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Research paper

Discovery of oral-available resveratrol-caffeic acid based hybrids inhibiting acetylated and phosphorylated STAT3 protein

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ABSTRACT

Constitutive activation of STAT3 has been found in a wide variety of cancers and demonstrated as a very attractive therapeutic target. Disrupting both acetylation and phosphorylation of STAT3 protein was hypothesized to greatly deactivate STAT3, therefore, treating cancers. To demonstrate the hypothesis, two series of novel resveratrol-caffeic acid hybrids were designed aiming to regulate both acetylation and phosphorylation of STAT3 protein, which is also the first report of the synthetic inhibitors simultaneously regulating two biological reactions of STAT3 to our knowledge. Most of these compounds were demonstrated with preferential antitumor activity with low IC_{50} values against two cancer cell lines. Particularly, compound **7d** was found as an excellent STAT3 inhibitor with over 50-fold better potency than resveratrol and caffeic acid. Meanwhile, the novel derivatives significantly inhibited the proliferation and induced the apoptosis of tumor cells. Molecular docking further disclosed the binding modes of STAT3 with the inhibitors. In addition, compound **7d** orally and significantly suppressed breast cancer 4T1 xenograft tumor growth *in vivo*, indicating its great potential as an efficacious drug candidate for human cancer therapy.

1. Introduction

Signal transducer and activator of transcription 3 (STAT3) is a cytoplasmic protein that plays important roles in oncogenic signaling pathways [1–3]. Constitutive activation of STAT3 has been found in a wide variety of cancers, including breast cancer, colon cancer, and other cancers, promoting it as a very attractive therapeutic target [4,5].

Cytokines, hormones, or growth factors bind to the cell membrane receptors leading to the activation of the receptors which then recruit the STAT3 protein [6], and thus cause the phosphorylation of STAT3 at tyrosine 705 residue (Tyr705). Two phosphorylated STAT3 proteins form homo-dimeric activated transcription factor complex through reciprocal binding of pTyr-SH2 domains. The p-STAT3 dimers translocate to the nucleus, where they bind to DNA and induce target genes expressions, such as Bcl-2, Cyclin D1 [4,7]. Apart from STAT3 phosphorylation at Tyr705, STAT3 is also acetylated at lysine 685 (Lys685). Converting Lys685 to arginine has been revealed to disrupt STAT3 dimerization, consequently abrogate STAT3 DNA-binding and transcriptional activation of oncogenes responding to cytokine stimulation [8]. Accordingly, acetylation of STAT3 is critical for stabling dimers, heightening transcription of cell growth-related genes, and promoting cell cycle progression [9,10]. Due to weak activity, significant toxicity, or poor bioavailability, none of STAT3 inhibitors reached the market yet. Therefore, discovery of novel potent and druggable STAT3 inhibitors is still highly valuable. Currently most of the known STAT3 inhibitors focus on the disruption of STAT3 phosphorylation and dimerization. One exception is resveratrol (RES) which targets on the acetylation of STAT3 and greatly diminishes the STAT3 activation [11,12]. Herein, we raised a hypothesis that inhibitor targeting both acetylated and phosphorylated STAT3 might significantly enhance its activity for chemoprevention and cancer therapy.

Natural products extracted from the food resources on account of their low toxicity and extensive application are excellent candidates that can be employed in designing anticancer drugs. Among them, RES, a naturally occurring phytoalexin abundant in red

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grapes and grape products, is one of the most famous natural product with anticancer, anti-oxidation and anti-inflammatory activities [11,13,14], which significantly inhibits STAT3 acetylation. Besides, RES inhibits Src tyrosine kinase and thereby blocks constitutive STAT3 activation in malignant cells [15]. Another dietary agent is caffeic acid (CaA), which is a phenolic compound primarily found in food plants [16]. It suppresses STAT3 phosphorylation to retard tumor growth [7,17]. The antitumor activity of a good deal of natural and synthetic compounds is also increased when the caffeic acid residues are introduced in the form of amides or esters [18]. Low toxicity makes these natural products extensively employed in cancer therapy.

Thus, two series of resveratrol-caffeic acid hybrids (Fig. 1) were designed, synthesized and biologically evaluated. The hybrids were validated to block both the acetylation and phosphorylation of STAT3, to inhibit malignancy proliferation and to induce the apoptosis of cancer cells. Furthermore, the IC_{50} value of compound **7d** was approximately 50-fold more active than RES. And it orally significantly suppressed breast cancer cells 4T1 xenograft tumor growth *in vivo*. In order to identify the possible binding mode, molecular docking studies were consequently performed. The results demonstrated the feasibility and efficiency of the strategy inhibiting two sites of the single STAT3 target for cancer therapy.

2. Results and discussion

2.1. Chemistry

The synthetic pathways of target compounds were outlined in Schemes 1–3. Firstly, the phosphonic acid precursor **(2)** was prepared from the commercially available starting material 4-Nitrobenzyl bromide **(1)** by the Arbuzov reaction [19], which was further reacted with 3,5-dimethoxybenzaldehyde **(3)** to offer compound **4** by the Horner-Emmons-Wadsworth olefination as described by Lion et al. [20]. The nitro group of compound **4** was reduced to the amine group by tin (II) chloride dihydrate under nitrogen and was converted to the respective (*E*)-4-(3,5-dimethoxystyryl) aniline **(5)** [21]. Finally, caffeic acid derivatives **6a-6o** were reacted with intermediate **5** to give the target compounds **7a-7o** in good yields [22]. Except for **6p**, which was substituted with two hydroxyl groups, **6p** was first protected with the acetyl groups and then deprotected. Finally, the target compound **10p** was synthesized as shown in Scheme 2 [23–25]. For the structure-activity relationship (SAR) study, compound **12a-12i** possessing ester linkage were also synthesized (Scheme 3).

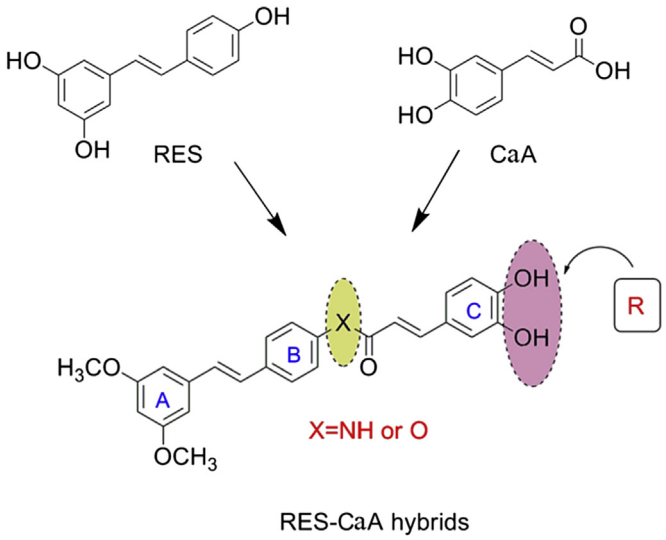

Fig. 1. Structures of resveratrol, caffeic acid and resveratrol-caffeic acid hybrids.

2.2. In vitro cell growth inhibitory activity

To explore the SAR, we evaluated the *in vitro* antitumor activity of the designed derivatives against human colonic carcinoma HT29 and human breast cancer MDA-MB-231 cell lines with RES and CaA as the positive controls using MTT assay. The IC_{50} results of **7a-7o, 10p** and **12a-12i** were illustrated in Table 1. Most compounds had lower IC_{50} values than the positive controls in the two cell lines. Compounds **7c** ($IC_{50} = 0.96$ μM for HT29) and **7d** ($IC_{50} = 2.14$ μM for MDA-MB-231) had the most potent antitumor activity among all. In comparison to the positive control RES ($IC_{50} > 100$ μM for HT29 and MDA-MB-231), both compounds were over 50-fold more active. In addition, HT29 cell line displayed higher sensitivity than MDA-MB-231 cell line for most compounds.

In general, the compounds which consist of amide linker (**7a-7o**) showed superior antitumor activities to compounds **12a-12i** possessing ester linkage. In addition, stronger electron-withdrawing substitutes (except trifluoromethyl group) had lower antitumor activities than electron-donating groups. Compounds with the methoxy group (such as **7c, 7d, 7e** and **12d**) or trifluoromethyl group (**7f, 12f**) exhibited strong anticancer activity. Moreover, the antitumor activity was significantly improved with the increased number of methoxyl group (**7b, 7c, 7d**). However, halogen substituents (**7i, 7m, 7n** and **12i**) resulted in a remarkable decrease of antitumor activity.

2.3. Compound 7d inhibited the STAT3 acetylation at lysine 685 and affected its specific genes expressions

To verify that STAT3 was the target of the designed compounds, western blot assays were performed to detect the abundances of acetylated and phosphorylated STAT3 (Ac-STAT3, p-STAT3). Because MDA-MB-231 cell line exhibits higher levels of acetylated STAT3, phosphorylated STAT3 and total STAT3 expressions than that of HT29 cell line (Fig. 2A), MDA-MB-231 cell line was selected to study the antitumor mechanism of compound **7d**. Exposing MDA-MB-231 cells to compound **7d** (1–5 μM) for 48 h led to dose-dependent inhibition of STAT3 acetylation at Lys685 residue, but had no effect on total STAT3 (Fig. 2B).

Mutating Lys685 of STAT3 is known to inhibit STAT3 acetylation. The mutation also up-regulates the expressions of several key tumor-suppressor genes in human cancers, including p53, PTPN6 (SHP-1), CDKN2A and DLEC1 [8,26,27]. Quantitative real-time RT-PCR was applied to assess the effect of compound **7d** on the mRNA expressions of the acetylated STAT3 downstream genes. We evaluated the mRNA levels of PTPN6 (SHP-1), CDKN2A and DLEC1 in MDA-MB-231 cells treated with compound **7d** for 48 h. Results indicated that compound **7d** increased the expressions of these tumor-suppressor genes which were related to STAT3 acetylation at Lys685 (Fig. 2C), thereby further demonstrated that **7d** inhibited STAT3 acetylation.

2.4. Compound 7d inhibited STAT3 tyrosine phosphorylation

The effect of compound **7d** (1–5 μM) on STAT3 phosphorylation at tyrosine 705 residue was also assessed in MDA-MB-231 cancer cells for 48 h. As shown in Fig. 2D, compound **7d** markedly decreased the phospho-Tyr705 STAT3 in a dose-dependent manner without affected the total STAT3, indicating that compound **7d** selectively decreased the level of p-STAT3 which was not due to a constitutional decrease of total STAT3 expression.