Acylshikonin Analogues: Synthesis and Inhibition of DNA Topoisomerase-I

Byung-Zun Ahn,* Kyong-Up Baik, Gi-Ryang Kweon,† Kyu Lim,† and Byung-Doo Hwang†

Colleges of Pharmacy and Medicine, Chungnam National University, 220 Gungdong Youseongku, Taejon 305-764, Korea

Received June 6, 1994®

Compounds bearing an acyl group of a various size at 1′-OH of shikonin were synthesized as acyl analogues of shikonin, which was isolated from the root of *Lithospermum erythrorhizon*, and evaluated for inhibitory effect on topoisomerase-I activity. A selective acylation at 1′-OH of shikonin in the presence of dicyclohexylcarbodiimide and 4-(dimethylamino)pyridine gave rise to a good yield of corresponding acylshikonin derivatives. In general, analogues with an acyl group of shorter chain lengths (C_2–C_6) exerted a stronger inhibitory action than those with longer chain lengths (C_7–C_{20}). While the halogen substitution at C-2 of the acetyl moiety failed to increase the inhibitory potency, the placement of double bonds in the acyl group (C_5–C_7) augmented the potency remarkably. Of the 32 derivatives evaluated, 15 compounds exhibited a higher inhibitory effect than shikonin. Noteworthy, the inhibitory potency of acetylshikonin, propanoylshikonin, and 4-pentenoylshikonin was approximately 4-fold greater than that of camptothecin. All these data suggest that the size of acyl moiety is important for the enhancement of potency, and the presence of olefinic double bonds is also beneficial.

Introduction

There are two major types of antitumor agents which are known to inhibit DNA topoisomerases. The well-studied class of DNA topoisomerase-I inhibitors are camptothecin analogues, some of which are being tested clinically as anticancer drugs against colon and other cancers.[1–5] However, some limitations such as poor water solubility and toxicity still exist. Meanwhile, the anthracycline compounds, highly effective in the treatment of human neoplastic diseases[6] such as leukemias and sarcomas, seem to inhibit topoisomerase-II as a primary target.[7] These compounds, however, also inhibit topoisomerase-I nonspecifically,[8] and moreover, the myocardial toxicity of these drugs limits their wider use in chemotherapy for prolonged periods.[9] For these reasons, efforts have been made to develop compounds showing a selective inhibition of the respective DNA topoisomerase.

Previously,[10] it was reported that shikonin, one of antineoplastic components isolated from *Lithospermi radix* (*Lithospermum erythrorhizon*, Boraginaceae), exhibited a strong cytotoxicity against L1210 cells. The following study[11] showed that acetylshikonin, another constituent of *Lithospermi radix*, possessed a good antitumor action (T/C value, 182%) in ICR mice bearing Sarcoma 180 cells. Since shikonin and acetylshikonin contain a substituted naphthazarine skeleton, which is present in the structure of anthracyclinones[12] and mitoxanthrone,[13] it was assumed that some part of antineoplastic action of these compounds might be related to the inhibition of DNA topoisomerases. Furthermore, the higher antitumor action of acetylshikonin, compared to shikonin, encouraged us to synthesize shikonin analogues with acyl moieties of various sizes.

Here, we report that shikonin and its analogues possessing short acyl chain lengths at 1′-OH are potent inhibitors of DNA topoisomerase-I as assayed by *in vitro* relaxation of supercoiled DNA.

Chemistry

The synthesis of acylshikonins has been carried out by a selective acylation at 1′-OH of shikonin in the presence of dicyclohexylcarbodiimide (DCC) and 4-(dimethylamino)pyridine (DMAP) and is outlined in Scheme 1. The reaction in a molar ratio of shikonin/DMAP (1:0.4) resulted in relatively good yields (30–92%) of 1′-acylshikonins [2-[1-(acyloxy)-4-methylpent-3-enyl]-5,8-dihydroxy-1,4-naphthoquinones]. The hydrogen bonding between phenolic OH and quinoid carbonyl seems to be a reason for hindered acylation at the phenolic OH's. Evidence for the acylation was mainly brought from the ^{1}H-NMR spectra; the signals at δ 12.30–12.60 ppm, which were attributed to protons at two phenolic OH's, was still present, but the signal at δ 2.38 ppm, due to the 1′-OH, disappeared. Instead, the signals at δ 2.15–7.70 ppm, corresponding to protons at C-2 in acyl moiety, appeared. The carbonyl group of the newly-formed acyl moiety could be also recognized from signals (δ 160–176 ppm) in the ^{13}C-NMR spectra.

Results and Discussion

Figure 1 shows that the DNA relaxation activity of topoisomerase-I, based on the densitometry measurement, was reduced by *n*-hexane extract of *L. erythrorhizon* in a concentration-dependent manner. In further studies, two active components were isolated from *n*-hexane extract and identified to be shikonin and acetylshikonin, which showed IC_{50} values of 208 and 45 μM (Figure 2), respectively. In a separate experiment, shikonin and acetylshikonin were tested for the inhibition of topoisomerase-II. However, these compounds exerted no significant inhibition of topoisomerase-II activity even at 500 μM. Thus, it appeared that shikonin and acetylshikonin exerted a preferential inhibition of topoisomerase-I. The higher potency of acetylshikonin, compared to shikonin, in the inhibition of DNA topoisomerase-I led us to examine analogues of acylshikonin bearing various acyl moieties; they included short

* To whom correspondence should be addressed at: College of Pharmacy, Chungnam National University, 220 Gungdong Yousungku, Taejon 305-764, Korea. Tel: (042) 821-5923. Fax: (042) 821-5903.
† College of Medicine.
® Abstract published in *Advance ACS Abstracts*, February 15, 1995.

0022-2623/95/1838-1044$09.00/0

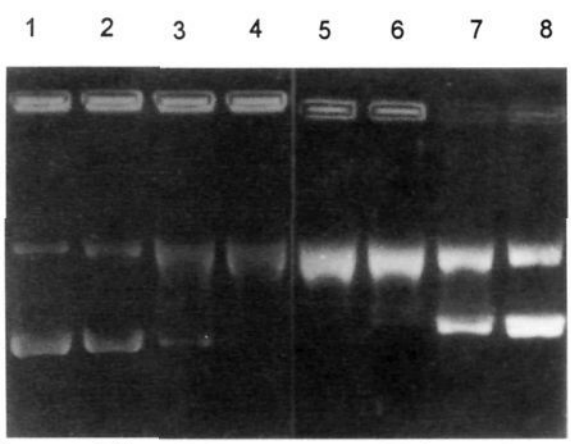


Figure 1. Effect of *n*-hexane extract of Lithospermi root on DNA topoisomerase-I from HeLa cells. The reaction mixture (20 μL) contained 50 mM HEPES (pH 7.0), 10 mM $MgCl_2$, 150 mM KCl, 0.75 μg of DNA, 1 mM DTT, 5 μg of BSA, and the enzyme. Where indicated, the *n*-hexane extract of various concentrations was included. Lane 1: no enzyme, no drug. Lanes 2–4: 0.5, 1, and 2 units of the enzyme. Lanes 5–8: 2 units of the enzyme, and 25, 50, 100, and 200 μg/mL of *n*-hexane extract.

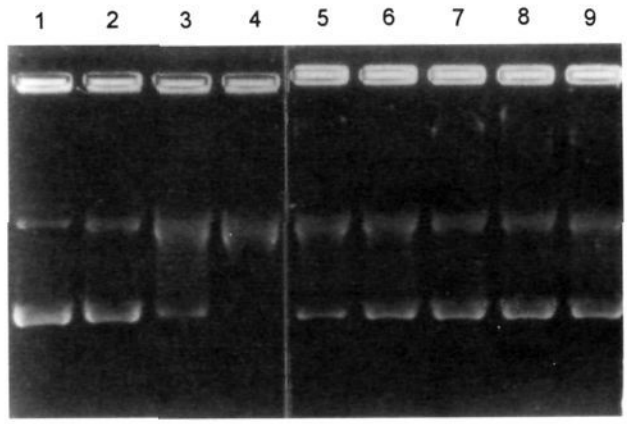
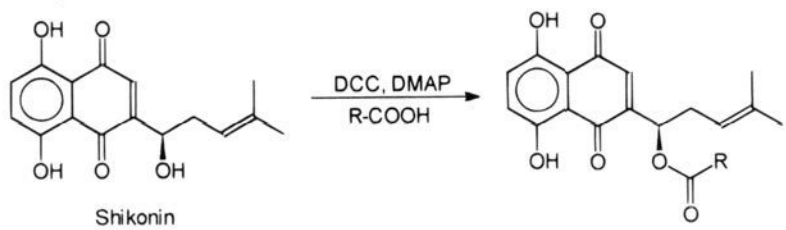


Figure 2. Effect of acetylshikonin on DNA topoisomerase-I from HeLa cells. The reaction mixture (20 μL) contained 50 mM HEPES (pH 7.0), 10 mM $MgCl_2$, 150 mM KCl, 0.75 μg of DNA, 1 mM DTT, 5 μg of BSA, and the enzyme. Where indicated, acetylshikonin of various concentrations was included. Lane 1: no enzyme, no drug. Lanes 2–4: 0.5, 1, and 2 units of the enzyme. Lanes 5–9: 2 units of the enzyme and 15, 30, 60, 120, and 240 μg/mL of acetylshikonin.

or long fatty acyl groups and benzoyl or phenylacetyl groups, as well as retinoyl moiety. When the synthetic analogues were evaluated for the inhibitory effect on topoisomerase-I activity, it was found that the inhibitory potency (IC_{50} value) was strongly dependent on the size of acyl substituent (Table 1). Of the compounds tested, acetylshikonin (**1**), *n*-propanoylshikonin (**4**), and 4-pentenoylshikonin (**8**) were the most potent inhibitors with similar IC_{50} values (40–45 μM). Moreover, these analogues (**1**, **4**, and **8**) were approximately 4-fold more potent than camptothecin (IC_{50}, 172 μM). Among the analogues (**4**, **5**, **7**, **10**, and **14**) with stretched and saturated acyl chains (C_3–C_7), the inhibitory potency seemed to decrease gradually with increasing size (*n*) of acyl moiety. And, the analogues (**17**–**20** and **26**–**30**) with chain lengths longer than *n*-heptanoyl group showed a negligible inhibition (IC_{50}, >625 μM). In addition, undecenyl- and retinoylshikonins had no remarkable activity.

Substitution on the acyl group was attempted in order to find a more potent inhibitor; the halogen substitution at C-2 of the acetyl moiety with an electron-withdrawing group such as chloro resulted in about a 3-fold decrease in inhibitory potency as seen in compounds **2** and **3**. Branching the *n*-propanoyl moiety of compound **4** by adding an α-methyl (**6**) also led to the diminishment of potency with respect to compound **4**, but a modest enhancement with respect to compound **5**. Noteworthy, the placement of double bonds in acyl group (C_5–C_7) contributed remarkably to augment the inhibitory action; IC_{50} values for *n*-pentanoyl-, *n*-hexanoyl-, and *n*-heptanoylshikonins were 161, 207, and 625 μM, respectively, and those for 4-pentenoyl-, *trans*-2-hexenoyl-, and 6-heptenoylshikonins were 40, 153, and 250 μM, respectively. The improvement of potency was more remarkable with dienes; compound **15** is more active than compound **16**. The introduction of a benzoyl group (**22**) on 1′-OH of shikonin resulted in a slight increase in potency as compared to shikonin, but the potency decreases with a phenylacetyl (**23**) or a diphenylacetyl moiety (**24**). Our present data show that among 32 synthetic analogues, 15 compounds are more potent inhibitors than shikonin. Moreover, 11 compounds expressed a greater inhibitory action than camptothecin as a prototype DNA topoisomerase-I inhibitor. There may be two important factors for the

Scheme 1. Synthesis of Acylshikonins



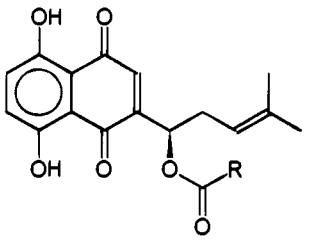
Shikonin
(isolated from *Lithospermum erythrorhizon*)

Acylshikonin

[DCC ; Dicyclohexylcarbodiimide
DMAP ; 4-Dimethylaminopyridine]

R-COOH : Alkyl & Alkenyl carboxylic acid (C_2-C_{12})
Fatty acid (C_{15}-C_{18})
Retinoic acid (*cis* & *trans*)

Table 1. IC_{50} Values[a] of Acylshikonin Analogues in the Inhibition of DNA Topoisomerase-I



compd. no	RCO-	IC_{50}[b] (μM)	compd no.	RCO-	IC_{50} (μM)
1	acetyl	45 ± 3[c]	**17**	*n*-octanoyl	>625
2	monochloroacetyl	137 ± 8[c]	**18**	*n*-nonanoyl	>625
3	trichloroacetyl	115 ± 6[c]	**19**	*n*-decanoyl	>625
4	*n*-propanoyl	44 ± 3[c]	**20**	*n*-dodecanoyl	>625
5	*n*-butanoyl	144 ± 10[c]	**21**	3-methyl-2-butenoyl	77 ± 6[c]
6	isobutanoyl	84 ± 5[c]			
7	*n*-pentanoyl	161 ± 10	**22**	benzoyl	153 ± 11
8	4-pentenoyl	40 ± 3[c]	**23**	phenylacetyl	222 ± 15
9	*trans*-2-pentenoyl	80 ± 5[c]	**24**	diphenylacetyl	>625
10	*n*-hexanoyl	207 ± 15	**25**	undecylenyl	>625
11	*trans*-2-hexenoyl	153 ± 11	**26**	stearoyl	>625
12	*trans*-3-hexenoyl	153 ± 15	**27**	palmitoyl	>625
13	2,4-hexadienoyl	130 ± 10[c]	**28**	oleoyl	>625
14	*n*-heptanoyl	625 ± 30	**29**	linoleoyl	>625
15	2,6-heptadienoyl	126 ± 6[c]	**30**	linolenoyl	>625
16	6-heptenoyl	250 ± 13	**31**	*trans*-retinoyl	>625
			32	*cis*-retinoyl	>625
shikonin		208 ± 10	camptothecin		127 ± 9

[a] See the Experimental Section for procedures. [b] IC_{50} (±SEM) is the concentration required to inhibit 50% of topoisomerase-I activity. Statistical analysis was done by Student's *t* test. [c] IC_{50} is significantly different from that of camptothecin; $p < 0.05$ ($n = 3$).

contribution of acyl moiety to the enhanced potency. One is the length of acyl moiety; acetyl- or *n*-propanoylshikonin is about 3 times more potent than *n*-butanoylshikonin. Another is the existence of olefinic double bonds in acyl chains; a similarity of IC_{50} values between 2,4-hexadienoylshikonin (**13**) and 2,6-heptadienoylshikonin (**15**) in comparison with the 3-fold difference of IC_{50} values between *n*-hexanoylshikonin (**10**) and *n*-heptanoylshikonin (**14**) reflects a considerable contribution of dienes. Although analogues bearing chain lengths longer than the heptanoyl moiety demonstrated a negligible effect, a role of these compounds as potential prodrugs may be possible in the cells containing esterases.

The chain length of acyl group for the maximal inhibition of topoisomerase-I was found to be two or three carbon atoms. These data suggest that there may be a size limitation of acyl moiety for the effective interaction of acylshikonins with topoisomerase-I and/or DNA. Although the mechanism for antitumor action of acetylshikonin was not examined here, it is assumed that the inhibition of DNA topoisomerase-I could be one of the mechanisms for its antitumor action. The support for the assumption may come from further studies on the relationship between the topoisomerase-I–inhibiting activity and the antitumor action of acylshikonins.

Experimental Section

General Experimental Procedures. Melting points were measured with an Electrothermal melting point apparatus and were uncorrected. IR spectra were recorded on a JASCO Report-100 spectrophotometer, and ^{1}H-NMR spectra were obtained using a Varian-Gemini 200 NMR spectrometer; the chemical shift values were expressed as ppm relative to TMS as an internal standard. High-resolution mass spectra were obtained on VG70-VSEQ (VG analytical) under standard conditions. Elemental analyses were determined by CE-2400 (Perkin-Elmer). Analytical thin-layer chromatography (TLC) was carried out on Merck precoated silica gel 60 F-254 plates. EM Kieselgel 60 (230–400 mesh ASTM) was used for column chromatography.

Isolation of Topoisomerase-I Inhibiting Substances from the Roots of *Lithospermum erythrorhizon*. The air-dried root (1 kg) was extracted twice with *n*-hexane (2 L) in a Soxhlet apparatus for 16 h at room temperature. The extract was concentrated under vacuum at 35 °C to give a dark viscous gum (9 g) after removal of the solvent. The gum was fractionated by silica gel column chromatography with a solvent system of *n*-hexane:dichloromethane (5:1 to 1:5), and five fractions exhibiting a significant inhibition of topoisomerase-I were obtained. Recrystallization of the second and third fractions gave crystals of acetylshikonin (250 mg) and shikonin (220 mg), respectively. The structures of those compounds were established by spectroscopic comparison with those of authentic substances.[14]

Synthesis of Acylshikonins. Shikonin isolated as mentioned above was used as starting material. Shikonin (1 mmol) was dissolved in 3 mL of anhydrous dichloromethane, and to the solution were added 1.05 mmol of DCC (dicyclohexylcarbodiimide), 0.25 mmol of DMAP (4-(dimethylamino)pyridine), and 1 mmol of organic carboxylic acid (99%, Aldrich, Milwaukee, WI) under nitrogen atmosphere in the ice bath. The resulting mixture was stirred for 3 h in the ice bath and then stirred for additional 1 h at room temperature. The reaction mixture was diluted with 20 mL of *n*-hexane and filtered. Then, the filtrate was concentrated to 4 mL *in vacuo*. The crude product was purified by silica gel column chromatography as described in the preceding part.

Determination of DNA Topoisomerase-I or -II Activity. DNA topoisomerase-I activity was measured by the relaxation of superhelical DNA.[15,16] The assay mixture (20 μL) containing 50 mM HEPES (pH 7.0), 150 mM KCl, 10 mM $MgCl_2$, 1.0 mM dithiothreitol, 0.05 mg/mL bovine serum albumin, 0.75 μg of supercoiled pBR322 DNA, and 5 μL of enzyme was used as control. The assay mixture containing test substance was the test group. Both control and test groups were incubated at 37 °C, and after 30 min of incubation, the reaction was terminated by the addition of 5 μL of a

solution consisting of 2% SDS, 20% glycerol, and 0.05% bromophenol blue. Electrophoresis was carried out over 1% agarose gel plates, equilibrated with TBE buffer (50 mM Tris base, 50 mM boric acid and 2.5 mM EDTA) and stained with 0.5 μg/mL ethidium bromide solution. The plates were photographed under UV light. The IC_{50} value is expressed as the concentration which caused 50% inhibition of the enzyme activity, corresponding to 50% inhibition of relaxation of supercoiled pBR322 DNA under the conditions used. In each test, camptothecin was used as a positive control, and the IC_{50} value of each acylshikonin analogue was compared with that of camptothecin. Each value is the mean (±SEM) of triplicate experiments. In a separate experiment, the activity of DNA topoisomerase-II from Hella cells was measured by the relaxation of supercoiled plasmid DNA. The inhibitory effect of shikonin or acetylshikonin (500 μM) was determined using the topoisomerase-II and supercoiled pUC19 DNA as substrate under conditions established earlier.[17]

Purification of Topoisomerase-I.[15,16] HeLa cells, purchased from ATCC, were grown in RPMI 1640 medium containing 5% fetal bovine serum. The cells were harvested and suspended in a solution consisting of 1 mM EDTA, 1 mM mercaptoethanol, 0.5 mM PMSF, 10% glycerol, and 50 mM phosphate buffer (pH 7.0) and then homogenized with Polytron homogenizer in the ice bath for 30 s. Phosphate buffer (0.2 M) was added to the homogenized solution to a final concentration of 0.05 M. After rehomogenization of this final solution for 30 s, the solution was kept for 1 h in the ice bath. The cooled solution was centrifuged (30000g, 30 min), and then the supernatant was subjected to phosphocellulose chromatography, which gave a partially purified enzyme preparation.

Acknowledgment. This study was supported by Korea Science and Engineering Foundation, The Research Center for New Drug Development and Cancer Research Center (KOSEF-SRC-56-CRC-24), Seoul National University.

Supplementary Material Available: Physical and chemical data for synthesized acylshikonin analogues (10 pages). Ordering information is given on any current masthead page.

References

(1) Hsiang, H. S.; Hertzberg, R. P.; Liu, L. F. Camptothecin Induces Protein-linked DNA Breaks via Mammalian DNA Topoisomerase I. *J. Biol. Chem.* **1985**, *260*, 14873–14878.
(2) Ohno, R.; Okada, K.; Masaoka, T. A. An Early Phase-II Study of CPT-11 a New Derivative Camptothecin for Treatment of Leukemia and Lymphoma. *J. Clin. Oncol.* **1990**, *8*, 1907–1912.
(3) Peter, J. H.; Pamela, J. C.; Leann, M.; Clinton, F. S.; Timothy, W. S.; Janet, A. H. Evaluation of 9-Dimethylaminoethyl-10-hydroxy-camptothecin against Xenografts Derived from Adult and Childhood Solid Tumors. *Cancer Chemother. Pharmacol.* **1992**, *31*, 229–239.
(4) Johnson, R. K.; McCabe, F. L.; Faucette, L. F.; Hertzberg, R. P.; Holdon, K. G. SK&F 10864, Water Soluble Analogs of Camptothecin with Broad-spectrum Activity in Preclinical Tumor Models. *Proc. Am. Assoc. Cancer Res.* **1989**, *30*, 623.
(5) O'Connor, P. M.; Kerrigan, D.; Bertrand, R.; Pommier, Y. 10,-11-Methylenedioxycamptothecin, A Topoisomerase-I Inhibitor or Increased Potency. *Cancer Commun.* **1990**, *2*, 395–400.
(6) Young, R. C.; Ozols, R. F.; Meyers, C. E. The Anthracycline Antineoplastic Drugs. *N. Engl. J. Med.* **1981**, *305*, 139–153.
(7) D'Arpa, P.; Liu, L. F. Topoisomerase-Targeting Antitumor Drugs. *Biochim. Biophys. Acta* **1989**, *989*, 163–177.
(8) Foglesong, P. D.; Reckord, C.; Swink, S. Doxorubicin inhibits Human DNA Topoisomerase-I. *Cancer Chemother. Pharmacol.* **1992**, *30*, 123–125.
(9) Buzdar, A. V.; Marcus, C.; Smith, T. L.; Blumenschein, G. R. Early and Delayed Cardiotoxicity of Doxorubicin. *Cancer* **1985**, *55*, 2761–2765.
(10) Lee, J. H.; Ahn, B. Z. Antineoplastic Natural Products and the Analogues(XI); Cytotoxic Activity against L1210 Cells of Some Raw Drugs from the Oriental Medicine and Folklore. *Korean J. Pharmacogn.* **1986**, *17*, 286–291.
(11) Kim, H.; Ahn, B. Z. Antitumor Effects of Acetylshikonin and Some Synthesized Naphtharazin on L1210 and S-180 Systems. *Yakhak Hoeji.* **1990**, *34*, 262–266.
(12) Bodley, A.; Liu, L. F.; Israel, M.; Seshadri, R.; Koseki, Y.; Giuliani, F. C.; Kirschenbaum, S.; Silber, R.; Potmesil, M. DNA Topoisomerase-II-mediated Interaction of Doxorubicin and Daunorubicin Congeners with DNA. *Cancer Res.* **1989**, *49*, 5969–5978.
(13) Murdock, K. C.; Child, R. G.; Fabio, P. F.; Angier, R. B. Antitumor Agents. 1. 1,4-Bis[(aminoalkyl)amino]-9,10-anthracenediones. *J. Med. Chem.* **1979**, *22*, 1024–1030.
(14) Mohammad, A.; Gallib, A. O. Shikonin Derivatives, Part VI. *Agric. Biol. Chem.* **1986**, *50*, 1651–1652.
(15) Liu, L. F.; Rowe, T. C.; Yang, L.; Tewey, K. M.; Chen, G. L. Cleavage of DNA by Mammalian DNA Topoisomerase-II. *J. Biol. Chem.* **1983**, *258*, 15365–15370.
(16) Liu, L. F.; Miller, K. G. Two Forms of DNA Topoisomerase from HeLa cell Nuclei. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *87*, 3487–3491.
(17) Chung, I. K.; Muller, M. T. Aggregates of Oligo(dG) Bind and Inhibit Topoisomerase-II activity and Induce Formation of Large Networks. *J. Biol. Chem.* **1991**, *266*, 9508–9514.

JM9403640