Pergamon

Bioorganic & Medicinal Chemistry Letters 12 (2002) 1375–1378

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Shikonin Derivatives: Synthesis and Inhibition of Human Telomerase

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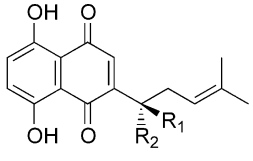
Received 10 January 2002; accepted 6 March 2002

Abstract—We synthesized DL-shikonin, shikonin, alkanin, and their cyclo-derivatives and acyl-derivatives. These compounds have low cytotoxicity, as well as inhibitory activity against the telomerase enzyme, except cyclo-derivatives. © 2002 Elsevier Science Ltd. All rights reserved.

Human telomeres are located at the end of eukaryotic chromosomes and consist of repeated TTAGGG sequences. Most of the telomeric DNA is double-stranded except the extreme terminal part which is 3′ single-stranded and overhangs over 200 nucleotides in length.[1] Telomere are considered to be essential for preventing aberrant chromosome fusion; degradation and recombination, and involvement in control of senescence, replication and the cell cycle clock.[2] Telomerase has the ability to maintain telomere length, by synthesizing further telomere repeats and adding them into the end of telomere.[3] Telomerase is present in the vast majority of cancer cells and is largely absent in normal somatic cells, so it is thought to play an important role in the maintenance of telomeres in cancer cells. The possibility of designing drugs with inhibitory activity against telomerase is emerging as an attractive strategy for chemotherapy.[4]

Telomerase is a multisubunit ribonucleoprotein complex which includes RNA component (hTR) and a reverse-transcriptase catalytic subunit (hTERT). Inhibitory strategies have mainly focused on three areas: antisense molecules (oligonucleotides, RNA molecules, ribozymes and peptide nucleic acid) directed against the hTR RNA component of telomerase, small molecule reverse transcriptase inhibitors (e.g., azidothymidine), and telomere itself (because it is the substrate on which telomerase acts[5]). Telomeres have both single-stranded DNA and protein components. In many organisms including human, the single-stranded DNA contains G-rich repeats which can fold into intramolecular (or intermolecular) quadruplex helix structures[6]; each G is Hoogsteen-hydrogen-bonded to another G in a square-planar arrangement called a G-quartet.[7] This structure hides the 3′-end of the DNA from telomerase and thereby inhibits telomere extension.[8] Therefore, small molecules that preferentially interact with and stabilize G-quadruplexes have been proposed as telomerase inhibitors.[9] The first small molecule inhibitor of human telomerase, anthraquinones, based on G-qudruplex interaction was identified by Hurley and co-workers.[10] They reported that the most potent inhibitor inhibited telomerase in cell free assays with IC_{50} of 23 μM. Subsequently, a series of planar aromatic molecules have now been shown to inhibit telomerase by this mechanism, including tetrapyridyl-substituted cationic porphyrins, perylene derivatives, acidines and fluorenones.[11]



1. R_1=OH, R_2=H
2. R_1=H, R_2=OH

Shikonin **1**, alkanin **2**, and their derivatives, found in most of the many traditional medicinal plants of the *Boraginaceae* family (mainly in the genus of *Alkanna, Lithospermum*), have been used as natural purple dyes since ancient times in China, Japan, and Europe.[12] They

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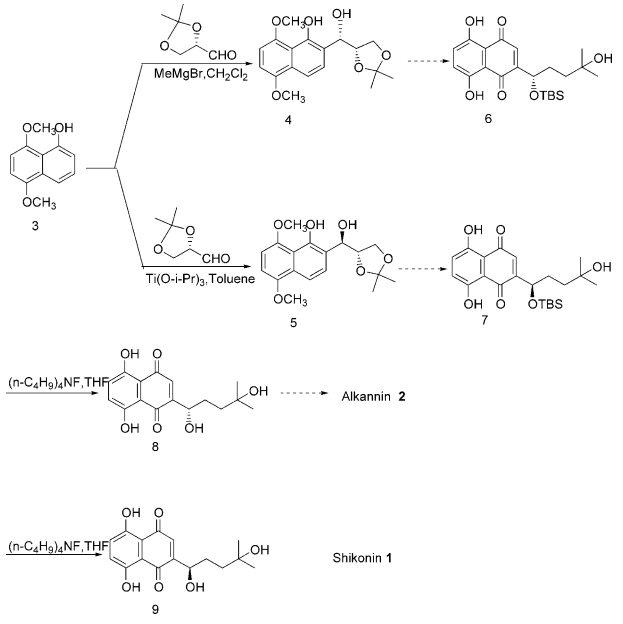
PII: S0960-894X(02)00158-0

exhibit many biological effects including anticancer activity.[13] During the past few years, these compounds have been the object of numerous biological studies. But their anticancer mechanism is still elusive. Recently, Ahn[14] reported that shikonin and its acyl derivatives have the inhibitory effects on Topoisomerase-I. They also reported these inhibitors have no activity against Topoisomerase-II (from Hella cells).[15]

Shikonin **1**, alkanin **2**, and their derivatives have the structural features of planar chromophores and short side chains. We examine the ability to inhibit the telomerase. Here we report synthetic and biological studies on shikonin, alkanin, DL-shikonin and their acyl-derivatives.

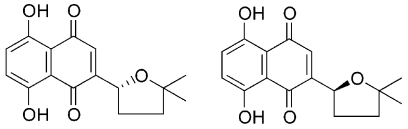
Several syntheses of DL-shikonin **25** have been reported in the literature.[16] We synthesized DL-shikonin by Reformatsky reaction.[17] Asymmetric syntheses of shikonin **1** and alkanin **2** are depicted in Scheme 1.

The chiral center may be established by direct C-arylation of D-2,3-isopropylideneglyceraldehyde with Mg-based or Ti-based naphthalenol **3** in highly diastereodivergent manner.[18] The starting material **3** can be efficiently synthesized from 1,4-dimethoxybenzene and Furan.[19] Treatment of **3** with EtMgBr, followed with D-2,3-isopropylideneglyceraldehyde, then subjected to ultrasonic wave at 0 °C for 3 h furnished the *threo* addition product **4** in diastereoisomeric excess of 90% and good yield (68%). Treatment **3** with Ti(O-*i*-Pr)$_3$, followed D-2,3-isopropylideneglyceraldehyde at 0 °C for 5 h, furnished the *erythro* addition product **5** in diastereoisomeric excess of 92% and good yield (70%). We used Cai's method[20] to construct the side chain and obtained compound **6** and **7**. After deprotection of *t*-butyldimethylsilyl (TBS) of compound **6** and **7** with tetrabutyl ammoniumfluoride (TBAF), we obtained compounds **8** and **9** which were converted into alkanin **2** and shikonin **1** in three steps.[16] Cycloshikonin **10** and



Scheme 1.

cycloalkanin **11** can be easily obtained by treating **6** and **7** with $BF_3 \cdot Et_2O$.[20]

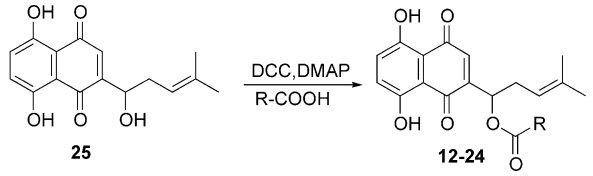


10 **11**

The syntheses of acyl DL-shikonin has been carried out by a selective acylation[14] with carboxylic acid at 1′-OH of DL-shikonin in presence of dicyclohexylcarbodiimide (DCC) and 4-(dimethylamino)pyridine (DMAP) in CH_2Cl_2 and outlined in Scheme 2.

The cytotoxicity of these compounds was evaluated by MTT[21] method using three human cancer cell lines: HL-60, A-549, P-388. Table 1 shows the relative cytotoxicity of these compounds at a concentration of 1 μM. The compounds with inhibitory rate of 100% indicate complete inhibition on the cell proliferation. The compounds with inhibitory rate higher than 85 or 50% are defined as having significant or slight cytotoxicity respectively, while those with inhibitory rate less than 50% are defined as having no cytotoxicity. These compounds have low cytotoxicity except **10** and **11**.

It is surprising that cycloshikonin or cycloalkanin have high cytotoxicity. As seen in Table 1, the chiral center of shikonin and alkanin has no effect on their activity. Compound **16** has high selective inhibitory effect on growth of human gastric carcinoma SGC-7901 and hepatocarcinoma BEL-7402 in vitro (Table 2).



25 **12-24**

Scheme 2.

Table 1. In vitro cytotoxicity of shikonin derivatives

Compd	RCO	A-549[a]	P-388	HL-60
1		+	+	+
2		+	+	+
25		+	+	+
10		—	+ + +	+ + +
11		—	+ + +	+ + +
12	Acetyl	+ +	+	+ +
13	*n*-Butanoyl	—	—	+
14	Benzoyl	—	—	–
15	*n*-Dodecanoyl	+	—	+
16	Iso-octanoyl	+ +	—	+
18	Undecylenyl	—	—	–
19	2-Naphthoxy acetyl	—	—	+
20	3-Indolacetyl	—	—	+
21	3-Nicotinoyl	—	—	+
22	Benzoxy acetyl	—	—	
23	1-Naphthoyl	—	—	+
24	2-Naphthoyl	—	—	+

[a](+ + +) total; (+ +) significant; (+) slight; (—) no inhibition.

Table 2. IC_{50} values of shikonin derivatives, in vitro cytotoxicity

Compd	IC_{50} (μM)[a]					
	K-562	A-549	SGC-7901	BEL-7402	MCF-7	WI-38
12	29	39	0.29	0.068	31	—
16	18	0.016	0.001	0.00017	250	—
24	31	390	49	67	93	—

[a](—) indicates no cytotoxicity.

Table 3. Human telomerase inhibition data IC_{50} values and *Taq* Inhibition data of shikonin derivatives

Compd	RCO	$^{Tel}IC_{50}$ (μM)	Taq inhibition[a]		
			10 μM[a]	20 μM	50 μM
12	Acetyl	37.3	—	—	—

[a](+++) total; (++) significant; (+) slight; (—) no inhibition.

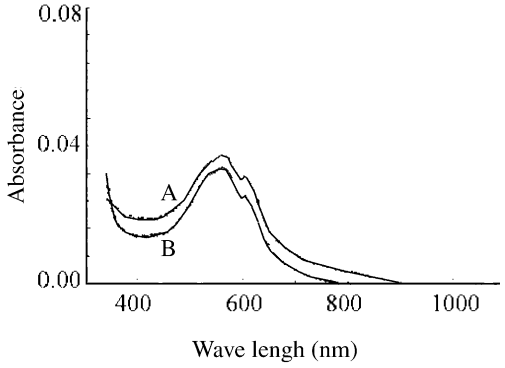


Figure 1. Visible absorption spectra of compound **25** (10 μM) (A) in phosphate buffer (K-BPES: 10 mM, KH_2PO_4/K_2HPO_4, 200 mM KCl, 0.1 mM EDTA) and in the presence of the G4-DNA d(TTAGGGT) (100 μM) (B).

These compounds were also evaluated in a modified telomeric repeat amplification protocol (TRAP)[22] for their ability to inhibit the extension of telomeres by human telomerase, using partially-purified enzyme extracted from human leukemia HL-60 cells. We examined their telomerase inhibitory activity at 50 μM and found that compounds **1**, **2**, **25**, **12**, and **24** can inhibit telomerase. In particular, compound **12** is the most potent one. As a prerequisite to the evaluation of compound **12** in the TRAP assay, we also describe the ability to inhibit the Taq enzyme (a thermophilic DNA polymerase used in the PCR-based TRAP assay) (in Table 3). Experiments gave a value of $^{Tel}IC_{50}$ and values are given in Table 3.

In order to verify the interaction of shikonin with G-quadruplex DNA, we monitored the changes in the visible spectrum of DL-shikonin **25** in the presence of the G4-DNA form of d(TTAGGGT).[23] As shown in Figure 1, the absorption spectrum of **25** undergoes significant hypochromicity, indicative of complex formation (very likely π–π stacking). The lack of isosbestic point indicates that there are probably multiple binding sites. The lack of red shift indicates that the structure of drug did not change much upon its binding to DNA. In

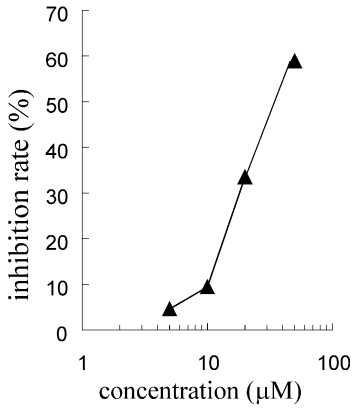


Figure 2. Dose–response plots for compound **12**, with telomerase inhibition measured by TRAP assay.

presence of normal DNA d(TTCTGTTGAATTAC GTTAAGCATGTAATAATT AACATGTAATGCA), **25** did not show similar interaction as in presence of G4-DNA.

These compounds may also have the G-quadruplex mechanism. Figure 2 shows, for a representative compound **12**, the dose–response plots for variation of telomerase inhibition with respect to concentration. A log-linear increase in inhibition was observed with increasing concentration.

We have identified a new class of compounds capable of inhibiting the telomerase at micromolar level. Though they are approximately 10-fold less active than the best G-quadruplex mediated inhibitors based on tricyclic chromophores.[24] It is notable that they have relatively low cytotoxicity compared to the other telomerase inhibitors and thus greater selectivity. Structure–activity studies on tricyclic inhibitors have previously demonstrated that the amido side chains were essential for activity. Suitable modification of the side chain structure would produce better inhibitors. Shikonin and its derivatives may present useful leads for the development of more potent telomerase inhibitors.

Acknowledgements

The authors thank Dr. Zheng Tao who kindly provided G4-DNA, We also thank Dr. Haiyong Han and Dr. Rongliang Lou for fruitful help and discussion.

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