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Synthesis and biological evaluation of novel pyrazolo[3,4-b]pyridines as cis-restricted combretastatin A-4 analogues

Xie-Er Jian[#], Fang Yang[#], Cui-Shan, Jiang, Wen-Wei, You, Pei-Liang, Zhao*

Guangdong Provincial Key Laboratory of New Drug Screening, School of Pharmaceutical Science, Southern Medical University, Guangzhou 510515, P.R.China

ABSTRACT: Twenty-six novel pyrazolo[3,4-b]pyridine-bridged analogues of combretastatin A-4 possessing 3,4,5-trimethoxylphenyl groups, were synthesized and evaluated for their antiproliferative and tubulin polymerization inhibitory activities. Preliminary biological evaluation demonstrated that some of the target compounds displayed significant antiproliferative effect against four different cell lines including MCF-7, MDA-MB-231, HeLa and Kyse150. The most active analogue **6n** was found to induce HeLa cells arrest in the G2/M phase in a dose-dependent manner. Molecular modeling studies indicated that derivative **6n** most likely occupies the colchicine site of tubulin. The initial results suggest that the 3,4,5-trimethoxyphenyl substituted pyrazolo[3,4-b]pyridine could serve as a promising scaffold for development of potent tubulin inhibitors as anticancer agents.

Keywords: Pyrazolo[3,4-b]pyridine; Synthesis; Tubulin polymerization; Antiproliferative activity.

*Corresponding author.E-mail: plzhao@smu.edu.cn (P.-L.Zhao)

# These authors contributed equally to this work.

Microtubules, are critical components of the eukaryotic cell cytoskeleton that maintain cell shape and play a significant role in cellular events such as supporting the process of mitosis and cell morphology and movement. [1-3] The aforementioned functions have made microtubules become an attractive target for development of clinically effective anticancer drugs. [4-6] A great deal of microtubule-targeting agents with diverse scaffolds have been investigated. [7-10] Among them, combretastatin A-4 (CA-4, Fig.1), one of the most active tubulin polymerization inhibitors, exerts outstanding antitumor activity with potencies in the low nanomolar on a wide variety of human cancer cells *in vitro* and *in vivo*, but isomerization of the cis isomer in CA-4 to the less active trans isomer and poor solubility have greatly hindered its clinical application. [11, 12]

Structure-activity relationship (SAR) studies indicated that the common features of these analogues consist of 3,4,5-trimethoxy substituents on the A ring and the cis-orientation of the aromatic A- and B-rings. Hence, incorporating the cis-olefin bridge of CA-4 into a heterocyclic ring to restrict the configuration has become a well-verified strategy to design potent CA-4 analogues. [13-16] For example, pyrazole analogue **2** was recently shown to effectively inhibit tubulin polymerization and to display strong cytotoxicity of the colon-26 adenocarcinoma cell line with an IC_{50} of 8.4 nM. [17] In addition, pyridine-bridged analogue **3** potently inhibited cancer cell survival and growth. [18] More recently, 3,5-substitutedpyrazolo[3,4-b]pyridine fused analogue **4** was also reported to possess excellent antiproliferative activities. [19]

In our previous efforts to search for novel tubulin inhibitors as anticancer agents, a series of CA-4 analogs with a locked cis-type bridge were designed and synthesized that showed remarkable anticancer activities. [20-24] In this study, as shown in Fig. **1**, we designed a class of novel 1,3-diarylpyrazolo[3,4-b]pyridine **5a-f** and **6a-t** as cis-restricted CA-4 analogues, by retaining the 3,4,5-trimethoxybenzene as A ring and incorporating the cis-olefin bridge into a fused-ring pyrazolo[3,4-b]pyridine. Herein we reported the synthesis, preliminary biological evaluation including antiproliferative, tubulin polymerization and cell cycle analysis, and molecular modeling

was also performed to explore the possible binding mode of target compounds.

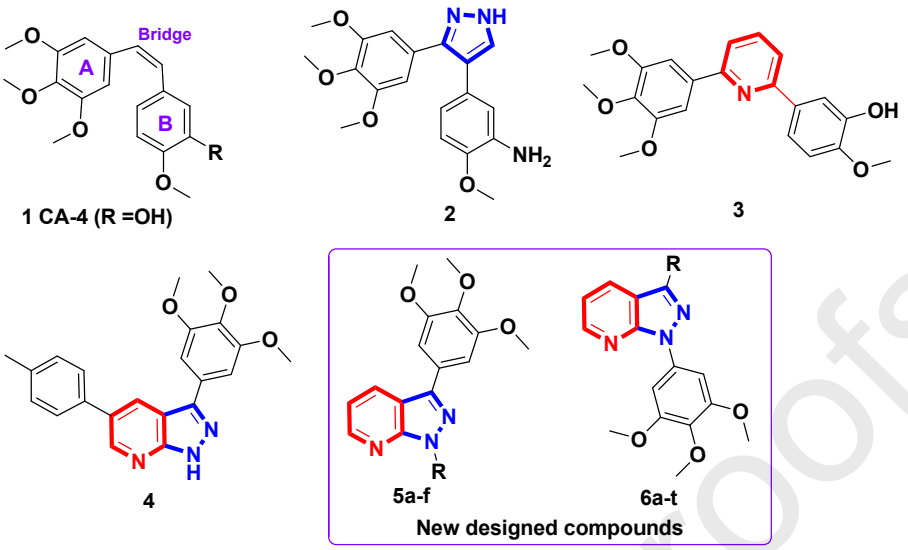
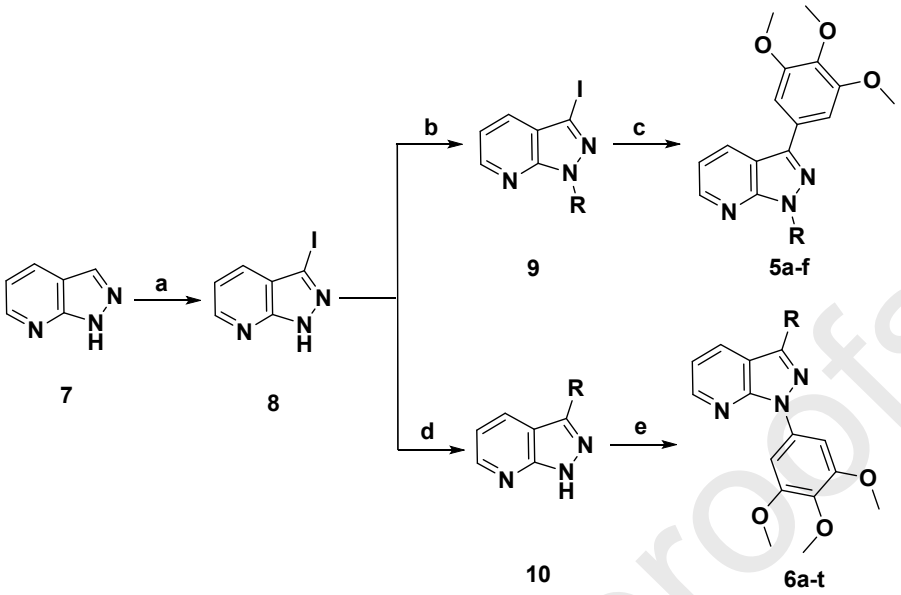


Fig. 1. Structures of selected tubulin inhibitors (**1**–**4**) with potent antiproliferative activity and our newly designed compounds (**5a**–**f**, **6a**–**t**).

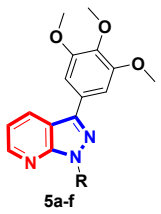
The general synthetic route for designed pyrazolo[3,4-b]pyridine derivatives **5a–f** and **6a–t** was illustrated in Scheme **1**. The key intermediate 3-iodo-1H-pyrazolo[3,4-b]pyridine **8** was prepared *via* iodination reaction of 1H-pyrazolo[3,4-b]pyridine **7** with *N*-iodosuccinimide (NIS) in the presence of potassium carbonate (K_2CO_3) as a base. Subsequently, intermediate **8** was treated with commercially available arylboronic acids using cupric acetate monohydrate as a catalyst, to give *N*-aryl pyrazolo[3,4-b]pyridines **9** which reacted with (3,4,5-trimethoxyphenyl)boronic acid, $Pd_2(dba)_3$, xantphos, and K_3PO_4 in a mixed solution of DMSO/H_2O under N_2 atmosphere, and provided 3-(3,4,5-trimethoxyphenyl) pyrazolo[3,4-b]pyridines **5a–f** in yields ranging from 75 to 85%. Finally, in a similar manner, *N*-(3,4,5-trimethoxyphenyl) pyrazolo[3,4-b]pyridines **6a–t** were generated from two step Suzuki coupling reactions, starting from the intermediate **8** with the appropriate arylboronic acids and (3,4,5-trimethoxyphenyl)boronic acid, respectively. The structures of the designed pyrazolo[3,4-b]pyridine derivatives **5a–f** and **6a–t** were identified by ^{1}H NMR, ^{13}C NMR, and HRMS spectroscopic techniques and the results were shown in the supplementary section.



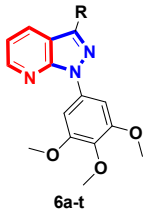
Scheme 1. Synthesis of the target compounds **5–6**. Reagents and conditions: (a) DMF, K_2CO_3, NIS, rt, 8h; (b) substituted benzyl chloride, K_2CO_3, DMF; or arylboronic acids, $Cu(OAc)_2$, Et_3N, THF, 80 °C, 24h; (c) DMF, H_2O, (3,4,5-trimethoxyphenyl)boronic acid, $Pd_2(dba)_3$, xantphos, K_3PO_4, 80 °C, 8-12h; (d) DMF, H_2O, arylboronic acids, $Pd_2(dba)_3$, xantphos, K_3PO_4, rt, 8-12h; (e) THF, (3,4,5-trimethoxyphenyl)boronic acid, $Cu(OAc)_2$, Et_3N, O_2, 80 °C, 24h.

All of the synthesized target compounds **5a–f** and **6a–t** were evaluated for their antiproliferative activities against a panel of four different human cancer cell lines, including MCF-7 (human mammary adenocarcinoma cells), MDA-MB-231 (human breast cancer cells), HeLa (human cervical cancer cells), and Kyse150 (human esophageal squamous cells) *in vitro* through standard MTT assay. Due to its nanomolar activity to a variety of cancer cells, CA-4 was used as a positive control in this study. The results of the designed compounds and the reference were shown in Table **1**. The IC_{50} value symbolizes the concentration causing 50% inhibition of cancer cell growth.

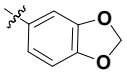
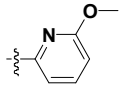
Table 1 Antiproliferative activities of designed compounds 5a–f, 6a–t against human cancer cell lines *in vitro*.



5a-f



6a-t

Comp.	R	*In vitro* cytotoxicity (IC_{50}±SD, μM)[a]			
		MCF-7	MDA-MB-231	HeLa	Kyse150
5a	4-$FC_6H_4CH_2$	>100	61.52±3.07	92.73±3.29	>100
5b	4-$CH_3C_6H_4CH_2$	>100	>100	>100	>100
5c	4-ClC_6H_4	>100	>100	>100	>100
5d	3,4-$(CH_3O)_2C_6H_3$	>100	>100	>100	>100
5e	4-$CH_3CO_2C_6H_4$	>100	>100	>100	>100
5f	4-$NH_2NHCOC_6H_4$	65.23±1.75	>100	29.66±0.57	>100
6a	4-FC_6H_4	76.66±2.59	56.38±3.89	51.39±4.29	41.12±1.73
6b	4-$CH_3C_6H_4$	>100	>100	>100	>100
6c	4-ClC_6H_4	97.63±1.55	84.33±4.13	>100	>100
6d	3,4-$(CH_3O)_2C_6H_3$	96.82±5.77	28.65±0.69	22.66±0.31	>100
6e	4-$CH_3CO_2C_6H_4$	>100	>100	>100	>100
6f	4-$NH_2NHCOC_6H_4$	>100	50.95±1.16	21.92±1.08	>100
6g	3,4-$Cl_2C_6H_3$	>100	>100	>100	>100
6h	4-CNC_6H_4	45.87±1.70	82.53±1.37	72.55±4.94	85.88±1.26
6i	5-F,2-$CH_3OC_6H_3$	>100	69.86±2.46	91.79±4.77	74.33±3.87
6j	2-$CH_3OC_6H_4$	>100	>100	>100	>100
6k	2-$CF_3OC_6H_4$	>100	60.46±4.91	78.85±3.32	>100
6l	3-$CH_3OC_6H_4$	86.92±2.04	86.66±0.70	85.04±5.28	>100
6m	4-F,2-$CH_3OC_6H_3$	87.64±1.86	46.98±0.78	58.33±2.40	87.35±0.96
6n	3-HO,4-$CH_3OC_6H_3$	27.22±2.31	27.04±6.42	18.08±1.48	62.82±2.52
6o	4-CH_3O,3-$NO_2C_6H_3$	28.45±3.49	37.28±0.45	23.50±2.97	>100
6p	4-$CH_3OC_6H_4$	>100	>100	>100	>100
6q	4-$NO_2C_6H_4$	>100	>100	84.52±1.94	>100
6r	3-NH_2,4-$CH_3OC_6H_3$	>100	>100	33.39±1.21	58.11±2.36
6s		80.11±1.99	79.65±1.36	38.86±1.71	58.85±6.56
6t		>100	>100	>100	>100
	CA4	0.041±0.013	0.017±0.07	0.003±0.001	0.60±0.01

[a] IC_{50} was tested after 48h of drug exposure. Each experiment was carried out at least three times. SD represents standard deviation.

Compounds **5a–f** possess 3,4,5-trimethoxyphenyl at the 3-position of the pyrazolo[3,4-b]pyridine ring, whereas their analogues **6a–t** were substituted by 3,4,5-trimethoxyphenyl on the 1-position of the core. This feature allowed us to explore the effect of the location of 3,4,5-trimethoxyphenyl group on antiproliferative activities. As illustrated in Table **1**, it is obviously to find that derivatives **6a–t** with 3,4,5-trimethoxyphenyl at position-1 showed more potent antiproliferative activities than the analogues **5a–f** possessing corresponding group at position-3 (for example, **5a** vs. **6a**, **5c** vs. **6c**, **5d** vs. **6d**), indicating that the 3,4,5-trimethoxyphenyl at position-3 was beneficial for antiproliferative activity. Among the series of **6a–t**, in agreement with CA-4, analogue **6n** with (3-hydroxy, 4-methoxy)phenyl group exhibited potent growth inhibitory activity against all the four tumor cell lines. On the contrary, electron-withdrawing groups such as fluorine (**6a**), chlorine (**6c**), carbomethoxy (**6e**), and cyano (**6h**) at the 4-position of the phenyl moiety, displayed much decreased antiproliferative activity, compared to derivative **6n**, while the compound **6t** with a 2-methoxypyridine substitution of (3-hydroxy, 4-methoxy)phenyl moiety, completely lost its inhibitory activity. These results again highlighted the importance of the (3-hydroxy, 4-methoxy)phenyl group in maintaining inhibitory potency against tumor cells.

Generally, significantly arresting cells in the G2/M phase is a typical feature of tubulin polymerization inhibitors. Hence, cell cycle analysis was carried out on HeLa cells for the highly active derivative **6n** to explore whether the antiproliferative activity was related to arrest the cell cycle. In this study, HeLa cells was treated with 9, 18, 36 μM concentrations of compound **6n** for 24h by flow cytometry. As depicted in Fig. **2**, with the increasing concentration, HeLa cells accumulated continuously in the G2/M phase, from 26.93% (9 μM) to 34.87% (18 μM) and then to 48.13% (36 μM) after 24h of treatment, which compared with 13.92% of the G2/M phase for untreated cells. These data suggested that compound **6n** arrested the cell cycle at the G2/M phase in a concentration-dependent manner.

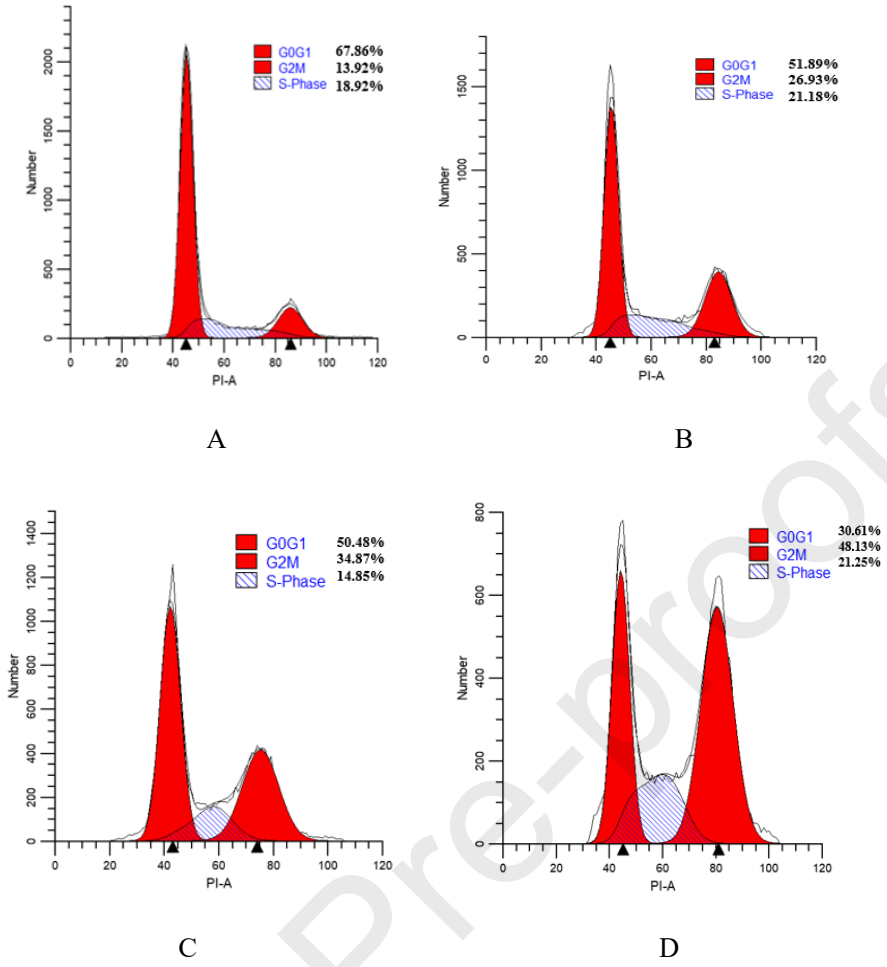


Fig. 2. Effect of compound **6n** on cell cycle in HeLa cells. Flow cytometry analysis of HeLa cells stained with propidium iodide and treated with **6n** for 24h. (A) Control; (B) **6n**, 9 μM; (C) **6n**, 18 μM; (D) **6n**, 36 μM.

To elucidate whether the newly pyrazolo[3,4-b]pyridines could inhibit tubulin polymerization *in vitro*, five representative analogues were selected to evaluate for their inhibitory activities at 10 μM concentration, meanwhile CA-4 was used as a positive control. As presented in Table **2**, some extent correlation with respect to antitubulin and antiproliferative activity was observed. Compound **6n**, one of the most active antiproliferative compounds in this series, also exhibited much higher antitubulin activity with 31% inhibition at a concentration of 10 μM, which indicated that this analogue most likely targeted at tubulin.

Table 2 Tubulin polymerization inhibitory activities of representative selected compounds

Comp.	R	Tubulin polymerization % inhibition [a]
6a	4-FC_6H_4	18
6d	3,4-$(CH_3O)_2C_6H_3$	14
6m	4-F,2-$CH_3OC_6H_3$	20
6n	3-HO,4-$CH_3OC_6H_3$	31
6o	4-CH_3O,3-$NO_2C_6H_3$	16
	CA-4	80

[a] Compounds were tested at a final concentration of 10 μM.

In order to investigate the binding features for this series of compounds, the representative derivative **6n** was chosed to perform the molecular docking study on the colchicine binding site of tubulin. As shown in Fig. **3**, **6n** occupied the colchicine binding site at the interface of α/β-tubulin, as expected, and the binding modes were quite similar with that of CA-4 in the co-crystallized tubulin structure (PDB: 5lyj). 1H-pyrazolo[3,4-b]pyridine framework of the compound **6n** was placed deeply in the β subunit of the tubulin, while the oxygen of trimethoxyphenyl formed a strong hydrogen bonding with βCys241 (2.1Å). In addition, the hydroxyl of the analogue **6n** established two H-bonds with βAsn258 (2.5 Å) and βMet259 (2.6 Å), respectively. These molecular docking results suggested that compound **6n** efficiently bound to the colchicine site of tubulin, which may be responsible for its tubulin polymerization inhibitory activities.

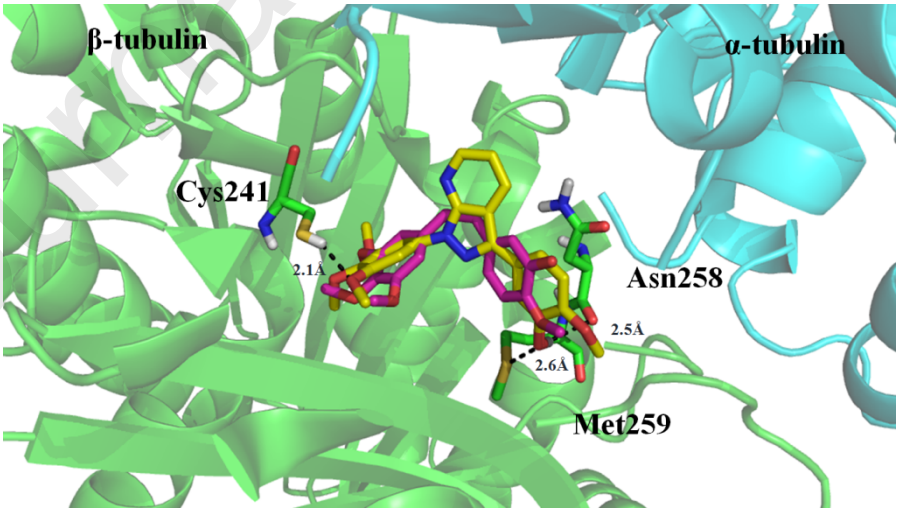


Fig. 3. Proposed binding model of compound **6n** (yellow) overlapping with CA-4 (magenta) in the colchicine binding site of tubulin. Subunits α and β of the tubulin heterodimer are shown as green and blue cartoon respectively. Potential hydrogen bonding interactions are indicated as black dashed lines. The main interacting residues are shown and labeled, including Cys241 (2.1Å), Asn258 (2.5Å) and Met259 (2.6Å). Final figure for docking pose was generated by PyMOL.

In conclusion, a series of novel 3,4,5-trimethoxyphenyl substituted pyrazolo[3,4-b]pyridine as cis restricted combretastatin A-4 analogues were designed and synthesized *via* a two-step Suzuki coupling reaction. Biological assays revealed that the most active derivative **6n** exhibited potent antiproliferative activities against four different cell lines including MCF-7, MDA-MB-231, HeLa and Kyse150. Furthermore, compound **6n** was shown to effectively arrest HeLa cells in the G2/M phase by flow cytometric analysis and inhibit tubulin polymerization. In addition, molecular docking study demonstrated that **6n** most likely occupied the colchicine binding site at the interface of α/β-tubulin, employing quite similar binding modes with that of CA-4. These observations indicated that 3,4,5-trimethoxyphenyl substituted pyrazolo[3,4-b]pyridine might be a promising scaffold for the development of novel tubulin inhibitors as anticancer drugs.

Acknowledgments

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A. Supplementary data

Supplementary data associated with this article can be found, inthe online version, at https://doi.org/10.1016/j.bmcl.2020.xx.xxx.

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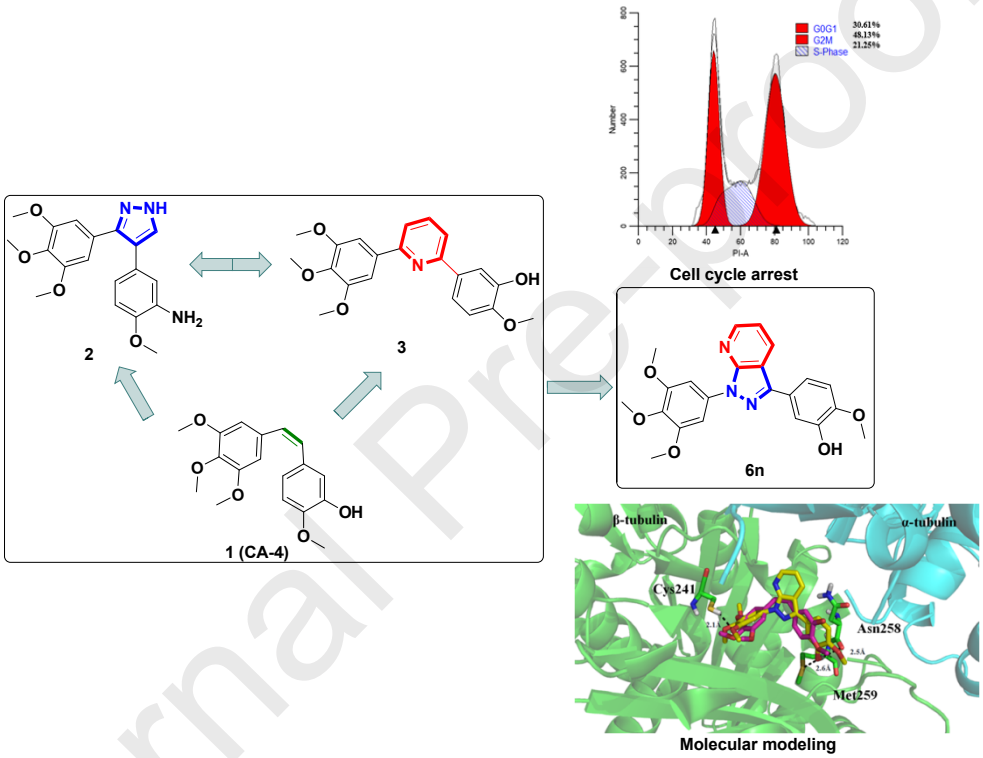
Guangdong Provincial Key Laboratory of New Drug Screening, School of Pharmaceutical Science, Southern Medical University, Guangzhou 510515, P.R.China



Declaration of interests

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

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: