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# Synthesis and biological evaluation of 7-*O*-modified oroxylin A derivatives

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ABSTRACT

Oroxylin A (5,7-dihydroxy-6-methoxyflavone) is a naturally occurring monoflavonoid isolated from the root of *Scutellaria baicalensis Georgi*, and exhibits potent anticancer activities in vitro and in vivo. In this study, we synthesized three series of oroxylin derivatives by connecting a nitrogen-containing hydrophilic, heterocyclic ring to the C7-OH via a varying length of carbon chain. All the derivatives were screened for anti-proliferative activities against three tumor cell lines. Some of the derivatives displayed higher activities compared to oroxylin A. The most potent antitumor compound, **5f**, also induced apoptosis in HepG2 cell. The difference of **5f** between the inhibiting rates of cell proliferation and the apoptotic rates indicated that **5f** was more likely to be a necrosis-inducing agent or both apoptosis/necrosis inducer.



Flavanoids belong to a big class of polyphenolic secondary metabolites in plants and in various common foods such as apple, onion, tea, and red wine. In addition to their biological roles in nitrogen fixation and chemical defense, flavanoids possess a broad range of pharmacological properties, including anti-atherosclerotic, anti-inflammatory, anticancer, anti-thrombogenic, antiviral and anti-osteoporotic effects,[1–6] and hence demonstrate considerable therapeutic potentials. However, many flavanoids have poor oral bioavailability and limited in vivo efficacy.[7]

Oroxylin A (**1**), 5,7-dihydroxy-6-methoxyflavone, is an O-methylated flavones found in the roots of traditional Chinese medicine *Scutellaria baicalensis*.[8] Recently, preliminary studies by our groups revealed the antitumor mechanisms of oroxylin A. Hu et al demonstrated that oroxylin A effectively induced programmed cell death and suggested that it could be a promising antitumor drug.[9] Yang et al reported that oroxylin A induced G2/M phase cell-cycle arrest via inhibiting Cdk7-mediated expression of Cdc2/p34 in human gastric carcinoma BGC-823 cells.[10] Sun et al showed that oroxylin A suppressed MDA-MB-435 human breast cancer cell invasion through down-regulating the expression of matrix metalloproteinase-2/9.[11] It was recently reported that oroxylin A had an antitumor effect on human cervical cancer HeLa cell line in vitro and in vivo.[12] All these reports suggest that oroxylin A has potent anticancer activities in vitro and in vivo.

Although various *aforementioned* in vitro studies have demonstrated therapeutic potentials of oroxylin A, it has very low oral bioavailability due to its extensive first-pass metabolism, mainly glucuronidation in gut.[13] Glucuronidation of oroxylin A would most likely happen at the 7-OH position due to the intra-molecular hydrogen bond on the 5-OH position. In order to block the 7-OH site from glucuronidation, and hence improve the oral bioavailability of oroxylin A, a nitrogen-containing hydrophilic, heterocyclic ring was introduced to the 7-OH via a certain length of carbon chain, and the antitumor activities of the derivatives were evaluated. The length of carbon chain and the amino substitution at the end of the chain was explored to investigate their impact on the biological activities.

Alkylation of **1**[14] was carried out by reaction with di-halogenated hydrocarbon in the presence of $K_2CO_3$ to afford intermediates **2**. Then primary or secondary amines were coupled with the halogenated hydrocarbons to afford the final products **3–5** (Scheme 1). Compounds in series I (**3d–3h**) contained a two-carbon alkyl chain between oroxylin A skeleton and heterocyclic moieties; compounds in series II (**4d–4h**) had a 3-carbon spacer; and compounds in series III (**5d–5h**) contained a 4-carbon spacer between oroxylin A and the substituent.[15]

The anti-proliferative activities of all the novel 7-*O*-alkylamino derivatives of oroxylin A were evaluated by MTT assay against three human tumor cell lines (HepG2, HCT-116 and BCG-823) with oroxylin A as a comparative control. All the treatments for MTT assay lasted for 48 h.

As shown in Table 1, the majority of the compounds displayed comparable or higher (lower $IC_{50}$ values) anti-proliferative activities than oroxylin A. Our results confirmed that the introduction of a side chains at 7-OH does reduce their activities against the three cancer cell lines.

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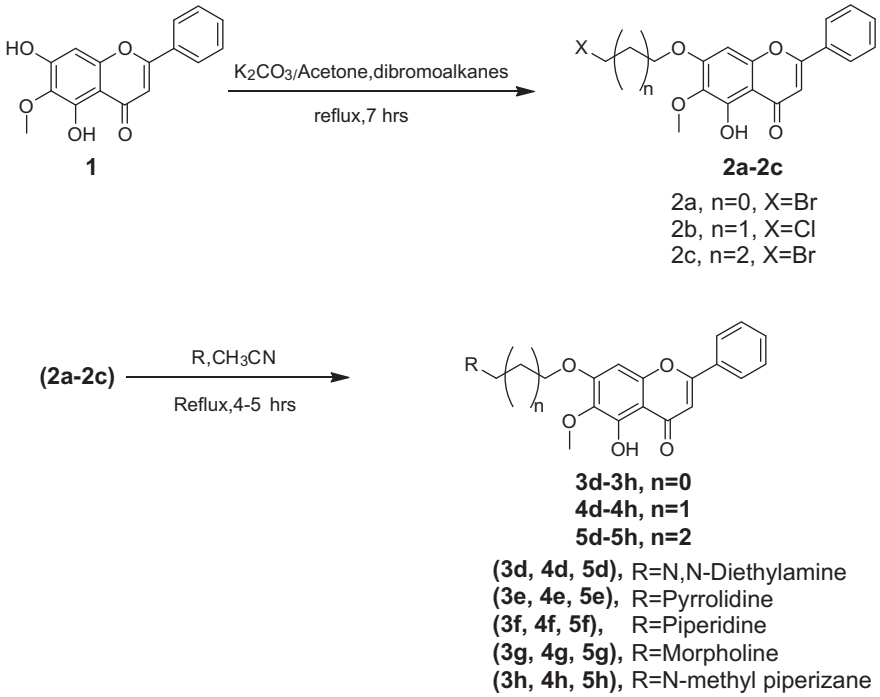


**Scheme 1.** Synthesis of 7-*O*-alkylamino derivatives of oroxylin A.

**Table 1**
Antitumor cell proliferation activities of 7-*O*-alkylamino derivatives of oroxylin A by MTT assay

| Compound | $IC_{50}$ value[a] (μM) | | |
|---|---|---|---|
| | HepG2 | HCT116 | BCG-823 |
| Oroxylin A | 22.7 | 33.9 | 25.5 |
| **3d** | 12.0 | 39.6 | 13.0 |
| **3e** | 9.7 | 23.4 | 22.7 |
| **3f** | 17.1 | 21.1 | 26.9 |
| **3g** | 67.6 | 56.3 | 38.2 |
| **3h** | 15.4 | 54.9 | 20.5 |
| **4d** | 18.0 | 23.4 | 3.5 |
| **4e** | 20.5 | 26.6 | 12.0 |
| **4f** | 6.8 | 22.1 | 9.7 |
| **4g** | 74.8 | 52.4 | 41.6 |
| **4h** | 30.4 | 42.5 | 42.3 |
| **5d** | 6.6 | 5.1 | 6.3 |
| **5e** | 4.7 | 1.4 | 9.5 |
| **5f** | 2.4 | 3.0 | 6.6 |
| **5g** | 50.6 | 32.4 | 26.8 |
| **5h** | 27.2 | 12.5 | 35.8 |

[a] The data shown were the mean from three parallel experiments ($n = 3$).

Compounds (**5d–5f**) containing a 4-carbon spacer were particularly potent with respect to cellular inhibition of all the three cell lines, with $IC_{50}$ value ranging from 1.42 to 9.52 μM, approximately 5- to 20-fold more potent than oroxylin A, except for **5g** and **5h**. Compound **5e** and **5f** were found to be the most potent, with $IC_{50}$ of 1.42 μM (HCT116) and 2.98 μM (HepG2), respectively. The results indicate that the length of chain between the terminal heterocyclic substitutes and oroxylin A is an important factor for their potency. However, with a morpholinyl or *N*-methyl piperazinyl group at the terminal of 7-*O*-alkyl oroxylin A, compound **5g** and **5h** (with $IC_{50}$ at 12.5–50.6 μM) displayed moderate levels of inhibition, which was similar to that of oroxylin A but over 4–25-fold (HepG2), 2–30-fold (HCT116), 3–6-fold (BCG823) less potent than **5d–5f**. The morpholinyl or *N*-methyl piperazinyl substitution in the compounds **3g–h** (with $IC_{50}$ at 15.4–67.6 μM) and **4g–h** (with $IC_{50}$ at 30.4–74.8 μM) also exhibited lower activities than substitutions with *N*,*N*-diethylamino (**3d** and **4d**), pyrrolyl (**3e** and **4e**) or a piperidyl (**3f** and **4f**) group, respectively, regardless to the length of carbon chain. It may be attributed to the volume of the side chain.

Based on our previous research, the antitumor effect of oroxylin A might be through apoptosis. In order to further explore the apoptosis induced by 7-*O*-alkylamino derivative of oroxylin A, Annexin V/PI staining assays were performed with compound **5f**, which was demonstrated the highest in vitro efficacy against HepG2 cells among the derivatives.

Apoptosis was determined by staining with Annexin V/PI labeling, as Annexin V can identify the externalization of phosphatidylserine during the process of apoptosis.[16] As shown in Figure 1, following the treatment with 1, 2 and 4 μM of compound **5f** for 72 h in HepG2 cells, the percentage of early apoptotic cells (right

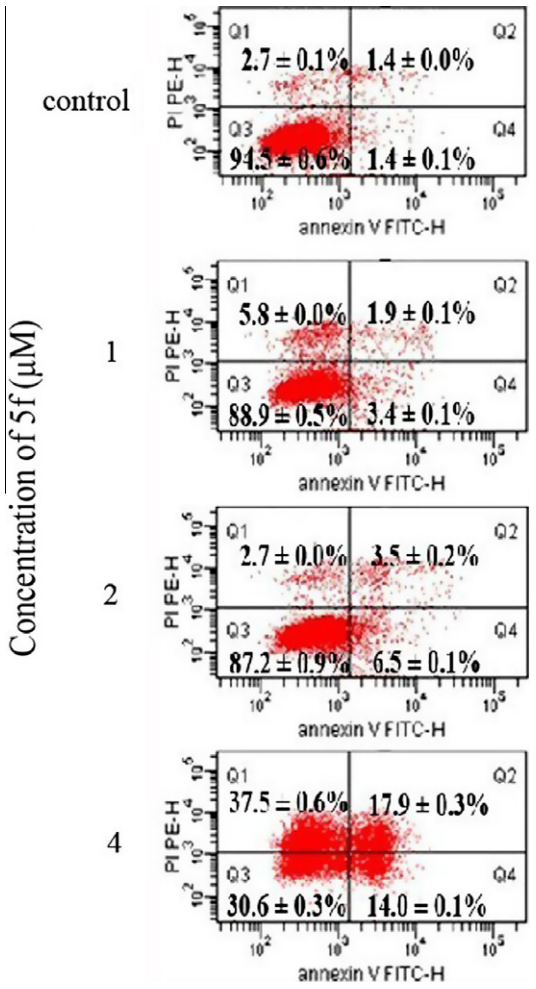


**Figure 1.** Compound **5f**-induced apoptosis in HepG2 cells. The cells were treated with **5f** for 72 h, and apoptosis was determined by Annexin V/PI double-staining assays.

low section of fluorocytogram) increased (from 1.4% to 14.0%), and the percentage of late apoptotic and necrotic cells (right upper section of fluorocytogram) were also increased (from 1.4% to 17.9%).

It is well-known that apoptosis can be characterized by morphological and biochemical changes in the cell nucleus, such as chromatin condensation and nuclear shrinking. In our experiments, the changes in nuclei in HepG2 cells by **5f** were observed under a fluorescent microscope. As shown in Figure 2, the control cells emitted blue fluorescence with consistent intensity, indicating that the chromatin was equivalently distributed in the nuclei. Following incubation with compound **5f** for 72 h, the HepG2 cells displayed chromatin congregated and karyopyknosis, which emitting bright fluorescence.

These above results demonstrate that compound **5f** is capable of inducing apoptosis in HepG2 cells. However, the value of MTT assay (represents cell viability) is much more efficient than that of Annexin V (represents cell apoptosis). The percentage of necrosis cells induced by **5f** is far away larger than that of apoptotic cells. Thus, we hypothesize that **5f** is more likely to be a necrosis-inducing agent or both apoptosis/necrosis inducer, which need for further studies in the future.

In conclusion, we have synthesized the novel 7-*O*-alkylamino derivatives of oroxylin A and assessed their biological activities in cellular proliferation assay. Among the three structural series of derivatives, the most active compounds (**5d–5f**) contained a 4-carbon spacer with appropriate amino substitutions such as *N*,*N*-diethylamino, pyrrolyl or piperidyl groups. A 4-carbon alkyl chain yields higher in vitro antitumor activities than the 2- and 3-carbon chains. Our results showed that compound **5f** exhibited strong antitumor effect in HepG2 cell line, involving with apoptosis induction. The difference of **5f** between the inhibition of cell proliferation and the apoptotic induction indicated that **5f** was more likely to be a necrosis-inducing agent or both apoptosis/necrosis inducer. Thus, the novel 7-*O*-alkylamino derivative of oroxylin A, compound **5f**, could be a promising antitumor candidate, and further in vitro and in vivo biological characters evaluations are warranted.

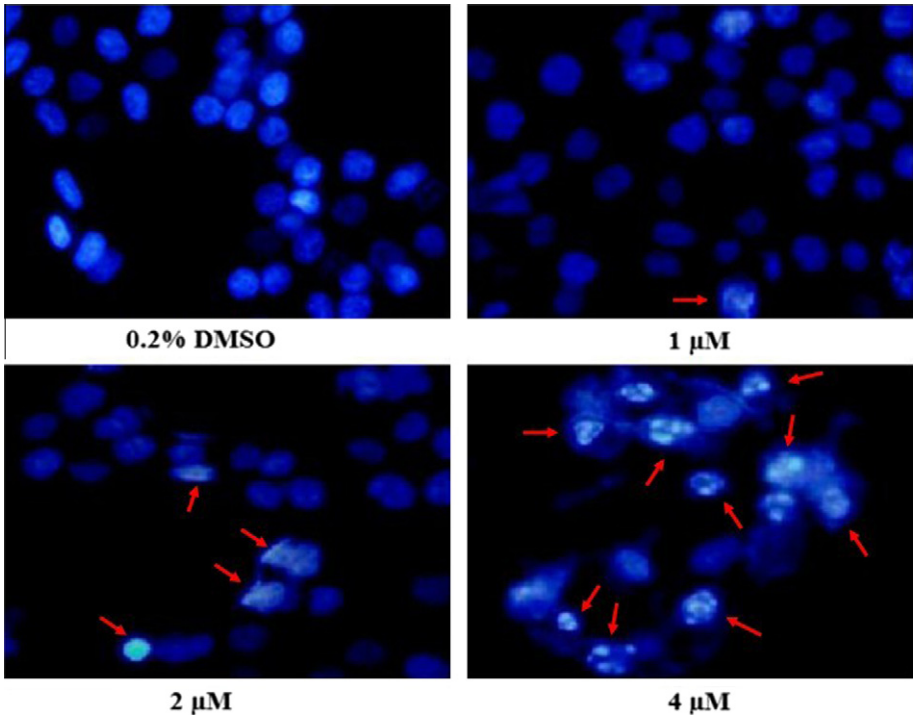


**Figure 2.** Fluorescent micrographs of DAPI staining (400×). The arrows indicate the apoptotic cells.

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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.11.117.

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15. *General procedures for the preparation of 7-O-alkylamino derivatives of oroxylin A*: (i) Procedures for the preparation of 7-*O*-halogenalkyl derivatives of oroxylin A (**2a**–**2b**): To a mixture of oroxylin A (**1**, 1.00 g, 3.52 mmol) and anhydrous potassium carbonate (1.46 g, 10.6 mmol) in 20 ml acetone, corresponding di-halogenated hydrocarbon (1,2-dibromo ethane for **2a**, 1-bromo-3-chloropropane for **2b**, 1,4-dibromo butane for **2c**) was added. The mixture was refluxed under a nitrogen atmosphere for 7–8 h. After completion of the reaction, potassium carbonate was filtered and washed with excess acetone ($2 \times 50$ ml). The combined acetone layer was concentrated under vacuum. The residue was purified by column chromatography on silica gel (60–120 mesh) to yield 7-*O*-alkyl oroxylin A (**2a**, **2b** and **2c**) in pure form. (ii) Procedures for the preparation of 7-*O*-alkylamino derivatives of oroxylin A: To a solution of halogenalkyl oroxylin A (**2a**, **2b** and **2c**) in 20 ml acetonitrile, the corresponding amine was added. The mixture was refluxed under a nitrogen atmosphere for 3–4 h. The reaction mixture was then concentrated under vacuum. The residue was purified by column chromatography on silica gel (60–120 mesh) to give the corresponding 7-*O*-alkylamino derivatives of oroxylin A (**3d**–**3h**, **4d**–**4h** and **5d**–**5h**) in yields of 60–85%.
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