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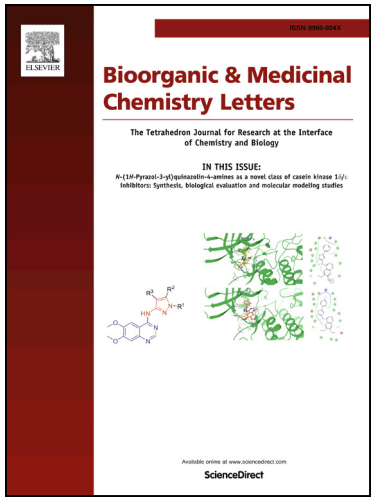

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Na Lv, Ming Sun, Chen Liu, Jiabin Li

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Design and synthesis of 2-phenylpyrimidine coumarin derivatives as anticancer agents

Na Lv [a,†], Ming Sun [a,†], Chen Liu [b], and Jiabin Li [c,*]

[a] *Department of Stomatology, First Affiliated Hospital of Anhui Medical University, Hefei 230032, China*
[b] *College of Basic Medicine, Anhui Medical University, Hefei 230032, China*
[c] *Department of Infectious, First Affiliated Hospital of Anhui Medical University, Hefei 230032, China*
***** Correspondence: jbahmu@yeah.net Tel.: +86-551-65161136
† These authors contributed equally to this work.

ABSTRACT A series of 2-phenylpyrimidine coumarin derivatives with potential telomerase-inhibiting activity was designed and synthesized. All of the compounds were screened for antiproliferative activity against CNE2, KB, and Cal27 cell lines *in vitro*. The results showed that most of the derivatives had a favorable effect on resisting tumor cell proliferation; compound 13, 3-(4-amino-5-oxo-5H-chromeno[4,3-d]pyrimidin-2-yl)phenyl 4-(dimethylamino)benzenesulfonate, exhibited the best activity. Flow cytometry revealed that compound 13 can inhibit CNE2 proliferation. Telomerase inhibition and *in vitro* antitumor activity were consistent among the compounds, but compound 13 showed the best telomerase-inhibiting activity and could inhibit telomere extension. Molecular docking results indicated that compound 13 bonded with telomerase reverse transcriptase (TERT) through multiple interactions, including hydrogen bonding and hydrophobic interactions. The results of the study provide further information on 2-phenylpyrimidine coumarins, expanding the types of telomerase inhibitors as the parent structures.

***Keywords*:** 2-phenylpyrimidine coumarin derivatives; antiproliferative; telomerase inhibitors

Telomerase, as a ribonucleoprotein enzyme with reverse transcriptase activity, can use its own RNA as a template to synthesize telomere DNA sequences [1-3]. Any abnormalities in telomerase structure and behavior are closely related to cell senescence, tumorigenesis and development [4, 5]. A large quantity of data confirms that more than 85% of human tumor cells have high levels of telomerase activity, but most somatic cells and benign tumor cells lack telomerase activity [6, 7]. Therefore, most scholars believe that the proliferation of some tumor cells can be effectively inhibited through the inhibition of telomerase activity [8, 9].

Coumarin, namely, benzopyrone, is a substance with an aromatic odor. Coumarin and its derivatives constitute important organic heterocyclic molecules with diverse physiological activities. As early as the 1960s, scientists found that coumarin compounds had antitumor metastasis effects [10]. As large quantities of coumarin compounds have been separated and synthesized, more and more coumarin compounds have been found to have antitumor effects [11-14] . In recent years, Liu's group reported on multiple series of coumarin derivatives, which were found to have favorable telomerase-inhibiting effects and inhibiting effects on cell proliferation in multiple tumors [15-17]. Aromatic sulfonate antitumor drugs are widely used in the clinic, and aromatic sulfonyl groups are important pharmacophores [18, 19]. Based on the above-mentioned studies and for the continuous extension of structural types of coumarin telomerase inhibitors, our research used 2-phenylpyrimidine coumarin as a parent nucleus. In addition, aromatic sulfonyl groups were introduced in the substituted phenyl meta-position, which was expected to increase the antitumor activity and interactions with telomerase. Then, a series of 2-phenylpyrimidine coumarin derivatives was obtained (**Figure 1**), and their antitumor effects and telomerase-inhibiting activities were evaluated.

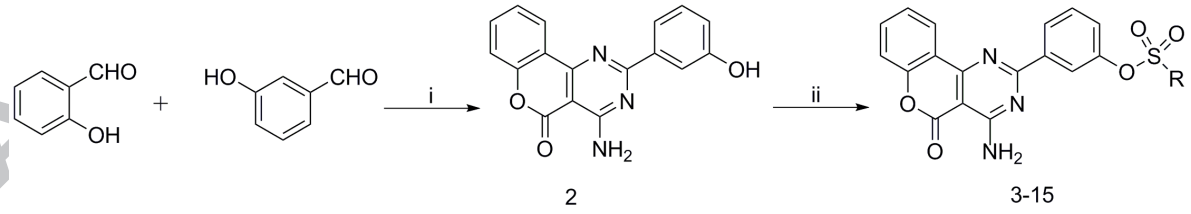


Figure 1. Synthesis of 3'-sulfonate-substituted 2-phenyl-benzopyranopyrimidine derivatives. Reagents and conditions: (i) $CNCH_2COOC_2H_5$, NH_4OAc, C_2H_5OH, reflux, 2 h and (ii) $ClSO_2R$, DMF, rt, and 5 h (R is shown in table 1).

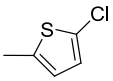
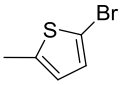
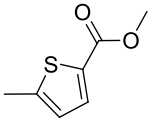
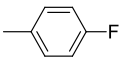
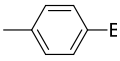
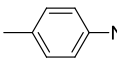
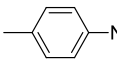
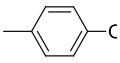
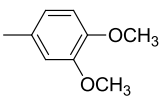
Figure 1 shows the synthesis route. Parent compound 2 was obtained through the "one-pot" method, namely, ethyl cyanoacetate, salicylic aldehyde, 3-hydroxybenzaldehyde, and ammonium acetate were put under reflux for 2 h [20]. During the reaction process, large

quantities of yellow solids precipitated, and then parent compound 2 was obtained through a simple after-treatment. Parent compound 2 can be directly applied in the next step without further purification, and it reacts with the substituted sulfonyl chloride to obtain the target product.

To test the antitumor activity of the synthesized compounds, we evaluated the activity of compounds 3–15 against human nasopharyngeal carcinoma cells (CNE2), human oral epidermoid carcinoma cells (KB), and human oral squamous cells (Cal27). The results are summarized in Table 1. Following the interactions between compounds and the cells, the results revealed that the most examined compounds showed potent activity against the CNE2 and Cal27 cell lines and moderate activity against the KB cell lines. Compounds 4, 8, 10, and 13 exhibited high activity against the CNE2 cell lines, with IC_{50} values of 4.24±0.17, 3.44±0.46, 2.34±0.21, and 1.92±0.13 μM, respectively. Compounds 8, 9, 10, and 13 possessed potent activity against the Cal27 cell lines, with IC_{50} values of 2.58±0.47, 4.91±0.87, 4.32±0.24, and 1.97±0.51 μM, respectively. Compounds 4, 8, and 13 showed high activity against the KB cell lines, with IC_{50} values of 5.62±0.35, 6.74±0.24, and 3.72±0.54 μM, respectively, which are comparable with the positive control doxorubicin (AMD).

Structure–activity relationship analysis showed that our examined compounds were divided into three series. Compound 1 has an alkyl moiety and no antiproliferative ability. Compounds 4–7 have thiophene moieties, while compounds 8–15 have aromatic-ring moieties; compounds 8–15 are better at resisting tumor cell proliferation being than compounds 4–7. In the aromatic-ring moiety series of compounds, the compounds substituted by halogen demonstrated moderate activity. Compound 13, which has N,N-dimethyl amino benzenesulfonyl moiety, had the best activity; its activity values (IC_{50}) for the CNE2, KB, and Cal27 cell lines were 1.92±0.13, 3.72±0.54, and 1.97±0.51 μM, respectively, and the values are comparable with those of potent ADM. Therefore, for this type of structural moiety, this 2-phenylpyrimidine coumarin derivative structure warrants further optimization as a potent anticancer agent.

Table 1. In vitro anticancer activity of the synthesized compounds [a].

Compound	R	In vitro anticancer activity, IC_{50} (μM) [b]		
		CNE2	KB	Cal27
3	–CH3	>100	>100	>100
4		4.24 ± 0.17	5.62 ± 0.35	10.12 ± 0.78
5		14.58 ± 1.98	20.86 ± 1.51	11.25 ± 0.99
6		18.64 ± 3.27	36.17 ± 1.41	18.34 ± 3.01
7		>100	38.36 ± 2.98	47.47 ± 3.26
8		3.44 ± 0.46	6.74 ± 0.24	2.58 ± 0.47
9		4.23 ± 0.18	11.59 ± 1.54	4.91 ± 0.87
10		2.34 ± 0.21	7.35 ± 0.16	4.32 ± 0.24
11		11.07 ± 1.45	15.83 ± 1.64	18.07 ± 2.21
12		16.25 ± 2.62	16.99 ± 1.60	3.54 ± 1.33
13		1.92 ± 0.13	3.72 ± 0.54	1.97 ± 0.51
14		16.57 ± 2.50	18.62 ± 1.98	9.63 ± 1.04
15		20.64 ± 2.21	29.72 ± 2.24	17.63 ± 1.85
ADM		2.12 ± 0.56	3.04 ± 0.87	1.56 ± 0.64

Negative control 0.1%DMSO, no activity.

[a] The data represented the mean of three experiments in triplicate and were expressed as means ± SD; only descriptive statistics were done in the text.

[b] The IC_{50} value was defined as the concentration at which 50% survival of cells was observed.

Next, flow cytometry was used to detect the effect of the compounds on inducing apoptosis in tumor cells. The Annexin V-FITC/PI double-staining method was used to evaluate the effect of compound 13 on inducing apoptosis of the CNE2 cells (**Figure 2**). Figure 2A shows that a blank control was not added to any of the compounds, and only 8.1% of the cells were in an apoptotic state. After compound 13 was added, and as its concentration increased, the number of apoptotic cells gradually increased; finally, the percentage of CNE2 cells in an apoptotic state had escalated to 54.9%. This result confirmed that compound 13 induced apoptosis in the CNE2 cells.

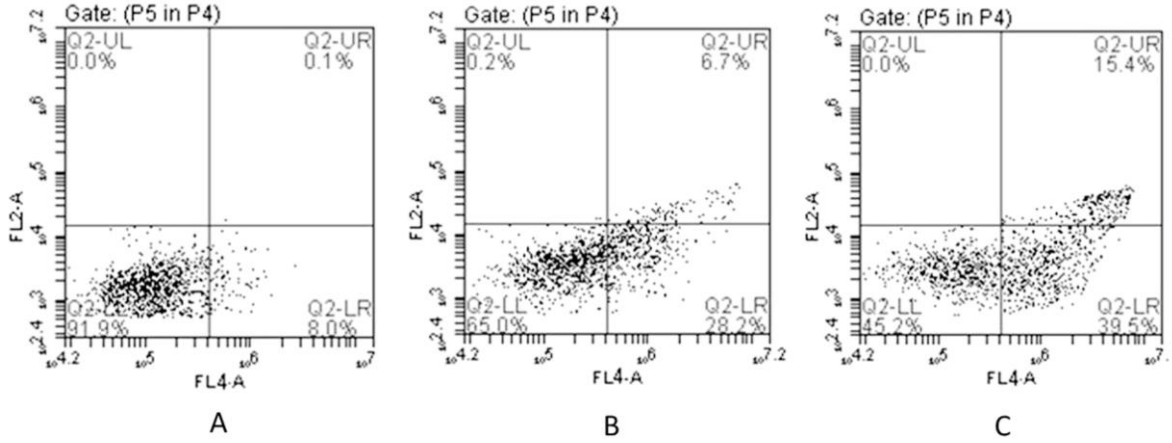


Figure 2. CNE2 cells were treated with no drug (A) and 0.5 μM (B) and 1 μM (C) of compound 13 for 4 days and then analyzed with flow cytometry.

Based on the discovered coumarin skeletal structure, the structural types of telomerase inhibitors were continuously extended. Our research evaluated the telomerase-inhibiting activities of some of the examined compounds using CNE2 cell extracts with ethidium bromide as the positive control. Results are shown in **Table 2**. Compounds 4, 8 13, and 15 show favorable telomerase-inhibiting activities, but compounds 3 and 12 had weak telomerase-inhibiting activity. These results are consistent with the *in vitro* antitumor effect of these compounds, and they indicate that the compounds may resist tumor cell proliferation by inhibiting telomerase activity.

Table 2. Inhibitory effects of selected compounds against telomerase [a].

Compound	IC_{50} (μM) telomerase [b]
3	58.64 ± 3.42
4	7.35 ± 0.96

8	3.16 ± 0.67
12	21.07 ± 2.32
13	0.82 ± 0.14
15	6.32 ± 0.56
Ethidium bromide	2.16 ± 0.28

Negative control 0.1%DMSO, no activity.
[a] The data represented the mean of three experiments in triplicate and were expressed as means ± SD; only descriptive statistics were done in the text.
[b] The IC_{50} value was defined as the concentration at which 50% survival of cells was observed.

Telomere length is jointly regulated by telomerase and the telomere-binding protein. Telomere length, a biological marker that measures cell senescence and apoptosis, is a good index to measure the biological characteristics of tumors [6, 21]. We conducted additional research on the length change of restrictive telomere fragments to further study compound 13's mechanism in inhibiting cell proliferation and inducing cell apoptosis. Telomere restriction fragment (TRF) experimental results (**Figure 3**) show that the CNE2 cells' telomere lengths after treatment with 1 μM of compound 13 for 8 days were shortened by 0.8 kb compared with the blank control group, and telomere shortening was also observed after treatment with 0.5 μM of compound 13 for 8 days. Telomere dysfunction can activate p53 to initiate cellular senescence or apoptosis to suppress tumorigenesis. In this study, senescence induction via compound 13 might be due to telomere-length shortening; this result is consistent with those previously reported for efficient telomerase inhibitors [6, 21]. Hence, compound 13 may inhibit cell proliferation by inhibiting telomere activity and inhibiting telomere length extension.

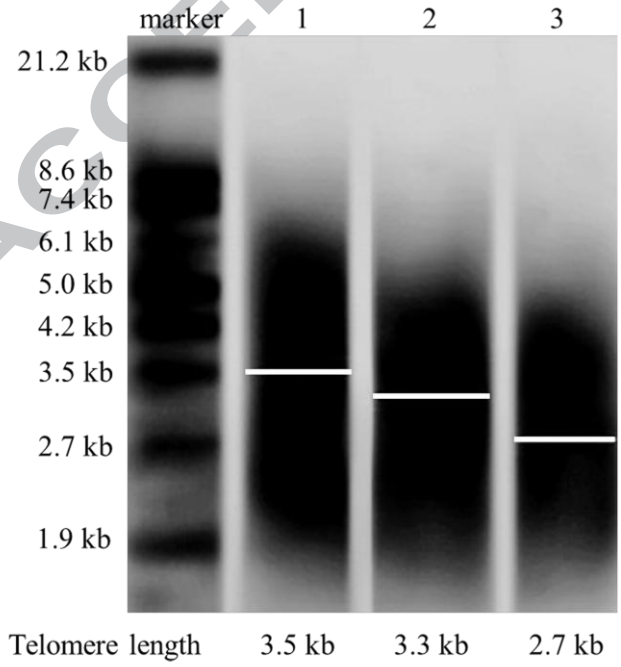


Figure 3. Effect of compound 13 on telomere length. TRF analysis of CNE2 cells treated with or without compound 13 for 8 days. Lane 1, with 0 μM compound 13; lane 2, with 0.5 μM compound 13; lane 3, with 1 μM compound 13.

To further examine the mechanism of the telomerase-inhibiting effects of 2-phenylpyrimidine coumarin derivatives and provide an idea for follow-up structural transformation, compound 13 and telomerase TERT (PDB code: 3DU6) [22], which includes ASP254 key active regions, were selected for the molecular docking experiment. The results are shown in **Figure 4**. In general, compound 13 could adaptively bind to catalytic subunits of telomerase and replace nucleotides as the active substrate. Multiple interactions, including hydrogen-bond interactions and hydrophobic interactions, can be seen in the figure. One hydrogen bond was formed between $–NH_2$ and ILE187 on the compound-substituted aromatic ring. Two hydrogen bonds were formed between the substituted amino group on the pyrimidine ring and ASP254. Another two hydrogen bonds were formed between two Os on the sulfonic group and ASP343. Furthermore, one pi–cation interaction was formed between the substituted aromatic ring and ASP254. These results show why compound 13 has favorable enzyme-inhibiting activity.

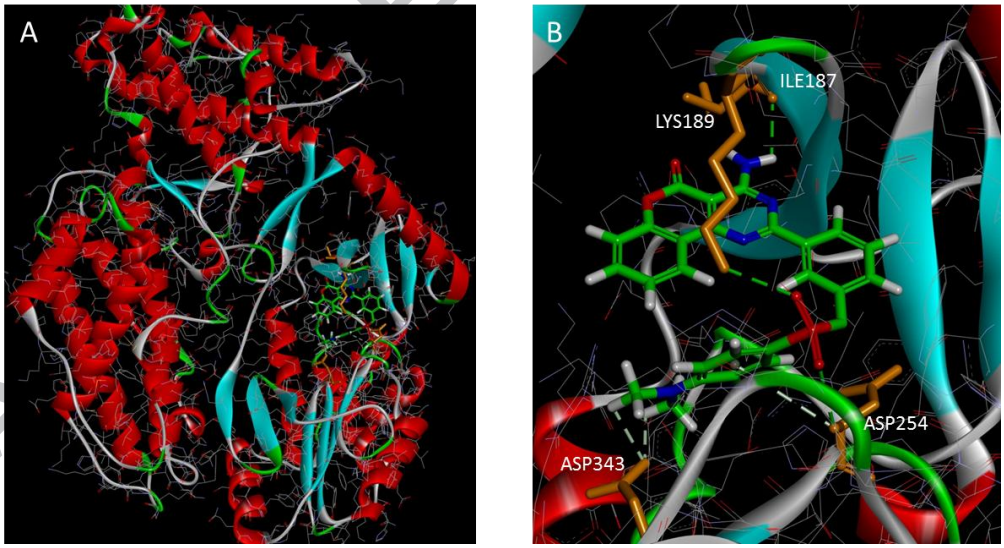


Figure 4. The binding mode between the active conformation of compound 13 and the protein TERT (PDB code: 3DU6). Entire view (A) and view of the active site cavity (B). The compound is rendered in green stick models, and the amino acid residues are rendered in golden sticks.

In summary, in our quest to find new, efficient compounds with telomerase-inhibiting activity and to extend the research on coumarin analogs, we designed new 2-phenylpyrimidine coumarin derivatives, followed by their chemical synthesis; we then performed biological evaluations of them. An *in vitro* antitumor study showed that these compounds have strong effects on resisting tumor cell proliferation. Among them, compound 13 exhibited strong inhibitory activity against the CNE2 cells, and it was revealed to have the most potent telomerase-inhibiting activity, with an IC_{50} value of 0.82±0.14 μM. Flow cytometric analysis indicated that cell proliferation was also arrested by compound 13, which was accompanied by a shortening of the telomere length. Molecular docking indicated that active compound 13 was nicely bound to the catalytic subunits of telomerase TERT through multiple patterns and could replace nucleotides as active substrates. The above results certify that 2-phenylpyrimidine coumarin derivatives have the potential to become lead compounds among antitumor drugs.

Acknowledgments

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A. Supplementary data

Supplementary data associated with this article can be found.

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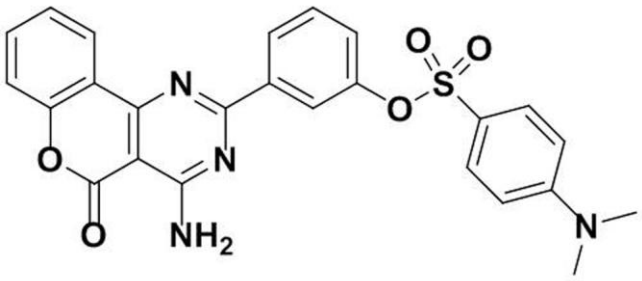

13 IC_{50}= 0.82 ± 0.14 μM