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Synthesis and evaluation of novel alkannin and shikonin oxime derivatives as potent antitumor agents

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ABSTRACT

A set of forty alkannin and shikonin oxime derivatives were firstly designed and synthesized. Their cytotoxicities against three kinds of tumor cells and a normal cell line were tested and compared with alkannin and shikonin. The cell-based investigation demonstrated that some oxime derivatives were more or comparatively effective to the lead compounds, especially their selective and excellent antitumor activities towards K562 cells with no toxicity in normal cells. We may conclude that oximate modification to the mother nucleus of alkannin and shikonin is an available approach to acquire potent antitumor agents.

Nearly half of the clinical anticancer drugs are either natural products or directly derived from naturally occurring lead compounds, such as paclitaxel, topotecan, irinotecan, vinblastine and colchicines.[1,2] Alkannin (*S*-isomer) and shikonin (*R*-isomer), extracted and identified, respectively, from the roots of *Alkanna tinctoria* in Europe and *Lithospermum erythrorhizon* in the Orient as a pair of enantiomers, have attracted great interest as hallmark molecules because of their fascinating biological activities.[2–6] Hundreds of alkannin and shikonin derivatives have been isolated, synthesized and evaluated as to their tumor inhibitory potency.[2–18] So far, most modifications have focused on the hydroxyl group in the side chain and some of these modified compounds have shown comparable or stronger cytotoxicities than their parent natural products in vitro,[4–13,16] such as β-hydroxyisovalerylshikonin, isovalylshikonin, acetyl-shikonin and so on. However, none of them enter into clinical trials because of their serious toxic effects, even though great tumor inhibitory effects were observed for many shikonin derivatives in cell culture studies.

The key pharmacophores of both alkannin and shikonin were found to be the naphthazarin ring and hydroxyl side chain. It has been demonstrated that the naphthazarin ring has a strong ability to generate reactive oxygen species (ROS) via the redox cycling and bio-reductive and Michael addition alkylation processes,[19–23] which induce the apoptotic death of many cancer cell lines indiscriminately cause damages to a wide variety of biological macromolecules, such as nucleic acids and proteins, not only in tumor cells but also in normal cells. In order to develop less toxic alkannin and shikonin derivatives, modifications of structures on naphthazarin ring besides the hydroxyl group of the side chain seem reasonable and practical. Part of the existing data displays that dimethylation of the naphthazarin ring for some compounds containing naphthazarin moiety will improve the antitumor activity.[14–18] However, adverse effects such as weight reduction, hypotrichosis and much bloody ascites were further observed in in vivo experiments. It is important to point out that *O*-dimethyl alkannin and shikonin derivatives have comparable level of ROS and alkylation to naphthoquinone compounds, and this should also be responsible for the unselective profile of cell damage. Therefore, only the modifications on the alcoholic hydroxyl group of the side chain and phenolic hydroxyl groups are insufficient to overcome drawbacks in the structure of alkannin and shikonin itself.

In this study, the modification of alkannin and shikonin was focused on the naphthoquinone moiety basing on the structures of *O*-dimethyl alkannin and shikonin derivatives. Many oxime compounds have recently exhibited satisfactory anticancer effects recently.[24–28] This research project presents a series of novel oxime alkannin and shikonin derivatives, both their synthesis and antitumor activities against different cancer cell lines in vitro.

A general synthetic route for *O*-dimethyl acylalkannin and acylshikonin oxime derivatives was illustrated in Scheme 1. (*R*)- or (*S*)-4-methyl-1-(1,4,5,8-tetramethoxynaphthalen-2-yl)pent-3-en-1-ol (**2**) was prepared in high optical purity (>99% ee) using 1,4,5,8-tetramethoxy-2-naphthaldehyde (**1**) as the starting material according to the procedures previously reported by our group.[29–31] Condensation of **2** with different kinds of carboxylic acid in the presence of dicyclohexylcarbodiimide (DCC), 4-dimethylaminopyridine (DMAP)

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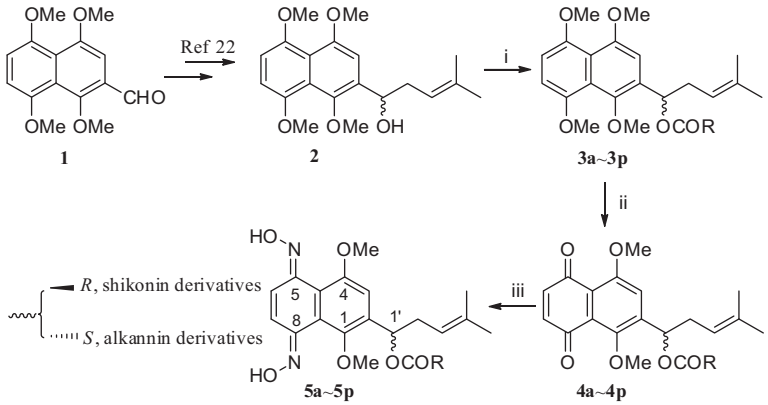


Scheme 1. Synthesis of the oxime derivatives **5a**–**5p**. Reagents and conditions: (i) RCO_2H/DCC/DMAP/CH_2Cl_2, rt, 1–12 h; (ii) CAN/H_2O/CH_2Cl_2, 0 °C, 15 min; (iii) $HONH_3Cl$/Py/EtOH, 50 °C, 8 h.

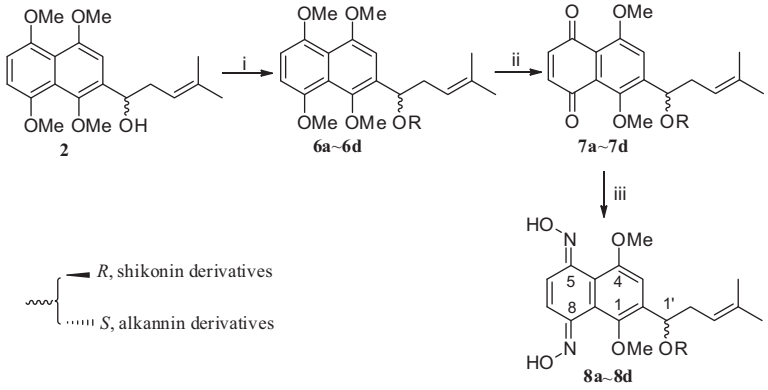
resulted in acyl derivatives (**3a**–**3p**) and subsequent oxidation with cerium (IV) ammonium nitrate (CAN) gave corresponding *O*-dimethyl alkannin and shikonin compounds (**4a**–**4p**).[17] Stirring at room temperature of compounds **4a**–**4p** with hydroxylamine hydrochloride respectively in the presence of pyridine subsequently produced alkannin and shikonin oxime derivatives (**5a**–**5p**).[32,33]

An appropriately designed route for the synthesis of ether derivatives of *O*-dimethyl alkannin and shikonin oxime derivatives was shown in Scheme 2. The nucleophilic substitution reaction of compound **2** with organic halides produced ether derivatives (**6a**–**6d**), and subsequent oxidation of **6a**–**6d** with CAN yielded key intermediates *O*-dimethyl alkannin and shikonin.[17] Targeted compounds **8a**–**8d** were easily obtained by the condensation reaction (Scheme 2) between **7a**–**7d** and hydroxylamine hydrochloride in the presence of pyridine.[32,33]

The in vitro cytotoxicities of the prepared alkannin and shikonin derivatives against MCF-7 (breast cancer), K562 (leukemia), DU145 (prostate cancer) and HSF (human skin fibroblasts) cells were evaluated by the standard MTT assay using alkannin and shikonin as reference compounds. Antitumor potencies of the forty compounds are displayed as IC_{50} values that were calculated by linear regression analysis of the concentration–response curves afforded for each compound. The results are summarized in Table 1.

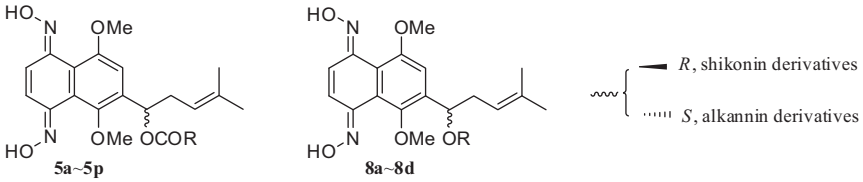
From the cytotoxic activity results of alkannin and shikonin derivatives shown in Table 1, we can see that most target oxime derivatives except shikonin compound **8a** displayed potent activity against three kinds of cancer cell lines. The potencies of some compounds were comparable to the lead compounds alkannin and shikonin, and some even better. Among these oxime derivatives, IC_{50} values of the most effective alkannin derivatives **5b** and **5e** against K562 were as low as 0.7 μM, which showed higher cytotoxicities than alkannin (IC_{50} = 1.3 μM). Meanwhile, the IC_{50} values of alkannin derivative **5e** against MCF-7 and DU145 were 7.5 and 19.3 μM, respectively. Almost all the IC_{50} values of other compounds in this category showed the same trend among tested cancer cell lines with the best inhibitory effect in K562, moderate in MCF-7, but little in DU145, reflecting excellent selectivity for a particular leukemia cell line. Moreover, it is exciting to note that none of the prepared compounds displayed cytotoxicity towards normal cell line HSF (IC_{50} >50 μM). Though great cytotoxicities on cancer cell lines (K562, MCF-7 and DU145) were observed for the lead compounds alkannin and shikonin, comparable inhibitory activities towards normal cell HSF and no selectivity was also observed between cancer and normal cells.

It can be observed from Table 1 that the side chain of oxime derivatives had a great effect on their cytotoxicities. Compounds with ester moieties in the side chain showed higher cytotoxicities than those with ether groups, especially for K562 cells. For example, alkannin derivative **5e** containing ester groups (IC_{50} = 0.7 μM) was approximately 20-fold more cytotoxic than relative ether compound **8d** (IC_{50} = 13.5 μM). For compounds **8a** with hydroxyl in the side chain, its cytotoxicity sharply decreased. It can be concluded that the cytotoxicities of resulting oxime derivatives are significantly correlated with the nature of the substituent group at 1′-position in the side chain. Within the series **5a**–**5p** which bear



Scheme 2. Synthesis of the oxime derivatives **8a**–**8d**. Reagents and conditions: (i) NaH/RBr/THF, 0–20 °C, 24 h; (ii) CAN/H_2O/CH_2Cl_2, 0 °C, 15 min; (iii) $HONH_3Cl$/Py/EtOH, rt, 12 h.

Table 1
In vitro inhibitory activity of alkannin and shikonin derivatives against DU145, MCF-7, K562 and HSF cell lines



Compd	Substituents	IC_{50} (μM)						
		DU145		MCF-7		K562		HSF
		R	S	R	S	R	S	R & S
5a	Methyl	25.1	17.1	20.9	8.1	9.3	5.3	>50
5b	3-Methylbut-2-enyl	11.3	18.1	6.0	1.6	1.4	0.7	>50
5c	3-Hydroxyisobutyl	28.9	32.8	26.0	8.2	9.5	4.7	>50
5d	Isopropyl	10.2	18.0	4.9	3.7	3.3	3.0	>50
5e	Isobutyl	27.7	19.3	16.7	7.5	2.1	0.7	>50
5f	Ethyl	12.3	20.6	7.1	3.1	5.4	3.7	>50
5g	Methylvinyl	18.0	19.5	4.9	2.4	3.1	2.6	>50
5h	Phenyl	19.4	16.9	4.5	2.8	1.9	1.6	>50
5i	2-Fluorophenyl	19.9	14.3	7.7	1.8	1.7	1.5	>50
5j	4-Fluorophenyl	18.4	17.0	3.6	1.5	1.5	1.3	>50
5k	2-Chlorophenyl	18.0	16.8	5.8	1.7	2.9	1.3	>50
5l	4-Chlorophenyl	16.8	21.4	6.6	2.4	2.1	1.3	>50
5m	4-Methoxyphenyl	16.3	16.9	6.3	1.9	2.5	2.1	>50
5n	4-Nitrophenyl	19.7	12.5	3.9	1.2	2.6	1.7	>50
5o	Pyridin-2-yl	12.1	20.7	7.6	6.3	3.8	3.6	>50
5p	Thiophen-2-yl	16.9	16.1	5.7	1.4	2.0	1.8	>50
8a	Hydrogen	>50	30.2	>50	21.9	42.7	13.8	>50
8b	Methyl	32.4	21.1	30.2	17.3	21.1	14.6	>50
8c	Ethyl	17.1	10.6	15.7	17.1	17.1	15.4	>50
8d	Isopentyl	26.3	30.8	16.2	17.6	12.4	13.5	>50
SK	Shikonin	16.0		1.8		0.7		1.2
AK	Alkannin	19.9		2.4		1.3		1.5

Data represent the mean values of three independent determinations.

ester groups in the side chain, the great majority of compounds displayed comparable activities to those of alkannin and shikonin. When the ester groups were linked with aliphatic hydrocarbon, their selectivities on three cancer cell lines and cytotoxicities were higher than those linked with aromatic hydrocarbon. Most alkannin oxime derivatives (*S*-isomer) showed generally better cytotoxic activities than corresponding shikonin derivatives (*R*-isomer), especially for MCF-7 and K562 cell lines, though there were some opposite outcomes in DU145 cell line. For synthesized ether derivatives (**8a**–**8d**), not only were their cytotoxicities on selective cancer cell lines not as good as ester compounds, but also no similar selectivities were observed as shown by the ester derivatives.

ROS, Michael addition and bioreductive alkylation were potential mechanisms leading to the cytotoxicity of shikonin compounds.[19,20,23] In this study, two representative compounds (*R*-**5b** and *S*-**5b**) and acetylshikonin (**SK-02**) (Fig. 1) were chosen to be tested and compared for their corresponding ROS and alkylation levels. A quantitative assay using single cells (MCF-7) analyzed by flow cytometry was adopted for testing ROS levels induced by these derivatives. DCFH-DA (2′,7′-dichloro-dihydrofluorescein diacetate; Sigma) was used as a ROS-capturing reagent in the method reported.[34] The data was summarized in Table 2. According to the data shown in Table 2, it is obvious that ROS generated by oxime derivatives significantly decreased. Compared to **SK-02**, which has the same side chain and was assumed as 100%, shikonin oxime derivative (*R*-**5b**) and alkannin oxime derivative (*S*-**5b**) decreased to 24.3% and 33.0%, respectively. Additionally, the alkylation level of oxime derivatives was tested and the results were summarized in Table 3. In this study, *p*-thiocresol, a kind of nucleophilic and reducing agent, was applied to react with the three compounds under the same conditions to determine their ability of alkylation. As shown in Table 3, for the two chosen oxime derivatives (*R*-**5b** and *S*-**5b**), both Michael addition and bioreductive alkylation pathways were blocked, and thus no alkylation product was obtained. Based on the ROS and alkylation data above, we conclude that the excellent cytotoxicities of these target oxime derivatives are not resulted from ROS and alkylation, but probably associated with other unknown mechanisms.

In summary, new series of oxime derivatives of alkannin and shikonin were synthesized and evaluated for their antitumor effects

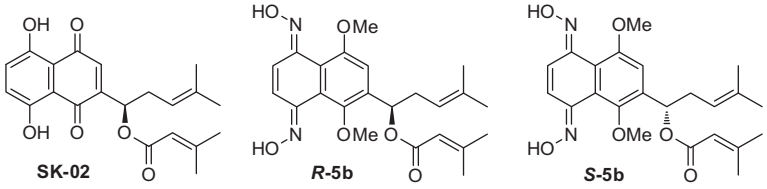


Figure 1. Structures of the selected three compounds for ROS and alkylation examination.

Table 2
Test of ROS level for the selected three compounds in MCF-7 cells by flow cytometer

Compd	Amount of ROS	Ratio to **SK-02** (%)	Ratio to control (%)
SK-02	134.8	100	414.8
R-**5b**	32.8	24.3	100.9
S-**5b**	44.6	33.0	137.2
Control	32.5	17.5	100

Table 3
Alkylation of the three compounds with *p*-thiocresol in 15 min

Compd	A	B	C (%)	Extent of reaction (%)
SK-02	—	—	100	100
R-**5b**	—	—	—	None
S-**5b**	—	—	—	None

A: Production of Michael addition.
B: Production of bioreductive alkylation.
C: Production of Michael addition and bioreductive alkylation.

against three cancer cell lines and against normal cells. Compared with the lead compounds alkannin and shikonin, all the oxime derivatives showed no cytotoxicity to the normal cells, while many of them exhibited comparable or stronger activity together with excellent selectivity towards K562 cells. The structure–activity relationships (SARs) results from these oxime derivatives showed that analogues with ester functional group at 1′-position of the side chain were associated with enhanced cytotoxic activity. Moreover, it was demonstrated that the excellent and selective antitumor activities of oxime derivatives were not ascribed to ROS and alkylation. Investigation into the potential antitumor mechanisms of this kind of compounds is ongoing in our lab.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.07.012.

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