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Short Communication

Finding a resveratrol analogue as potential anticancer agent with apoptosis and cycle arrest

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ABSTRACT

Resveratrol has been extensively studied as the anti-cancer agent. A variety of resveratrol analogues have been developed with structural modification to improve its bioactivity. In this work, resveratrol analogues, compound **1**-**4**, were designed and synthesized with the Stille–Heck reaction. These results showed compound **1**–**4** had better anticancer effect than that of parent resveratrol. Especially compound **1** ((E)-4,4′-(ethene-1,2-diyl)bis(3-methylphenol)) displayed the excellent cytotoxicity and high selectivity. The mechanism research indicated compound **1** inhibited cell proliferation by binary paths of cell cycle arrest in S phase regulated by cyclin A1/A2 and apoptosis induction mediated by Bax/Bcl2 in a prooxidant manner.

Carcinogenesis is a multistep and heterogeneous process, which involves the DNA damage and imbalance of homeostasis resulting in cells escaping the restraints into uncontrolled growth. Cancer has been one of the most common non-communicable chronic diseases, ranking as the leading cause of death worldwide.[1] Therefore, the prevention and treatment of cancer is of great importance.

Natural products, as potential sources of new drugs, provide diversified and promising drug lead scaffolds, which have been the hotspot in new drug discovery.[2] Resveratrol (trans-3,5,4′-trihydroxy-trans-stilbene, RES) with unique skeleton and pharmacological properties, has been increasing prominence.[3] Many studies have reported RES shown the anti/pro-oxidation, anti-inflammation, anti-microbial effects, especially anticancer activities via targeting oxidative systems, inducing cell-cycle arrest, apoptosis or autophagy.[4,5] However, the poor cytotoxicity and low bioavailability limit its practical application.[6] Therefore, researchers often modify the structure to improve its anticancer bioactivity.[7]

RES is consisted of two phenolic rings connected by a styrene double bond with trans-stilbene skeleton. Up to now, the modifications of RES mainly focus on the change fans-into cis-, the reduction of carbon-carbon double bonds, the elongation of the conjugated chains, and the substitution of amino, methoxyl, hydroxyl, or other groups on the benzene ring.[7–11] Halogen, methyl and trifluoromethyl played the important roles in improving drug activities.[9,11] Hence, in this work, we designed and synthesized the compound **1**–**4** (Fig. 1) based on (1) structural modification of methyl, chlorine, fluorine, trifluoromethyl and (2) group introduction at benzene ring in the adjacent position of the double bond with increase of dihedral angle for improving ability of recognition and bond between drug and proteins. We anticipated to obtain molecules with better anticancer activities through the above structural modification strategies. Interestingly, These results showed the synthesized analogues of resveratrol have better anticancer effect than that of parent resveratrol, and compound **1** ((E)-4,4′-(ethene-1,2-diyl)bis(3-methylphenol)) displayed the best cytotoxicity with excellent selectivity. Consequently, the action mechanism research demonstrated the compound **1** inhibited cell proliferation by cell cycle arrest and apoptosis.

Firstly, the compounds **1**–**4** were synthesized based on the classic one-step Stille–Heck tandem reaction (Fig. 1B, for details see the Supporting Information). With the compounds **1**–**4** in hand, the cytotoxic activities of the resveratrol and compound **1**–**4** were firstly investigated by MTT assay in cancer cell lines (HeLa, MCF-7

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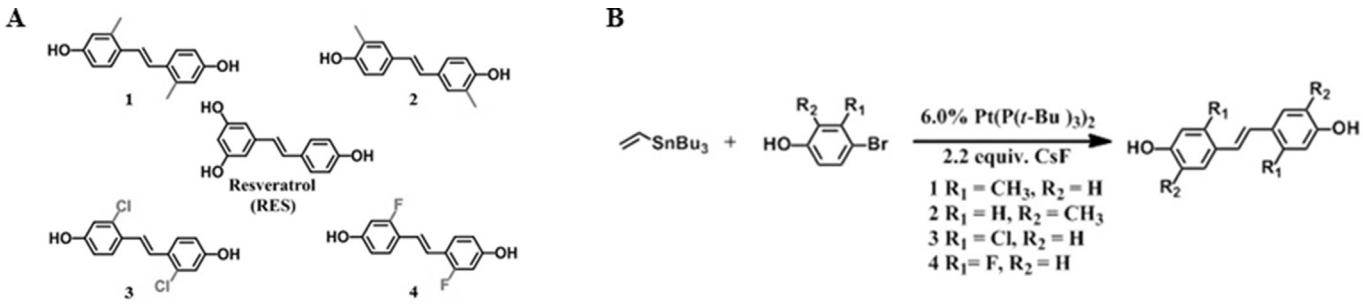

Fig. 1. The structure and the synthesis of compounds **1**–**4**.

and H1299) and normal cells (MRC-5). Exhilaratingly, compound **1**–**4** exerted more obvious cytotoxicity in HeLa and MCF-7 cells compared to RES, which meant the drug modification strategy was effective (Table S1). Compound **1** showed the most excellent cytotoxicity among the remain compounds, especially in HeLa cells with the IC_{50} value of 10.72 μM, and showed markedly less cytotoxicity in normal cells simultaneously (Figure S1). However, the compound **2** didn't exhibit the excellent cytotoxicity and selectivity like that of compound **1**. Therefore, the change of dihedral angle of molecular structure and the increase of methyl group's lipid solubility played an important role for improving biological activities of drug molecule.

Favorable activity and good selectivity of compound **1** encouraged us to further investigate its antiproliferation mechanism. As we know, carcinogenesis is a complex and multi-stages process, and may be associated with multiple mechanisms, especially the disorder of cell cycle or the deregulation of apoptosis in response to DNA damage or cellular perturbations, resulting in the abnormal proliferation.[12,13]

Firstly, the cell cycle arrest with incubation of compound **1** was investigated in HeLa. Flow cytometry result revealed that S phase proportion increased significantly at 10 μM after treatment for 24 h/48 h (Fig. 2A and B), which confirmed that compound **1** arrested cell cycle progression in S phase. The precis and strict regulation of the key checkpoints of the cell cycle, G1/S, intra-S, and G2/M, is closely related to the cyclins. Based on the fact that cyclin A mediated S phase progression, the western blot was performed to detect its level. After incubation with compound **1** for 24 h, the expression of cyclin A1 and cyclin A2 decreased dramatically at concentration 16 μM (Fig. 2C), which validated compound **1** induced cell cycle arrest in S phase to inhibit cellular proliferation by down-regulation the expression of cyclin A1 and cyclin A2 in HeLa cells.

Besides, the induction of apoptosis is another valid mechanism to inhibit the cell proliferation. To further explore the contribution of apoptosis to the inhibition process, the apoptotic effect of compound **1** was detected. As shown in Fig. 2D, the apoptotic effect was found at concentration 8 μM and showed concentration-dependent

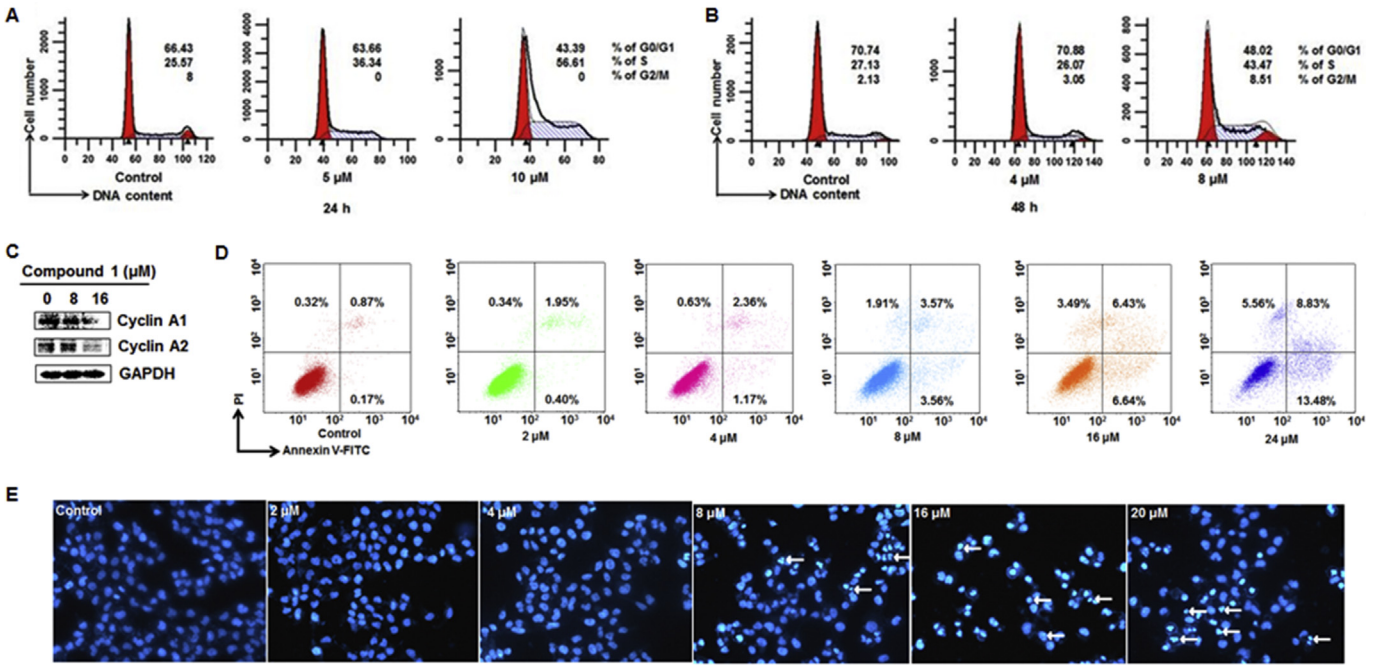

Fig. 2. Compound **1** inhibited tumor cell proliferation by both arresting cell cycle in S phase and apoptosis. (A) HeLa cells were treated with gradient concentration (control, 5 μM and 10 μM, respectively) for 24 h. (B) HeLa cells were treated with gradient concentration (control, 4 μM and 8 μM, respectively) for 48 h. (C) Compound **1** down-regulated the expression of cyclin A1 and cyclin A2. (D) HeLa cells were incubated with indicated concentration (control, 2 μM, 4 μM, 8 μM, 16 μM and 24 μM, respectively) for 48 h. The percentage shown in each quadrant 4–1–2–3 represents the proportion of early apoptotic cells, late apoptotic cells, necrotic and normal cells, respectively. (E) DAPI fluorescence observation showed apoptosis induction by compound **1** in HeLa. HeLa cells were treated with compound **1** at different concentration for 24 h, the apoptosis characteristic was observed using fluorescent microscope after nuclear staining with DAPI. The white arrow indicates nuclear chromatin condensation and the formation of apoptotic bodies.

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characteristics as incubation time extended to 48 h. In addition, apoptosis is characterized by cell shrinkage, chromatin condensation, nuclear fragmentation and the formation of apoptotic bodies, which were observed by fluorescence microscopy with DAPI staining. After 24 h of incubation with compound **1**, DAPI staining showed typical apoptosis characteristics with chromatin condensation and nuclear fragmentation (arrow) of HeLa cells at above concentration 8 μM (Fig. 2E). The result confirmed compound **1** inhibited HeLa cells proliferation by inducing apoptosis.

Excessive ROS accumulation may cause oxidative damage in response to chemotherapeutic stimuli, which may lead to DNA damage and cell death by apoptosis induction.[14] Therefore, the ROS level was detected to explore the apoptosis mechanism. Interestingly, the DCF fluorescent intensity increased obviously with the increase of compound **1** concentration (Fig. 3A). In addition, the cell survival rate was improved after ROS scavengers, including glutathione (GSH) and N acetyl L cysteine (NAC), which were separately added in the cells combined with compound **1** (Fig. 3B). Next, the $O_2\bullet^-$-specific fluorescence probe dihydroethidium (DHE) and dihydrorhodamine 123 (DHR 123 detects H_2O_2) were utilized to analyze the ROS species. As shown in Fig. 3C, the fluorescence intense of DHE increased about 23.0% after compound **1** treatment at concentration 24 μM for 6 h, whereas the fluorescence of DHR 123 showed little variation. Obviously, ROS, especially $O_2\bullet^-$, accumulation ascribed to compound **1** which may enhance cytotoxicity by oxidative stress leading to a disruption of cell signaling. To investigate the source of ROS, we determined the expression of mitochondrial complex V. The results displayed its expression was decreased with incubation of compound **1**, which lead to ROS level increasement with reduction of ATP synthesis (Fig. 3D). In addition, increased intracellular Ca^{2+} and elevated ROS levels promote each other.[15] Therefore, we tested the intracellular free Ca^{2+} with the dye Fluo-3 AM. The result showed that the fluorescence increased steadily in a compound **1** concentration dependent manner, which indicated that the intracellular free Ca^{2+} was associated with compound **1**-induced apoptosis (Fig. 3E). Thus, this is the other important reason for increase of ROS levels.

To further investigate the mechanism of apoptosis, we analyzed the expression of apoptosis-related proteins including p53, p21, Bax and Bcl-2 by western blot. After compound **1** treatment for 24 h, the pro-apoptotic protein Bax up-regulated, while the anti-apoptotic protein Bcl-2 down-regulated accordingly (Fig. 3F). Obviously, the increased Bax/Bcl-2 ratio promoted apoptosis. Subsequently, the Bax inhibitor peptide V5 (BIP V5), which also increased the expression of Bcl-2, was utilized to further verified the role of Bax in apoptosis induced by compound **1**. The result showed that the apoptosis effect started to reverse (Fig. 3G). However, the level of p53, the key regulator in apoptosis induction, was not altered significantly (Fig. 3F). Meanwhile, the cell apoptosis state didn't relieve after compound **1** cotreatment with pifithrin-β (PFTβ), an inhibitor of p53 protein (Fig. 3G), which further evidenced that the apoptosis induced by compound **1** was p53-independent.

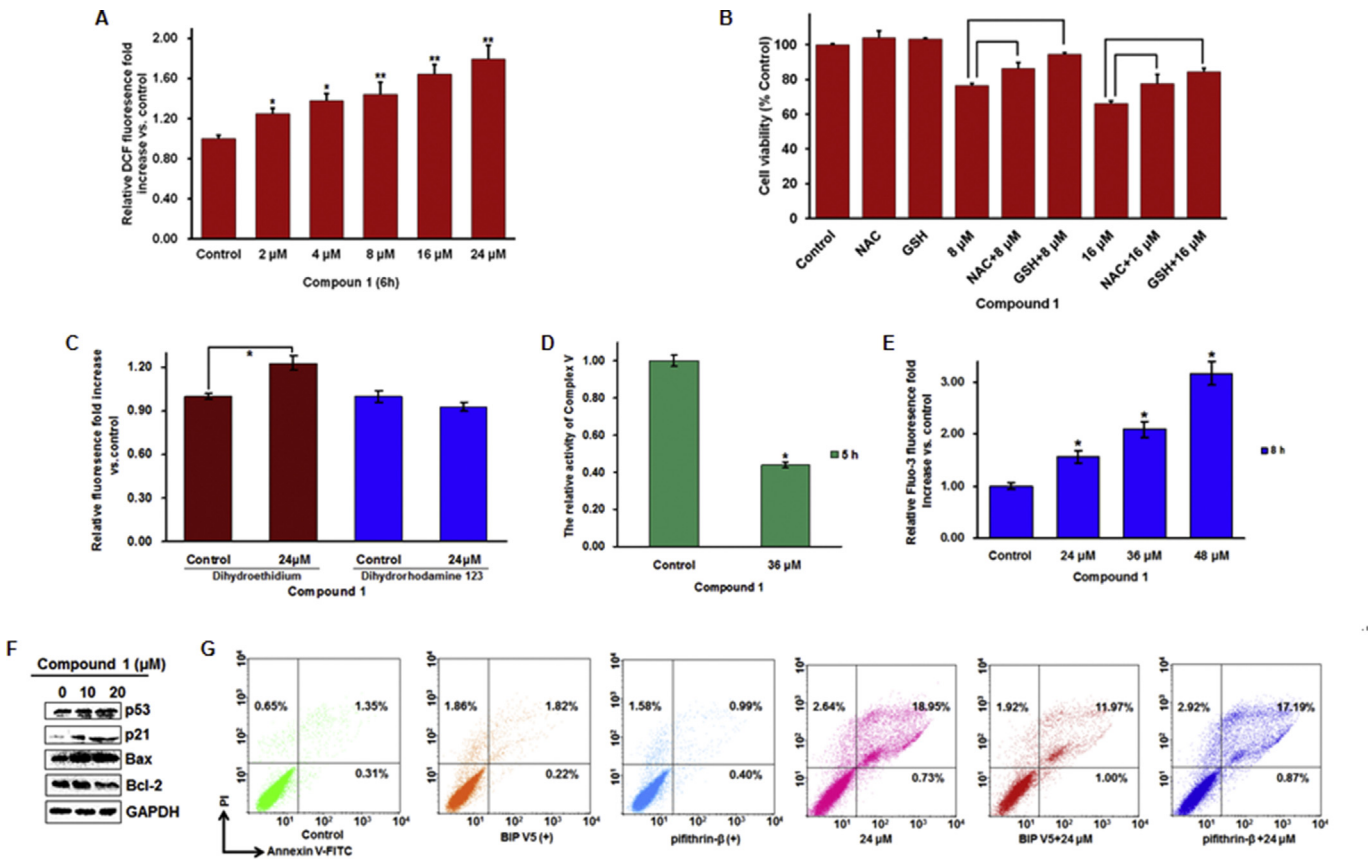

Fig. 3. Apoptosis induction by compound **1** was attribute to the triangular interaction among increased ROS, complex V inhibition and intracellular free Ca^{2+} stimulation as well as the increased Bax/Bcl-2 ratio in p21 dependent manner. (A) ROS level obviously increased after compound **1** treatment for 6 h. (B) The cell viability increased after treatment of ROS scavengers (GSH 10 mM and NAC 10 mM) combined with compound **1** (10 μM and 20 μM, respectively) for 28 h. (C) The $O_2\bullet^-$ level increased after compound **1** treatment for 6 h at 24 μM, but there was no difference in H_2O_2 between treated and untreated samples. (D) The activity of mitochondrial complex V decreased significantly after compound **1** (36 μM) treatment for 5 h. (E) The concentration of intracellular free Ca^{2+} increased remarkably in a concentration-independent manner after compound **1** treatment for 8 h. (F) The expression of tumor suppressors p53, p21, pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2 were analyzed. (G) Bax inhibitor BIP V5 relieved compound **1**-induced apoptosis, while the p53 inhibitor pifithrin-β didn't change the state. The asterisks (*) represents the significant differences ($P < 0.05$) between compared groups. The differences analyzed by ANOVA using SPSS 22.0.

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Nonetheless, the tumor suppressor p21 was significantly increased (Fig. 3F). Based on the above study, the apoptosis induction by compound **1** may be attributed to p21 in p53-independent upregulation of Bax and downregulation of Bcl-2. On the other hand, p21 is also a negative regulator of the cell cycle by inhibiting cyclin−cyclin dependent kinase (CDK) complexes in response to various cellular signals to inhibit cells proliferation.[16] Considering that compound **1** blocked cell cycle in S phase by down-regulation of cyclin A1/A2 in HeLa cell as our testing shows, it is suspected that compound **1** induced cell cycle arrest in S phase in a p21-dependent manner. Therefore, compound **1** inhibited tumor cell proliferation by binary paths of cell cycle arrested in S phase and apoptosis.

In summary, in this work, we synthesized a series of drug molecules with resveratrol as the parent molecule based on methyl, halogen and trifluoromethyl modification. The results showed compound **1** possessed the best cytotoxicity and highest selective anticancer potential in our test. Therefore, the tactics is effective for improving biological activities based on structural modification of methyl, chlorine, fluorine, trifluoromethyl and group introduction at benzene ring in the adjacent position of the double bond with increase of dihedral angle. The mechanism research displayed tumor cell death was mediated by a binary pathway including cell cycle arrest in S phase and apoptosis. On one hand, compound **1** cause cycle arrest mainly by regulating expression of cyclin and CDK in a p21-dependent manner. On the other hand, the compound **1** can inhibit the expression of mitochondrial complex V causing a decrease in ATP synthesis to lead increase of ROS levels. Meanwhile, the increase of intracellular Ca^{2+} and the increase of ROS promote each other, which had contributed significantly to the increase in ROS levels. Up-expression of p21 protein with higher levels of ROS can obviously increase Bax/Bcl-2 ratio, which lastly causes apoptosis. The related detailed molecular mechanism is still under further study in our laboratory. This study provides basic research for designing effective anti-tumor drugs.

Conflict of interest

All the authors declare that they have no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jphs.2020.03.007.

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