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Synthesis and biological activity of novel shikonin analogues

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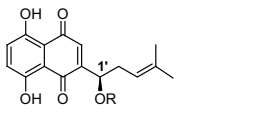
ABSTRACT

A series of shikonin analogues with side chain variants have been synthesized and evaluated for antitumor activity. These novel analogues show a broad spectrum of in vitro cytotoxicity against various cancer cell lines. Additionally, some analogues were also found to have the ability to decrease the expression level of HIF-1α in breast cancer cells MDA-MB-231 under hypoxia. The features of these analogues suggest their potential in cancer therapy.

Shikonin (**1a**, Fig. 1), isolated from the roots of the traditional oriental herb *Lithospermium erythrorhizon*, is a natural product that has a long history in treating burns, inflammations, wounds, and ulcers in Far East and Europe.[1] Over the past few decades, its potential as a drug candidate for cancer treatment has evoked a lot of research interest. A number of studies show that shikonin and its derivatives display significant cytotoxic activity against various cancer cells both in vitro and in vivo.[2,3] The mechanism of action of this class of compounds, however, has not been fully elucidated. A 'redox cycle' seems to be the primary cause of cytotoxicity in which a semiquinone radical is thought to play a central role towards cell damage. The bioreductive alkylation mechanism may be another plausible explanation for their cytotoxic behavior.[3] Moreover, several research groups confirmed that shikonin and its derivatives could induce DNA cleavage and cell apoptosis by strongly interacting with both topoisomerase I and II, which suggest that these DNA enzymes might be the molecular targets for the naphthoquinone compounds.[4–7]

Ahn and coworkers reported that acylated shikonin derivatives, for example, acetylshikonin (**1b**, Fig. 1) and isobutyrylshikonin (**1c**, Fig. 1), are more potent inhibitors of topoisomerases than the parent compound shikonin.[4] Recently, we demonstrated that 2-furoylshikonin (**1d**, Fig. 1), another derivative of shikonin, is a mixed topoisomerase I and topoisomerase II inhibitor, possesses potent inhibitory effects on tumor growth both in vitro and in vivo.[7] Based on our in vitro screening data on the shikonin derivatives, we found that those derivatives with methyl and isopropyl substituted at 1′ position exhibit more potent in vitro inhibitory activity against various cancer cell lines than other alkyl-substituted counterparts.[8] Besides, we found that shikonin (R-isomer), its S-isomer and its racemic compound did not show a significant difference in in vitro inhibitory activity again various cancer cell lines.[8] As a continuing part of our project, we designed and synthesized a series of simplified versions of shikonin derivatives with the structures shown in Figure 2. Herein we report the synthesis and biological activity studies of a series of shikonin derivatives with arylsulfonamides side chains (**2**, **3**, Fig. 2).

The synthetic route to shikonin analogues **2a–k** is shown in Scheme 1. Compound **4**, prepared by following known procedures,[9] was first converted to ketone **6** via Grignard addition to Weinreb amide **5**.[10–12] Next, reductive amination of ketone **6**,[13] followed by Boc protection, afforded **7**. The key intermediate **9** was then obtained in two steps via oxidation of **7** using PhI(OCOCF3)2 to provide naphthoquinone **8**[10,14] along with subsequent



1a Shikonin R=H
1b Acetylshikonin R=$COCH_3$
1c Isobutyrylshikonin R=$COCH(CH_3)_2$
1d 2-furoylshikonin R=

Figure 1. The structures of shikonin and its bioactive derivatives.

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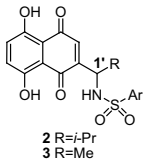
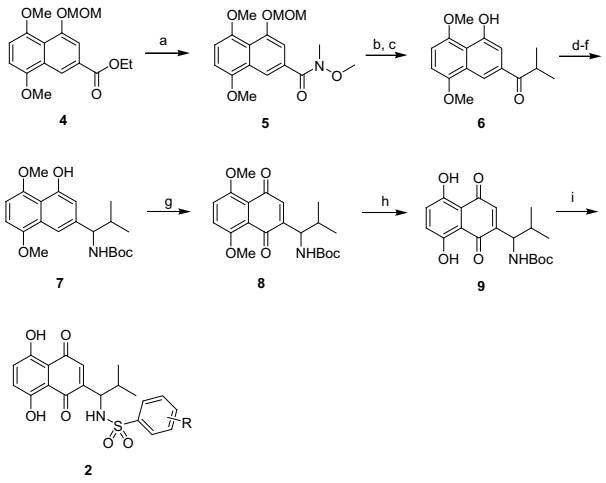


Figure 2. The structures of new simplified shikonin analogues.

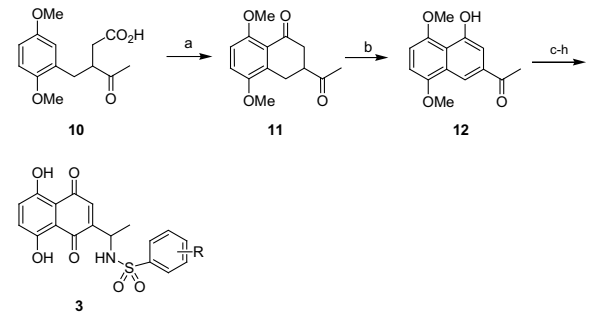


Scheme 1. Synthesis of analogues **2a–k**. Reagents and conditions: (a) *N*,*O*-dimethylhydroxylamine hydrochloride, *i*-PrMgBr, THF, −10 °C, 92%; (b) *i*-PrMgBr, THF, −10 °C°, 53%; (c) concd HCl, MeOH, reflux, 83%; (d) hydroxylamine hydrochloride, NaOAc, MeOH, reflux; (e) H_2, 10% Pd/C, concd HCl, EtOH, rt; (f) $NaHCO_3$, Boc_2O, Et_3N, MeOH, 0 °C, 58% (from **6** to **7**); (g) $C_6H_5I(OCOCF_3)_2$, CH_3CN, 0 °C, 71%; (h) AgO, 6 N HNO_3, acetone, 0 °C, 59%; (i) HCl/EtOAc, rt, $ArSO_2Cl$, *i*-Pr_2NEt, CH_2Cl_2, THF, rt, 33–67%.

demethylation.[15] Finally, deprotection of **9** and arylsulfonation of the resulting amine with substituted phenylsulfonic chlorides gave analogues **2a–k**.

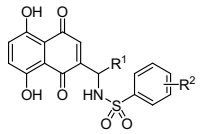
To add an additional element of diversity, we sought to synthesize a set of compounds **3a–k** with a modified alkyl group adjacent to amine. Since the preparation of ketone **6**, the important intermediate in the synthesis of **2**, required seven steps, a more concise strategy, in which ketone **12** was an intermediate instead of **6,** was employed (Scheme 2). The starting material **10**, prepared in two steps using a modified known procedure,[16] was cyclized to naphthalenone **11**, and then subsequent aromatization[17] provided ketone **12** in moderate yield. With ketone **12** in hand, analogues **3a–k** were prepared in a same manner as sulfonamide **2**.

Analogues **2** and **3** were tested for in vitro inhibitory activity against two human cancer cell lines, cervical carcinoma cells



Scheme 2. Synthesis of analogues **3a–k**. Reagents and conditions: (a) PPA, 60 °C, 60%; (b) 10% Pd/C, biphenyl, decalin, reflux, 66%; conditions for (c) to (h) are the same as those for (d) to (i) in Scheme 1.

Table 1
In vitro inhibitory activity of shikonin analogues **2a–k** and **3a–k** on HeLa[a] and HL60[b] cells



Compound	R^1	R^2	IC_{50} on HeLa[c] (μM)	IC_{50} on HL60[c] (μM)
2a	i-Pr	4-Me	4.85	NT[d]
2b	i-Pr	2-Me	0.79	0.19
2c	i-Pr	4-OMe	8.95	NT
2d	i-Pr	2,4-OMe	2.75	0.40
2e	i-Pr	4-F	6.65	NT
2f	i-Pr	4-Cl	8.65	NT
2g	i-Pr	4-Br	7.65	NT
2h	i-Pr	3-Br	0.86	0.79
2i	i-Pr	2-Br	8.31	0.34
2j	i-Pr	4-$COCH_3$	3.82	0.11
2k	i-Pr	4-CO_2Et	0.85	0.15
3a	Me	4-Me	5.34	0.03
3b	Me	2-Me	4.49	0.07
3c	Me	4-OMe	3.76	0.02
3d	Me	2,4-OMe	19.5	0.50
3e	Me	4-F	6.41	0.09
3f	Me	4-Cl	0.81	0.25
3g	Me	4-Br	5.21	0.10
3h	Me	3-Br	6.35	0.16
3i	Me	2-Br	6.20	0.12
3j	Me	4-$COCH_3$	5.76	0.26
3k	Me	4-CO_2Et	2.26	0.12
VP16			0.98	0.82
shikonin			13.39	0.44

[a] Inhibitory activity was determined by SRB assay.[18]
[b] Inhibitory activity was determined by MTT assay.[19]
[c] The IC_{50} values were means calculated from three independent experiments.
[d] NT, not tested.

(HeLa) (Table 1) and promyelocytic leukemia cells (HL60) (Table 1). The result showed that most of new analogues display higher inhibitory effects than those of the positive controls on HL60, except for **2h** which showed slightly lower inhibitory activity than shikonin (0.79 μM vs 0.44 μM). The IC_{50} values against HL60 of some analogues, such as **3a** and **3c**, were even less than 50 nM. As for HeLa cells, these new derivatives also showed significant inhibitory activity with IC_{50} values ranging from several micromolar to sub-micromolar, while overall activity was not so remarkable compared with VP16. Based on the data, the size of R^1 and the position and electronic effect of R^2 did not seem to have a direct correlation with inhibitory activity.

Analogues **2k**[20] and **3f**[20] were selected for further evaluation for inhibitory activity against other tumor cell lines, including breast cancer (MDA-MB-231), colon cancer (HCT116), lung cancer (A549), rhabdomyosarcoma (RH30), oral epidermoid cancer (KB-3-1 and KB/VCR) and leukemia (K562) (Table 2). Both **2k** and **3f** exhibited potent inhibitory activity on these cancer cell lines with the average IC_{50} value of 8.6 μM and 7.3 μM, respectively. In addition, it was noteworthy that **2k** and **3f** also showed significant effects on KB/VCR, the vincristine-selected multidrug-resistant (MDR) subline, with IC_{50} of 13.2 μM and 12.4 μM, respectively. Because resistance to chemotherapy is an important cause for treatment failure in cancers,[21] this result implies to some extent the potential value of these series of analogues on anticancer therapy.

Hypoxia has long been recognized as a common feature of solid tumors. Hypoxia-inducible factor 1α (HIF-1α) is the most important transcript factor in response to intracellular oxygen pressure. Almost undetectable under normoxia, it is enhanced to accumulate significantly in a hypoxic environment. HIF-1α is

Table 2
In vitro cytotoxicity of compounds **2k** and **3f** on cell lines originated from different tissues[a]

Compound	IC_{50} (μM)[b]								
	MDA-MB-231	HCT116	A549	RH30	KB-3-1	KB/VCR	HT29	K562	Mean
2k	4.7	3.8	11.4	2.6	11.8	13.2	2.8	18.2	8.6
3f	4.5	3.2	8.3	1.9	6.9	12.4	1.3	19.9	7.3

[a] The cytotoxic effects were determined by MTT assay on K562 and by SRB assay on the other cell lines.
[b] The IC_{50} values were means calculated from three independent experiments.

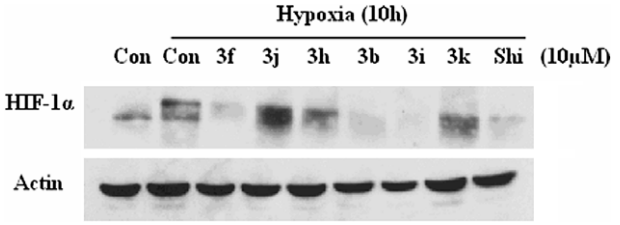


Figure 3. Compounds **3b**, **3f**, **3h**, **3i**, and shikonin decrease HIF-1α protein accumulation. 5×10^5 MDA-MB-231 cells were treated with 10 μM of compound respectively and exposed to hypoxia (1% O_2) for 10 h. Then the cells were collected and detected for HIF-1α and β-actin by Western blotting.[23]All data shown were representative of three independent experiments. Con, control; Shi, shikonin.

correlated with increased patient mortality in many different cancers including brain, breast, cervix, colon, ovary, lung cancer, etc. Genetic manipulations that increase its expression in human cancer cells have been shown to increase tumor growth, angiogenesis, and metastasis. HIF-1α has been validated as a therapeutic target, and a growing number of novel compounds were found to have biological activity.[22] Here we also found shikonin and its analogues could significantly reduce HIF-1α protein accumulation induced by hypoxia in breast cancer cells MDA-MB-231 (Fig. 3). It was the first time that naphthazarine compounds were found to have such biological activity. HIF-1α would remain as the potential antitumor target for shikonin derivatives with further mechanism studies underway.

In conclusion, a series of novel analogues of shikonin with arylsulfonamide side chains (**2** and **3**) were synthesized and tested for their in vitro antitumor activity. Most of the analogues exhibited significant inhibitory activity on HeLa and HL60 with IC_{50} values lower than the lead compound shikonin. The potential value of these new analogues in cancer treatment was corroborated by the moderate to high inhibitory activity of analogues **2k** and **3f** on diversified human cancer cell lines, including MDR cell line KB/VCR. Moreover, shikonin and some of its analogues were found to decrease the expression level of HIF-1α in breast cancer cells MDA-MB-231, which indicates a potential antitumor target for naphthazarine compounds. Therefore, shikonin analogues with arylsulfonamide-containing side chains deserve further evaluation as a new class of anticancer agents.

Acknowledgments

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20. Analytical data for **2k** and **3f**. **2k**: a red crystal, mp 173–175 ℃; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.77 (d, J = 6.9 Hz, 3H), 1.06 (d, J = 6.9 Hz, 3H), 1.39 (t, J = 7.2 Hz, 3H), 2.11–2.21 (m, 1H), 3.92 (t, J = 9.0 Hz, 1H), 4.34 (q, J = 7.2 Hz, 2H), 5.56 (d, J = 9.0 Hz, 1H), 6.68 (s, 1H), 7.12 (dd, J = 9.6 Hz, 2H), 7.73 (d, J = 8.4 Hz, 2H), 7.90 (d, J = 8.4 Hz, 2H), 12.27 (s, 1H), 12.50 (s, 1H); HRMS: Calcd for $C_{23}H_{23}NO_8S$ 473.1144, Found 473.1143. **3f**: mp 151–153 ℃, a red crystal. ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 1.52 (d, J = 7.2 Hz, 3H), 4.45–4.46 (m, 1H), 5.49 (d, J = 9.3 Hz, 1H), 6.85 (s, 1H), 7.16 (dd, J = 9.9 Hz, 2H), 7.27 (d, J = 8.4 Hz, 2H), 7.64 (d, J = 8.4 Hz, 2H), 12.34 (s, 1H), 12.49 (s, 1H); HRMS: Calcd for $C_{18}H_{14}ClNO_6S$ 407.0230, Found 407.0231.
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23. Western blotting: eighty percent confluenced cells (5×10^5 cells/well) were treated with tested compounds for the indicated times under hypoxic or normoxic conditions. After the medium was discarded, the cells were lysed in 1× SDS gel loading buffer [50 mM Tris–HCl (pH 6.8), 100 mM DTT, 2% SDS, 0.1% bromphenol blue, 10% glycerol], and then boiled for 10–15 min. The same amounts of cell lysates were resolved on 10% SDS–polyacrylamide gels, and the proteins were electrotransferred to Hybond-C nitrocellulose membranes (Amersham, Piscataway, NJ). The blots were incubated with the indicated primary antibodies, then washed and incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies. Immunoreactivity was visualized using the ECL Plus Western Blotting Detection System (GE Healthcare, UK).