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Research paper

Phosphoramidate protides of five flavones and their antiproliferative activity against HepG2 and L-O2 cell lines

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ABSTRACT

A series of flavone-7-phosphoramidate derivatives were synthesized and tested for their antiproliferative activity in vitro against human hepatoma cell line HepG2 and human normal hepatic cell line L-O2. Compound **8d**, **16d** and **17d**, incorporating the amino acid alanine, exhibited high inhibitory activity on HepG2 cell line with IC_{50} values of 9.0 μmol/L, 5.5 μmol/L and 6.6 μmol/L. The introduction of acyl groups played a pivotal role in the selective inhibition toward human hepatoma HepG2 cells, except for compound **8a**, **9a** and **16b**. Compound **8d**, **16d** and **17d** could significantly induce G2/M arrest in HepG2 cells. Specially, Compound **16d** could lead early apoptosis in HepG2 cells.

1. Introduction

The study of the flavonoids dated back to the 17th century and since then, various discoveries have been made regarding their biological functions [1]. Chrysin, apigenin, luteolin, daidzein and genistein (Fig. 1), polyphenolic compounds available in foods and traditional Chinese medicines of plant origin, belong to the flavone subclass of flavonoids usually occurring as glycosylated forms. These five flavones demonstrate versatile biological activities, such as antibacterial activities [2], antioxidant [3], anti-inflammatory [4], antiviral [5], and antitumor [6–11]. Some reports have been dedicated to the improvement of the anticancer activities and the structure-activity relationships of these flavones' derivatives [12,13].

A strategy known as 'phosphoramidate protide' was introduced by McGuiganet al. as a means of improving the therapeutic potential of a prototype drug for HIV-1 [14,15]. Traditionally, phosphoramidate groups are covalently linked to nucleosides in 'phosphoramidate protide'. Phosphoramidate chemistry has also been applied to nucleoside analogues to generate a new class of anti-cancer agents, which overcome diverse resistance mechanisms [16]. The potency of the compounds varies with the individual components (aryl, ester, and amino acid) of the phosphoramidate moiety. Our interest is focused on the application of phosphoramidate chemistry to the five flavones and the change in the antiproliferation in vitro. In this regard, herein we report the synthesis of a series of amino acid-based flavone phosphoramidates. Subsequently, structurally related flavone phosphoramidates were evaluated in vitro against human hepatoma cell line HepG2 and human normal hepatic cell line L-O2.

2. Results and discussion

2.1. Chemistry

To expand the structural diversity of title compounds, we prepared a series of flavone-7-phosphoramidate derivatives by altering the amino acid and flavone moieties. The structures of target compounds were confirmed on the basis of NMR and mass spectrum. The target compounds were prepared using phosphorochloridate chemistry [17,18]. The key reagent to prepare flavone derivatives is the phenyl aminoacyl phosphorochloridate **2a** ~ **2e** (Scheme 1). It was prepared using two reagents: an amino acid ester and a phenyloxy phosphorodichloridate. The first reagent, an amino acid ester, can be prepared by esterification of the appropriate amino acid via standard esterification methods [19]. Phenyl

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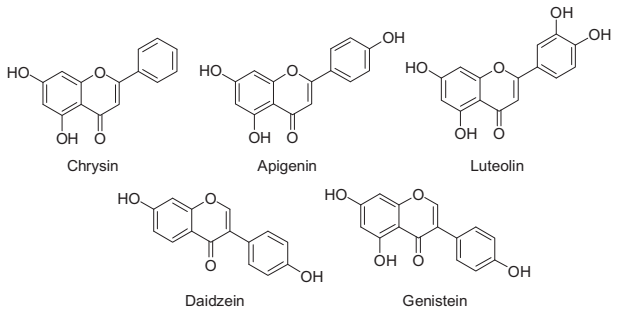
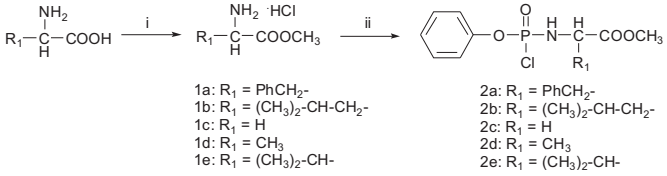


Fig. 1. Structures of five flavones.



Scheme 1. Synthesis of phenyl aminoacyl phosphorochloridates (**2a**–**e**). Reagents and conditions: (i) $SOCl_2$, CH_3OH, −10 °C; (ii) phenyl phosphorodichloridate, TEA, anhydrous CH_2Cl_2, −70 °C.

phosphorodichloridate is commercially available. The phenyl aminoacyl phosphorochloridates were formed by reaction of phenyl phosphorodichloridates with appropriate amino acid esters in the presence of triethylamine [17]. As noted in Table 1, we varied the amino acid from phenylalanine to leucine, glycine, L-alanine and valine. Because of their limited stability, the crude product of compounds **2a** ~ **2e** was directly used as materials in the ProTide syntheses.

Flavone-7-phosphoramidate derivatives were synthesized from phenyl aminoacyl phosphorochloridate and different kinds of flavone. The preparation of flavone-7-phosphoramidate derivatives is illustrated in Scheme 2. The synthetic route for isoflavone-7-phosphoramidate derivatives is outlined in Scheme 3. In order to favor the 7-regioselectivity of the phosphorylation with the phenyl aminoacyl phosphorochloridates, apigenin, luteolin, daidzein and genistein were acetylated with Ac_2O/pyridine and then selectively deprotected on 7-OH group with PhSH/imidazole [20]. Selectively deacylation on 7-OH group was optimized in mixture solvents of N-methyl pyrrolidone/THF (1:3) with short reaction time and good yield (74.8%–85.0%).

Each aminoacyl phosphorochloridate (**2a** ~ **2e**) was reacted with the 7-OH group of compounds chrysin, **6**, **7**, **14** and **15**. The phosphorochloridates **2a** ~ **2e** were allowed to react with chrysin or compound **7** in the presence of TEA to generate the desired compounds **3a** ~ **3e** and **9a** ~ **9e** in THF. But to compounds **6**, **14** and **15**, the reaction lasted more than 4 days when catalyzed by TEA in THF. When the reaction system of K_2CO_3/acetone was used, the phosphoramidate derivatives (**8a** ~ **8e**) syntheses were completed within six hours [21]. The reaction between phosphorochloridates **2a** ~ **2e** and isoflavone derivatives **14** or **15** was very slow using K_2CO_3/acetone. So microwave reaction was used to shorten the reaction time. The synthetic conditions of the acetoxyflavones-7-phosphoramidate derivatives were summarized (Table S1). Finally, the deacylation conditions were screened [22–26] and pyrrolidine was used to deacetylate [27] (Table S2). While deacylating compounds **10a** ~ **10e** and **11a** ~ **11e** using pyrrolidine, the product became complex and difficult to be purified. Therefore, daidzein and genistein were tried to react with phosphorochloridates **2a** ~ **2e** under microwave-assistant condition and turned to be successful (Scheme 3).

2.2. Biological studies

Among the screened compounds, compound **8d**, **16d** and **17d** from acetoxyflavone-7-phosphoramidate series were found to be active in cell based cytotoxicity screening at less than 10 μM concentration. These three compounds were selected for detailed mechanistic investigations.

2.2.1. Compounds inhibit cell proliferation in HepG2 and L-O2 cell lines

Cytotoxicity effects of compounds in HepG2 (hepatocellular carcinoma) cell lines and L-O2 (human liver) cell lines were analyzed by using MTT assay. Most of the compounds inhibited cellular proliferation in HepG2 and L-O2 cell lines. The IC_{50} values (μM) for 48 h incubation were reported (Table 1). Among the 39 tested derivatives, 28 flavone-7-phosphoramidates showed inhibition of HepG2 cells with IC_{50} values less than 100 μmol/L. The results revealed that most of the synthetic compounds exhibited moderate inhibition of cell proliferation. Compound **8d** (IC_{50} 9.0 ± 1.7 μM)、**16d** (IC_{50} 5.5 ± 1.3 μM) and **17d** (IC_{50} 6.6 ± 1.3 μM) displayed higher potency. Interestingly, these three compounds were all L-alanine phosphoramidates. This is consistent with the most potent gemcitabine Protides [16]. In addition, the acetyl group plays an important role to increase the cytotoxicity toward tumor cells HepG2 (Table 1), except for compounds **8a**, **9a** and **16b**. Especially, acetylation for isoflavone-7-phosphoramidates improved the selectivity between HepG2 and L-O2 more than six fold.

2.2.2. Compound *8d*, *16d* and *17d* induces G2/M phase cell cycle arrest in HepG2 cell line

Apigenin, daidzein and genistein were previously shown to induce G2/M cell cycle arrest in human oesophageal adenocarcinoma cells [7], breast cancer cells [28–30], colon cancer cells [31,32], T24 human bladder cancer cells [33], and human malignant glioma cells [34]. In view of the above-mentioned effects on cell growth, it was needed to examine whether the flavone-7-phosphoramidate derivatives **8d**, **16d** and **17d** were able to induce G2/M phase arrest in HepG2. Treatment of HepG2 cell line with compound **8d**, **16d** and **17d** at IC_{50} concentration induced cell cycle arrest in G2/M phase (Fig. 2). Control cells showed 16.4% cells in G2/M phase which was increased to 72.6%, 98.1% and 96.6% by the treatment with compound **8d**, **16d** and **17d**, respectively. Apigenin could increase T24 human bladder cancer cells in G2/M phase to 37.94% at the concentration of 160 μM and incubated for 24 h, in contrast with control (14.45%) [33]. Daidzein could cause cell cycle arrest at G2/M phase (21.8%–38.9%, 22.7% for control) in human breast cancer MCF-7 at various concentration of 1–100 μM and incubated for 72 h [30]. Genistein could also induce cell cycle arrest at G2/M phase (58%, 34% for control) in human malignant glioma LNT-229 cell line at the concentration of 100 μM and incubated for 24 h [34]. Compared with the literature, daidzein derivative **16d** and genistein derivative **17d** almost completely arrested the cell cycle at G2/M phase. We could infer confidently that the action of apigenin, daidzein and genistein on cell cycle was increased by the introduction of phosphoramidate groups dramatically.

2.2.3. Compound 16d leads early apoptosis in HepG2 cell line

The effects on cell growth and cell cycle progression of the flavone-7-phosphoramidate derivatives **8d**, **16d** and **17d** were better than that of flavones. So the apoptosis was further measured by double staining of cells with Annexin-V/PI and quantified by