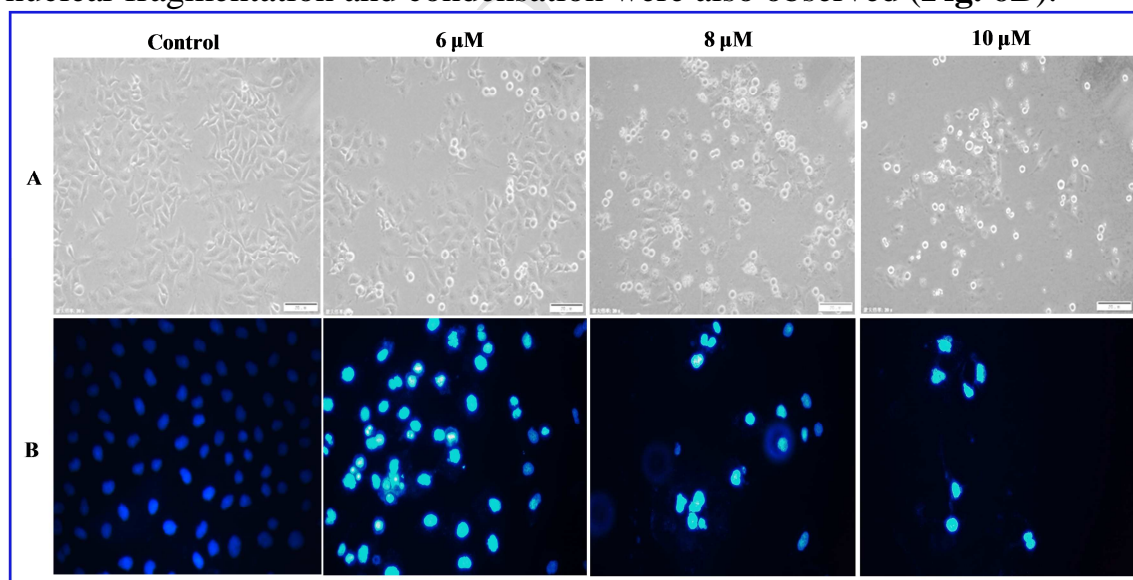

Fig. 6. (A)The morphological changes of cells were observed under an inverted microscope (magnification,×400).(B) MGC-803 cells were incubated with indicated concentrations of 1035 for 48 hours and then stained with DAPI. The stained nuclei were then observed under a fluorescentmicroscope (magnification,×400).

2.6. Apoptosis detection by flow cytometry

The effect of compound **11E** on the apoptosis was investigated using the propidium iodide (PI) and Annexin V-FITC biparametric cytofluorimetric analysis. After treatment with different concentrations of compound **11E** (0 μM, 6 μM, 8μM, 10 μM) for 48 h, MGC-803 cells were stained with PI and FITC, and then analyzed by the flow cytometry [31]. As illustrated in **Fig. 7**, compound **11E** induced apoptosis of MGC-803 cells in a concentration-dependent manner. Specifically, the percentage of apoptotic cells was about 3.3% for the control group. When treated with high concentration (10 μM) of compound **11E**, around 54.8% of apoptosis rate was observed. While the late apoptosis rate of MGC-803 cells was not changed significantly with increasing concentrations.
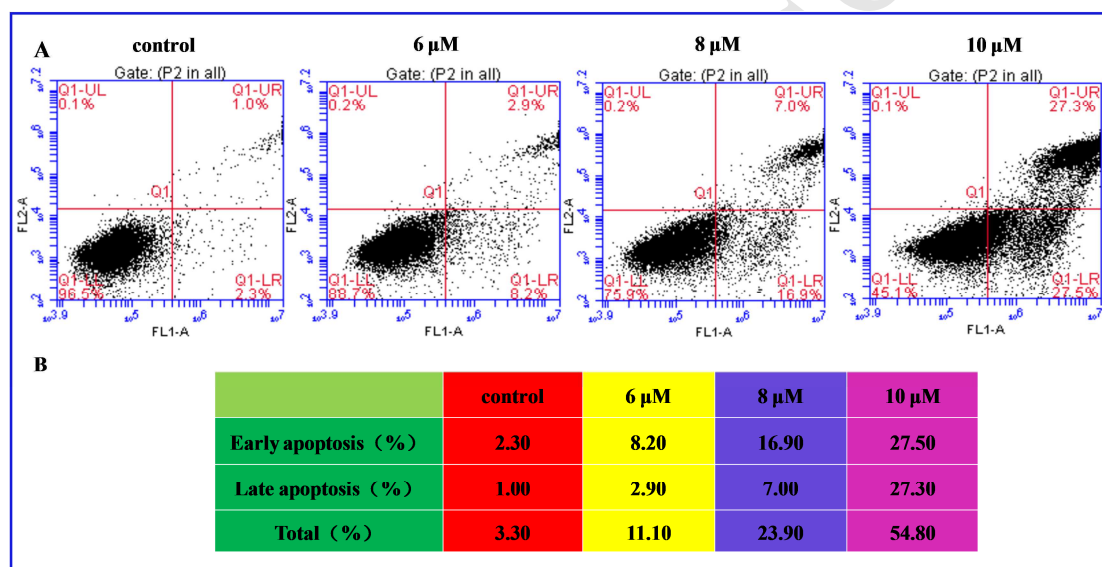

	control	6 μM	8 μM	10 μM
Early apoptosis（%）	2.30	8.20	16.90	27.50
Late apoptosis（%）	1.00	2.90	7.00	27.30
Total（%）	3.30	11.10	23.90	54.80

Fig. 7. Compound **11E** induced apoptosis of MGC-803 cells in a concentration-dependent manner.

2.7. *11E decreased the membrane potential (ΔΨ) of the mitochondria.*

The decrease of mitochondrial membrane potential through multiple mechanisms including deficiency of oxidizable substrates for the blockage of respiration, the mitochondria, or uncoupling of the inner membrane, followed by cytochrome c release from the mitochondria and expression changes of apoptosis-related proteins (e.g. Bax, Bcl-2, caspase3/9, parp, etc) have been observed during apoptosis process. The remarkable apoptosis induced by compound **11E** promoted us to explore whether this compound had an effect on the mitochondrial membrane potential. MGC-803 cells were incubated at the indicated concentrations (0 μM, 6 μM, 8μM, 10 μM) for 48 h, then stained and analyzed by flow cytometry. As shown in **Fig. 8**, compound **11E** caused the mitochondrial

membrane potential decrease in a concentration-dependent manner. The percentage of cells at 10 µM was 42.9%, significantly higher than that of the control group (4.1%).
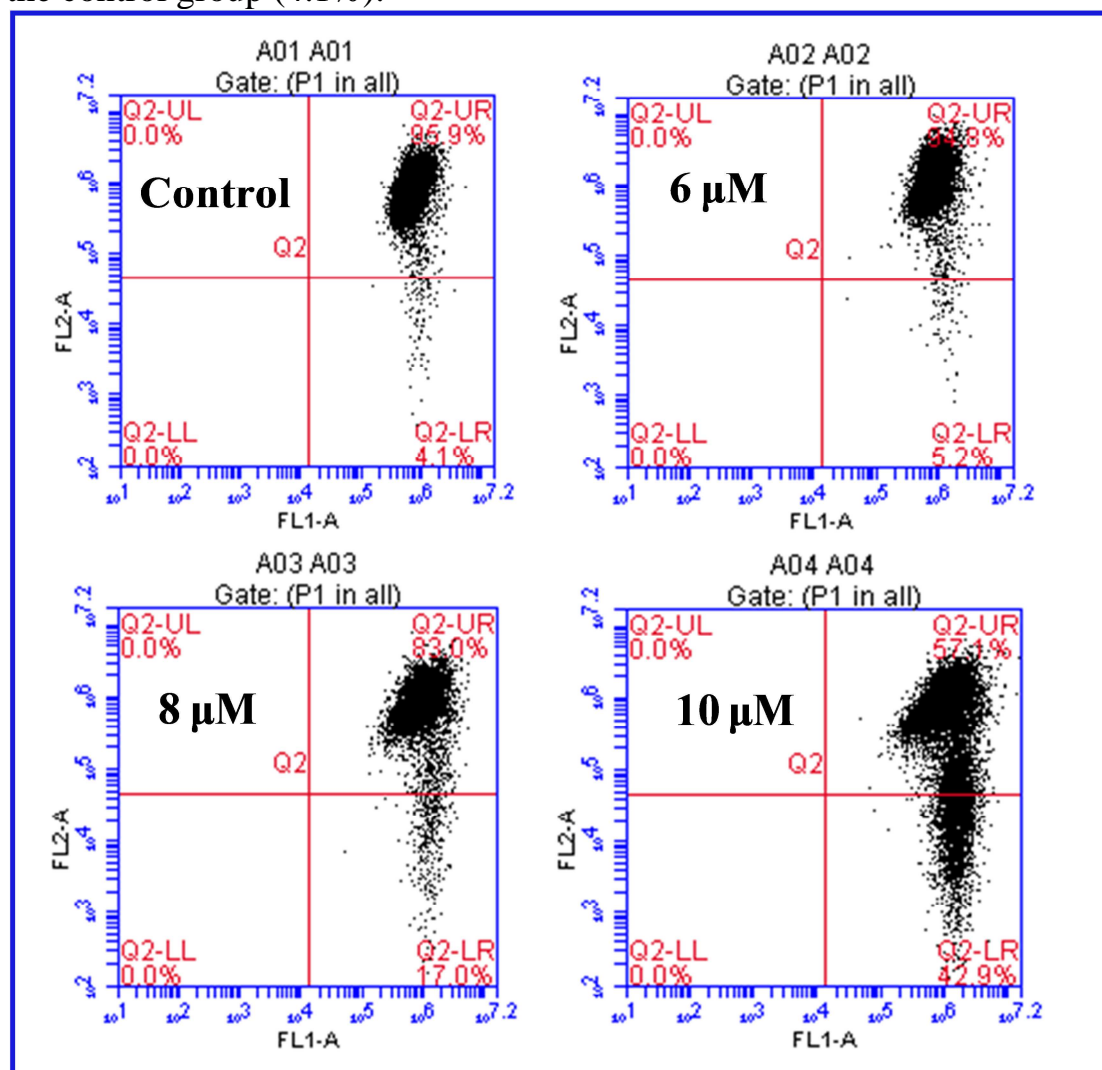

Fig. 8. MGC-803 cells were treated with vehicle control and various concentrations of **11E** (0µM, 6µM, 8µM, and 10µM) for 48 hours. The mitochondria membrane potential was measured by JC-1 dye retention using flow cytometry.

2.8. *11E affected apoptosis-related protein expression.*

Proteins in Bcl-2 family are key regulators of apoptosis and overexpression of the prominent pro-survival Bcl-2 family members like Bcl-2 and Bcl-xL is a common feature responsible for deregulation of apoptosis in human adenocarcinomas, which make tumor cells resistant to conventional cancer therapeutic agents and thereby resulting in hindrance to cancer cell death [32, 33]. Caspases are cysteine-dependent, aspartate-directed proteases that have been shown to play an important part in the apoptotic cascade of events and caspase 3 and caspase 9 are one of the most important effectors [34]. Poly (ADP ribose) polymerase (PARP),

the first caspase substrate identified, is a particularly interesting death substrate, and its cleavage is now recognized as a hallmark of apoptosis [35].

To further investigate the mechanism of apoptosis, western blot analysis was performed in MGC-803 cells by treatment with compound **11E** (0µM, 6µM, 8µM, and 10µM) for 48 h. As shown in **Fig. 9**, treatment of MGC-803 cells with compound **11E** resulted in increased expression of Bax, Cleaved-Caspase 9, Cleaved-Caspase 3, Cleaved PARP and DR4 in a concentration-dependent manner. Meanwhile, the expression of apoptotic-related proteins (Bcl-xl and Bcl-2) decreased accordingly. Based on these results, 1,2,3-triazole-5,6-diphenyl-1,2,4-triazine **11E** was a potent apoptosis inducer.
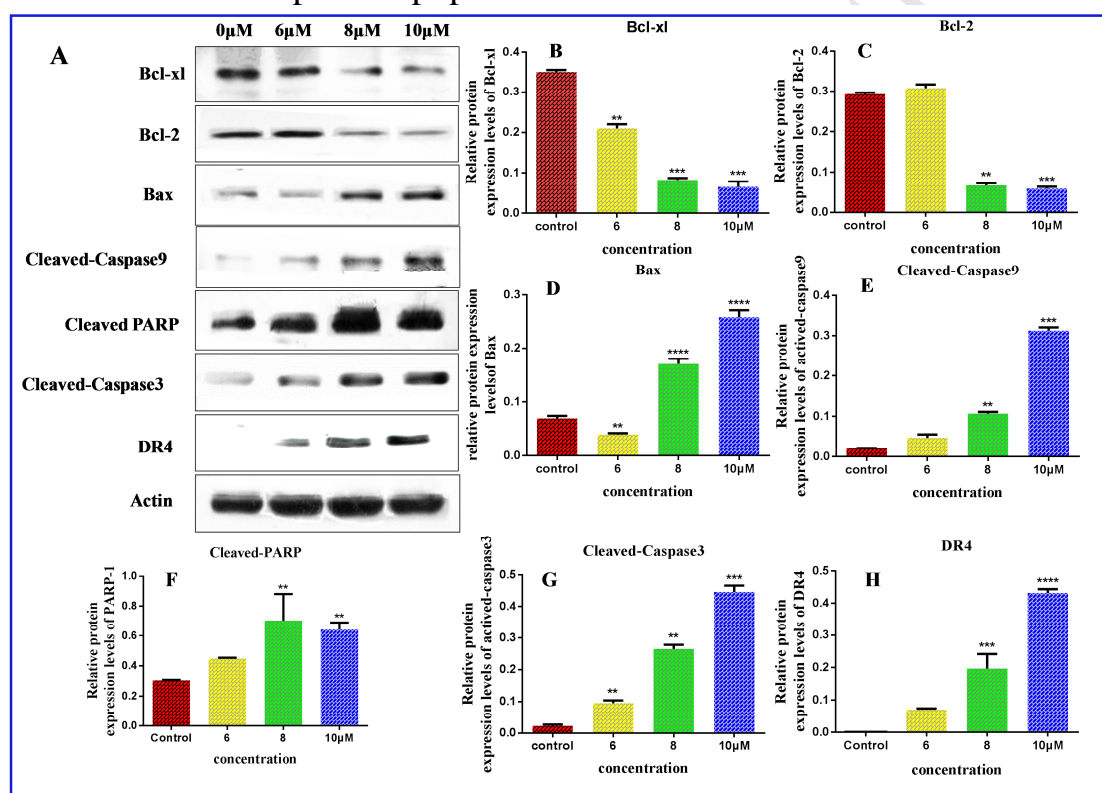

Fig. 9 Expression changes of apoptosis related proteins. (A) compound **11E** induced expression changes of apoptosis related proteins in MGC-803 cells; (B-H) Statistical analysis of protein expression levels. The results shown were representative of three independent experiments. **: p < 0.01 verse control , ***: p < 0.001 verse control, ****: p < 0.0001 verse control.

3. Conclusions

Following our previous work, we designed a series of novel 1,2,3-triazole-5,6-diphenyl-1,2,3-triazole derivatives based on molecular hybridization strategy. Majority of hybrids possessed moderate to good growth inhibition against the tested cancer cells. Particularly, compound

11E exhibited more potent antiproliferative activity than well-known antitumor drug 5-Fu against all three cancer cell lines. The preliminary SAR illustrated that 1,2,3-triazole as a reported antitumor scaffold could play potential synergistic effect for 1,2,3-triazole-5,6-diphenyl skeleton. These hybrids in this work might serve as bioactive fragments and lead compounds for developing more potent cytotoxic agents.

The mechanism investigation showed that compound **11E** can inhibit the colony formation, halted cell cycle progression at the G2/M phase, induce morphological changes and lead to apoptosis. Further mechanisms of apoptosis indicated that 1,2,3-triazole-5,6-diphenyl-1,2,3-triazole **11E** induced MGC-803 cells apoptosis probably through mitochondrial pathway by the decrease of mitochondrial membrane potential and regulation the expression of apoptosis-related proteins. In summary, novel 5,6-diphenyl-1,2,3-triazole derivative bearing a 1,2,3-triazole moiety **11E** was a potential apoptosis inducer, which can be used as a lead compound for the development of potent cancer chemotherapeutic agents in the drug discovery process.

## 4. Experimental section

4.1. General

Reagents and solvents were used without special treatment. Melting points were determined on an X-5 micromelting apparatus and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker 400 MHz and 100 MHz spectrometer, respectively. High resolution mass spectra (HRMS) of all derivatives were recorded on a Waters Micromass Q-T of Micromass spectrometer by electrospray ionization (ESI).

### 4.2. General procedure for the synthesis of compounds **10A-10C**

5,6-Diphenyl-1,2,4-triazine-3-thiol **9** was prepared by a reported method [1]. To a solution of **9** (0.5 mmol, 1.0 eq) and bromide (0.5 mmol, 1.0 eq) in acetone (10 mL) was added Et$_3$N (0.75 mmol, 1.5 eq), the mixture was refluxed for about 3~4 h. Upon completion, EtOAc and H$_2$O were added. The aqueous layer was extracted with EtOAc for several times; the combined organic layers were washed with H$_2$O for several times to remove the acetone, and then washed with brine, dried over MgSO$_4$ and evaporated to give the products. The residue was purified with column chromatography (hexane: EtOAc = 7:1) to obtain analogue **10A-10C**.

### 4.2.1. 1-((5,6-diphenyl-1,2,4-triazin-3-yl)thio)propan-2-one (**10A**)
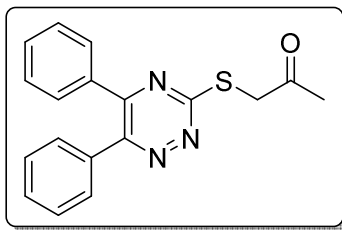
Light yellow solid; Mp:102~104 ^{0}C; Yield: 35%. ^{1}H NMR (400 MHz, Acetone-d^6) δ 7.75 – 7.27 (m, 10H), 4.32 (s, 2H), 2.35 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d^6) δ 201.49, 170.52, 156.47, 155.24, 136.66, 136.40, 131.60, 130.75, 130.33, 130.10, 129.28, 129.23, 41.54, 29.11. HR-MS (ESI): Calcd. $C_{18}H_{16}N_3OS$, $[M+H]^+$ m/z: 322.1018, found: 322.1014.

4.2.2. 5,6-diphenyl-3-(prop-2-yn-1-ylthio)-1,2,4-triazine (10B)
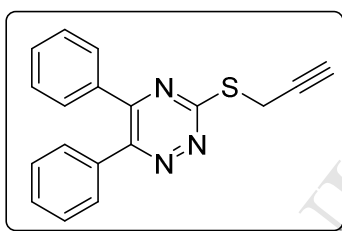
Yellow solid; Mp:96~99 ^{0}C; Yield: 63%. ^{1}H NMR (400 MHz, Acetone-d^6) δ 7.65 – 7.36 (m, 10H), 4.19 (d, J = 2.6 Hz, 2H), 2.77 (t, J = 2.6 Hz, 1H). ^{13}C NMR (100 MHz, Acetone-d^6) δ 169.89, 156.57, 155.45, 136.63, 136.35, 131.68, 130.83, 130.35, 130.16, 129.32, 129.25, 80.13, 72.65, 19.47. HR-MS (ESI): Calcd. $C_{18}H_{14}N_3S$, $[M+H]^+$ m/z: 304.0909, found: 304.0908.

### 4.2.3. 4-((5,6-diphenyl-1,2,4-triazin-3-yl)thio)butanoic acid (10C)
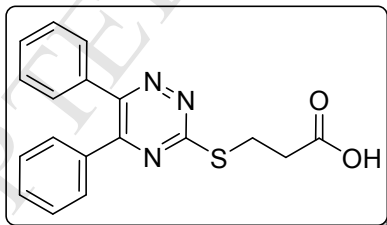
Light yellow solid; Mp:172~174 ^{0}C; Yield: 34%. ^{1}H NMR (400 MHz, Acetone-d^6) δ 7.56 – 7.13 (m, 10H), 3.44 (dd, J = 9.4, 4.5 Hz, 2H), 2.80 (t, J = 6.9 Hz, 2H). ^{13}C NMR (100 MHz, Acetone-d^6) δ 173.04, 171.06, 156.54, 155.20, 136.80, 136.55, 131.58, 130.76, 130.35, 130.09, 129.31, 129.26, 34.39, 26.42. HR-MS (ESI): Calcd. $C_{18}H_{16}N_3O_2S$, $[M+H]^+$ m/z: 338.0965, found: 338.0963.

### 4.3. General procedure for the synthesis of compounds 11A-11L.

Compound **10B** (1 mmol), azide derivatives (1 mmol), $CuSO_4.5H_2O$ (0.2 mmol) and sodium ascorbate (0.1 mmol) were dissolved in THF/H_2O (5 ml/5 ml) to stir for 8 h at room temperature. Upon completion, the precipitated product was filtered off and washed with ethanol to afford the crude product, which was purified with column chromatography (hexane: EtOAc = 9:1) to obtain analogue **11A-11L**.

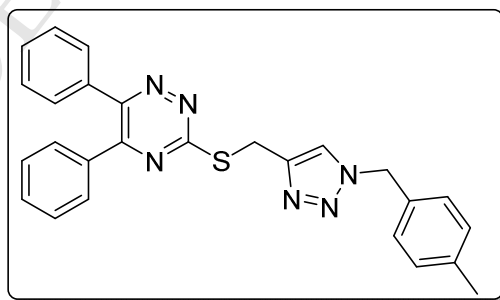### 4.3.1. 3-(((1-(4-chlorobenzyl)-1H-1,2,3-triazol-4-yl)methyl)thio)-5,6-diphenyl-1,2,4-triazine (**11A**)
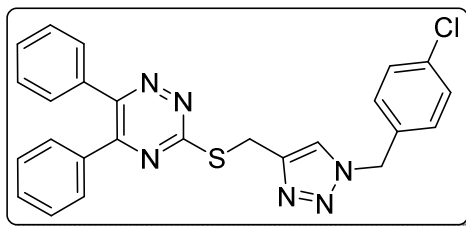
Light yellow solid; Mp:120~122 ^{0}C; Yield: 52%. ^{1}H NMR (400 MHz, Acetone-d^6) δ 7.97 (s, 1H), 7.60 – 7.28 (m, 14H), 5.58 (s, 2H), 4.66 (s, 2H). ^{13}C NMR (100 MHz, Acetone-d^6) δ 170.68, 156.46, 155.21, 144.89, 136.72, 136.40, 135.91, 134.53, 131.64, 130.77, 130.65, 130.34, 130.15, 129.73, 129.34, 129.27, 124.06, 53.41, 26.05. HR-MS (ESI): Calcd. $C_{25}H_{20}ClN_6S$, [M+H]$^+$m/z: 471.1160, found: 471.1159.

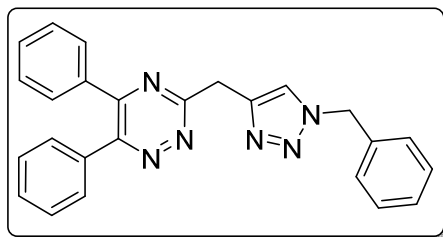### 4.3.2. 3-(((1-(4-bromobenzyl)-1H-1,2,3-triazol-4-yl)methyl)thio)-5,6-diphenyl-1,2,4-triazine (**11B**)
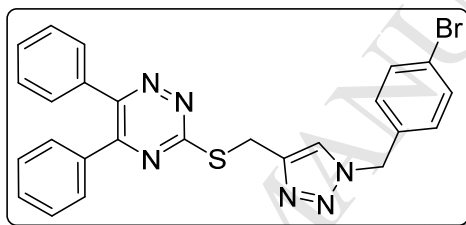
Light yellow solid; Mp:132~134 ^{0}C; Yield: 41%. ^{1}H NMR (400 MHz, Acetone-d^6) δ 7.97 (s, 1H), 7.60 – 7.32 (m, 12H), 7.24 (d, J = 8.4 Hz, 2H), 5.57 (s, 2H), 4.66 (s, 2H). ^{13}C NMR (100 MHz, Acetone-d^6) δ 170.68, 156.47, 155.21, 144.91, 136.71, 136.40, 136.38, 132.73, 131.64, 130.94, 130.77, 130.34, 130.15, 129.35, 129.27, 124.08, 122.65, 53.46, 26.04. HR-MS (ESI): Calcd. $C_{25}H_{20}BrN_6S$, [M+H]$^+$m/z: 515.0657, found: 515.0654.

4.3.3. 3-(((1-(4-methylbenzyl)-1H-1,2,3-triazol-4-yl)methyl)thio)-5,6-diphenyl-1,2,4-triazine (**11C**)
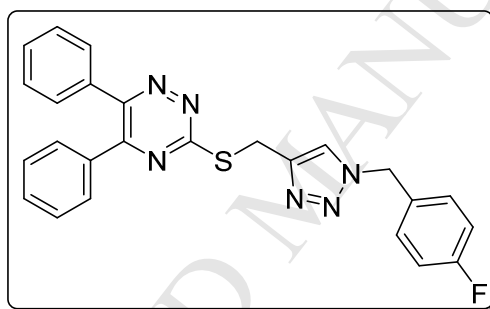

Light yellow solid; Mp:119~121 ^{0}C; Yield: 64%. ^{1}H NMR (400 MHz, Acetone-d^6) δ 7.90 (s, 1H), 7.60 – 7.32 (m, 10H), 7.15 (dd, J = 22.4, 8.0 Hz, 4H), 5.50 (s, 2H), 4.65 (s, 2H), 2.27 (s, 3H). ^{13}C NMR (100 MHz, Acetone-d^6) δ 170.73, 156.46, 155.18, 144.69, 138.82, 136.73, 136.42, 133.95, 131.62, 130.77, 130.34, 130.27, 130.14, 129.33, 129.27, 128.92, 123.81, 54.03, 26.09, 21.11. HR-MS (ESI): Calcd. $C_{26}H_{23}N_6S$, [M+H]$^+$m/z: 451.1708, found: 451.1705.

4.3.4. 3-((1-benzyl-1H-1,2,3-triazol-4-yl)methyl)-5,6-diphenyl-1,2,4-triazine (11D)
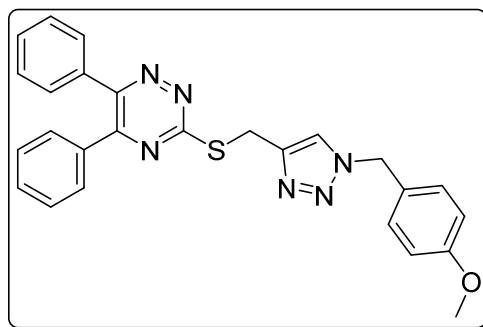

Light yellow solid; Mp:130~132 ^{0}C; Yield: 72%. ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.50 (s, 1H), 7.47 – 7.20 (m, 13H), 7.12 (dd, J = 6.6, 2.9 Hz, 2H), 5.38 (s, 2H), 4.57 (s, 2H). ^{13}C NMR (100 MHz, CDCl$_3$) δ 168.68, 154.50, 152.85, 143.41, 133.98, 133.76, 133.38, 129.79, 128.61, 128.31, 128.10, 127.85, 127.47, 127.44, 127.31, 126.79, 121.52, 52.94, 24.38. HR-MS (ESI): Calcd. C$_{25}$H$_{21}$N$_6$S, [M+H]$^+$m/z: 437.1549, found: 437.1548.

### 4.3.5. 3-(((1-(4-fluorobenzyl)-1H-1,2,3-triazol-4-yl)methyl)thio)-5,6-diphenyl-1,2,4-triazine (11E)

Light yellow solid; Mp:126~128 ^{0}C; Yield: 64%. ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.50 (s, 1H), 7.41 (dd, J = 12.7, 5.1 Hz, 4H), 7.37 – 7.32 (m, 2H), 7.31 – 7.27 (m, 2H), 7.24 (d, J = 7.8 Hz, 2H), 7.11 (dd, J = 8.6, 5.2 Hz, 2H), 6.90 (dd, J = 11.9, 5.3 Hz, 2H), 5.33 (s, 2H), 4.56 (s, 2H). ^{13}C NMR (100 MHz, CDCl$_3$) δ 168.80, 162.97, 160.50, 154.67, 153.05, 143.71, 134.13, 130.01, 129.44, 128.91, 128.77, 128.52, 128.25, 127.65, 127.50, 121.64, 115.11, 52.33, 24.52. HR-MS (ESI): Calcd. C$_{25}$H$_{20}$FN$_6$S, [M+H]$^+$m/z: 455.1456, found: 455.1454.

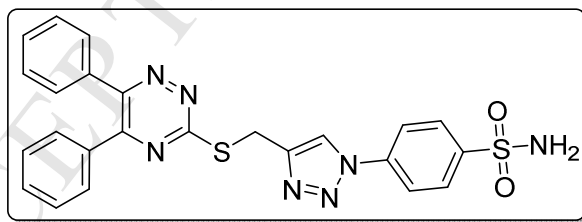### 4.3.6. 3-(((1-(4-methoxybenzyl)-1H-1,2,3-triazol-4-yl)methyl)thio)-5,6-diphenyl-1,2,4-triazine (11F)

Light yellow solid; Mp:108~111 ^{0}C; Yield: 65%. ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.46 (s, 1H), 7.45 – 7.20 (m, 10H), 7.08 (d, J = 8.6 Hz, 2H), 6.75 (d, J = 8.7 Hz, 2H), 5.30 (s, 2H), 4.56 (s, 2H), 3.69 (s, 3H). ^{13}C NMR (100 MHz, CDCl$_3$) δ 168.90, 158.83, 154.65, 153.02, 143.46, 134.18, 133.96, 129.96, 128.79, 128.56, 128.48, 128.28, 127.62, 127.49, 125.55, 121.45, 113.41, 54.28, 52.67, 24.57. HR-MS (ESI): Calcd. C$_{26}$H$_{23}$N$_6$OS, [M+H]$^+$m/z: 467.1657, found: 467.1654.

4.3.7. (E)-3-(4-(4-(((5,6-diphenyl-1,2,4-triazin-3-yl)thio)methyl)-1H-1,2,3-triazol-1-yl)phenyl)-1-(4-methoxyphenyl)prop-2-en-1-one (11G)
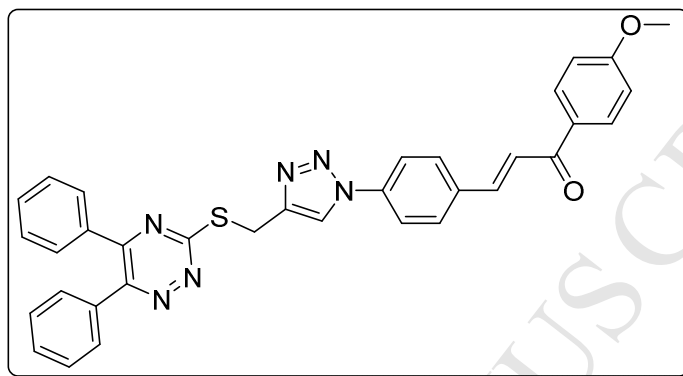
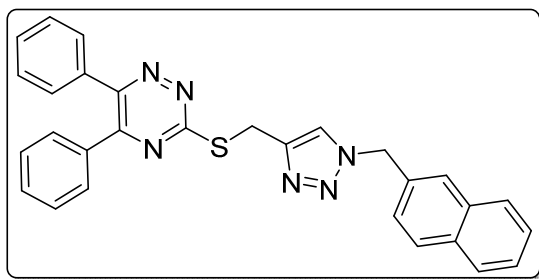Light yellow solid; Mp:162~164 ^{0}C; Yield: 61%. ^{1}H NMR (400 MHz, CDCl$_3$) δ 8.09 (s, 1H), 7.96 (d, J = 8.8 Hz, 2H), 7.68 (d, J = 15.6 Hz, 1H), 7.56 – 7.23 (m, 15H), 6.90 (d, J = 8.8 Hz, 2H), 4.67 (s, 2H), 3.80 (s, 3H). ^{13}C NMR (100 MHz, CDCl$_3$) δ 187.09, 162.66, 140.89, 136.50, 135.90, 134.06, 133.96, 130.32, 130.09, 129.92, 129.68, 129.24, 128.80, 128.55, 128.28, 127.67, 127.64, 127.58, 127.52, 122.62, 120.64, 120.11, 118.38, 112.94, 54.52, 24.45. HR-MS (ESI): Calcd. C$_{34}$H$_{27}$N$_6$O$_2$S, [M+H]$^+$m/z: 583.1919, found: 583.1916.

4.3.8. 4-(4-(((5,6-diphenyl-1,2,4-triazin-3-yl)thio)methyl)-1H-1,2,3-triazol-1-yl)benzenesulfonamide (11H)

Light yellow solid; Mp:236~238 ^{0}C; Yield: 56%. ^{1}H NMR (400 MHz, DMSO-d^6) δ 8.85 (s, 1H), 8.06 (d, J = 8.8 Hz, 2H), 7.99 (d, J = 8.8 Hz, 2H), 7.58 – 7.33 (m, 12H), 4.77 (s, 2H). ^{13}C NMR (101 MHz, DMSO-d^6) δ 168.94, 155.63, 154.18, 144.64, 143.71, 138.39, 135.08, 134.87, 130.74, 129.60, 129.25, 129.19, 128.36, 128.33, 127.37, 122.02, 120.12, 24.63. HR-MS (ESI): Calcd. C$_{24}$H$_{20}$N$_7$O$_2$S$_2$, [M+H]$^+$m/z: 502.1127, found: 502.1120.

4.3.9. 3-(((1-(naphthalen-2-ylmethyl)-1H-1,2,3-triazol-4-yl)methyl)thio)-5,6-diphenyl-1,2,4-triazine (11I)

Light yellow solid; Mp:136~138 ^{0}C; Yield: 69%. ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.80 – 7.63 (m, 3H), 7.60 (s, 1H), 7.53 (s, 1H), 7.52 – 7.23 (m, 10H), 7.20 (dt, J = 11.5, 3.8 Hz, 3H), 5.54 (s, 2H), 4.57 (s, 2H). ^{13}C NMR (100 MHz, CDCl$_3$) δ 168.85, 154.68, 153.02, 143.70, 134.15, 133.91, 132.15, 132.12, 130.90, 129.94, 128.75, 128.47, 128.27, 128.07, 127.61, 127.45, 126.92, 126.73, 126.29, 125.66, 125.64, 124.24, 121.80, 53.32, 24.57. HR-MS (ESI): Calcd. C$_{29}$H$_{23}$N$_6$S, [M+H]$^+$m/z: 487.1709, found: 487.1705.

4.3.10. 7-(3-(4-(((5,6-diphenyl-1,2,4-triazin-3-yl)thio)methyl)-1H-1,2,3-triazol-1-yl)propoxy)-3-(4-methoxyphenyl)-4H-chromen-4-one (11J)
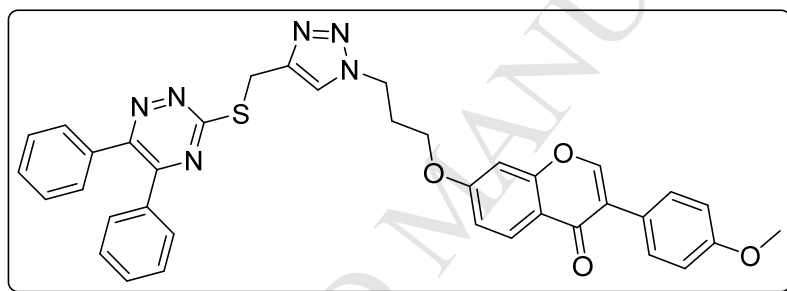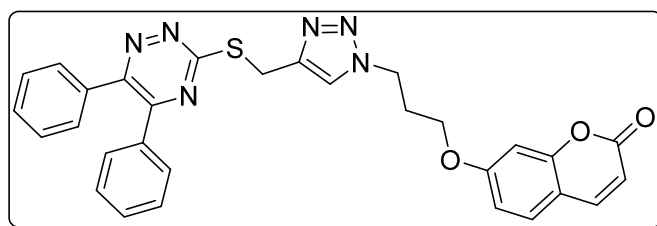

Light yellow solid; Mp:162~164 ^{0}C; Yield: 70%. ^{1}H NMR (400 MHz, CDCl$_3$) δ 8.10 (d, J = 8.9 Hz, 1H), 7.81 (s, 1H), 7.63 (s, 1H), 7.42 (dd, J = 14.5, 7.9 Hz, 6H), 7.29 (ddt, J = 21.4, 15.3, 7.7 Hz, 6H), 6.94 – 6.81 (m, 3H), 6.70 (d, J = 2.2 Hz, 1H), 4.59 (s, 2H), 4.46 (t, J = 6.8 Hz, 2H), 3.96 (t, J = 5.7 Hz, 2H), 3.76 (s, 3H), 2.41 – 2.24 (m, 2H). ^{13}C NMR (100 MHz, CDCl$_3$) δ 174.73, 168.86, 161.60, 158.56, 156.76, 154.78, 153.11, 151.09, 143.32, 134.10, 133.88, 130.03, 129.09, 128.79, 128.54, 128.24, 127.66, 127.51, 126.88, 123.85, 123.13, 122.24, 117.66, 113.57, 112.93, 99.73, 63.80, 54.32, 45.93, 28.60, 24.52. HR-MS (ESI): Calcd. C$_{37}$H$_{31}$N$_6$O$_4$S, [M+H]$^+$m/z: 655.2128, found: 655.2127.

4.3.11. 7-(3-(4-(((5,6-diphenyl-1,2,4-triazin-3-yl)thio)methyl)-1H-1,2,3-triazol-1-yl)propoxy)-2H-chromen-2-one (11K)

Light yellow solid; Mp:127~129 ^{0}C; Yield: 68%. ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.63 (s, 1H), 7.53 (d, J = 9.5 Hz, 1H), 7.48 – 7.23 (m, 11H), 6.75 – 6.63 (m, 2H), 6.17 (d, J = 9.5 Hz, 1H), 4.60 (s, 2H), 4.46 (t, J = 6.8 Hz, 2H), 3.94 (t, J = 5.7 Hz, 2H),

2.33 (p, J = 6.4 Hz, 2H). ^{13}C NMR (100 MHz, CDCl$_3$) δ 168.88, 160.48, 160.01, 154.76, 153.11, 143.39, 142.25, 134.13, 133.91, 130.05, 128.81, 128.55, 128.25, 127.90, 127.67, 127.52, 123.97, 122.17, 112.41, 111.88, 111.47, 100.63, 63.80, 46.00, 28.64, 24.52. HR-MS (ESI): Calcd. C$_{30}$H$_{25}$N$_6$O$_3$S, [M+H]$^+$m/z: 549.1712, found: 549.1709.
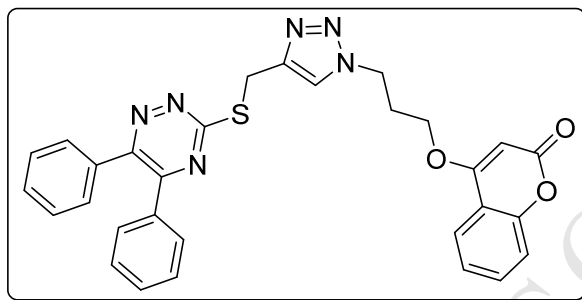
4.3.12. 4-(3-(4-(((5,6-diphenyl-1,2,4-triazin-3-yl)thio)methyl)-1H-1,2,3-triazol-1-yl)propoxy)-2H-chromen-2-one (11L)

Light yellow solid; Mp:131~132 ^{0}C; Yield: 70%. ^{1}H NMR (400 MHz, DMSO) δ 8.17 (s, 1H), 7.67 (ddd, J = 15.6, 8.3, 4.1 Hz, 2H), 7.54 – 7.28 (m, 12H), 5.85 (s, 1H), 4.63 (s, 2H), 4.58 (t, J = 6.7 Hz, 2H), 4.22 (t, J = 5.7 Hz, 2H), 2.43 – 2.26 (m, 2H). ^{13}C NMR (100 MHz, DMSO) δ 169.24, 164.70, 161.53, 155.54, 154.09, 152.68, 142.89, 135.14, 134.89, 132.71, 130.80, 129.64, 129.31, 129.22, 128.43, 128.36, 124.05, 123.77, 122.98, 116.33, 115.05, 90.54, 66.78, 46.69, 28.67, 24.89. HR-MS (ESI): Calcd. C$_{30}$H$_{25}$N$_6$O$_3$S, [M+H]$^+$m/z: 549.1715, found: 549.1709.

4.4. Biological testing.

The MTT assay, Clonogenicity assay, analysis of cell cycle distribution, Hoechst 33258 staining, detection of apoptosis and mitochondrial membrane potential were carried out following our previously reported method [36]. The Western blot analysis was performed according to the methods previously published [37]. Statistical analysis was performed according to the our reported methods [22]. Therefore, no details are given here.

Acknowledgment

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Highlights

• SAR for this 5,6-diaryl-1,2,4-triazine scaffold is explored.

• **11E** inhibited colony formation and arrested cell cycle at G2/M phase.

• **11E** caused morphological changes and decreased mitochondrial membrane potential.

• 5,6-Diaryl-1,2,4-triazine hybrids were discovered as novel apoptosis inducers.