Available online at www.sciencedirect.com
SCIENCE DIRECT
Bioorganic & Medicinal Chemistry 14 (2006) 2048–2051
Bioorganic & Medicinal Chemistry

Inhibition of cellular proliferation by diterpenes, topoisomerase II inhibitor

Shohei Miyata,[a,*] Li-Yan Wang,[b] Chisato Yoshida[a] and Susumu Kitanaka[c]

[a]*Department of Chemistry, College of Humanities and Sciences, Nihon University, Sakurajosui, Setagaya-ku, Tokyo 156-8550, Japan*
[b]*Department of Natural Products Chemistry, Shenyang Pharmaceutical University, 103 Wenhua Road, Shenhe District Shenyang 110016, PR China*
[c]*College of Pharmacy, Nihon University, 7-7-1 Narasinodai, Funabashi, Chiba 274-8555, Japan*

Received 20 September 2005; revised 31 October 2005; accepted 31 October 2005
Available online 28 November 2005

Abstract—We examined the effects of 12 terpene compounds derived from the roots of *Euphorbia kansui* on the proliferative activity of *Xenopus* embryo cells. Eight of these compounds showed significant inhibition of cellular proliferation even at low concentrations, while four of them needed to be present at higher concentrations to inhibit cellular proliferation. In order to define the mechanism of inhibition of cellular proliferation by these compounds, the effects of diterpene compounds on the activity of topoisomerase II were measured. Most of the diterpene compounds that inhibited cellular proliferation also inhibited topoisomerase II activity.

1. Introduction

In vitro, long-term culture of cancer cells with anti-cancer drug candidates has been widely adopted by researchers to screen specific anti-tumor drugs. Whereas because screening with *Xenopus* embryonic cells uses a simple salt solution and the results can be obtained overnight, it is rapid, and the procedure is simpler.[1] We isolated some inhibitory compounds of the embryonic cell from the dried roots of the plant *Euphorbia kansui*[2,3] and demonstrate that these compounds also inhibited the proliferation of cancer cells.[1,4] Some of these compounds were shown to inhibit chromosome condensation and segregation.[4] Among those compounds, which inhibited cellular proliferation and chromosome condensation and segregation, were some which also inhibited the activity of topoisomerase II.[5,6] We then purified the many terpene compounds and examined the effects of these compounds on cellular proliferation and topoisomerase II activity. In *Escherichia coli* and yeasts, topoisomerase II has been implicated in many biological functions, such as DNA replication, transcription, chromosome condensation, and recombination.[7–9] In mammalians, however, the biological functions of topoisomerase II have not been as clearly identified as in *E. coli* and yeasts, perhaps because of the absence of topoisomerase II mutants. For this reason, specific topoisomerase II inhibitors have been applied to various biological systems. There are two well-characterized modes of action of drugs acting against the eukaryotic topoisomerase II. A large number of topoisomerase II inhibitors exert their cytotoxic effects by stabilizing the formation of covalent complexes between topoisomerase II and DNA.[10,11] In recent years, a diverse group acting on other steps in the catalytic cycle of topoisomerase II has been reported, and many of these drugs are widely used as anti-cancer drugs.[12,13] Although many of these drugs inhibited both topoisomerase II activity and cellular proliferation, the mechanisms by which these drugs affect cell growth still remain unclear.[5,6] To determine the mechanism of regulation of cellular proliferation, we first compared the inhibitory activity of many of these diterpene compounds against cellular proliferation to their inhibitory activity against topoisomerase II activity.

2. Results and discussion

2.1. Inhibition of cell proliferation

We studied the effects of compounds (**1**–**12**) on the proliferative activity of embryonic cells (Table 1). All

Keywords: *Euphorbia kansui*; Diterpene; Inhibition of cell proliferation; Inhibition of topoisomerase II; *Xenopus* embryo cell.
* Corresponding author. Tel.: +81 3 3329 1151; fax: +81 3 5317 9433; e-mail: miyata@chs.nihon-u.ac.jp

0968-0896/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2005.10.059

Table 1. Effects of diterpene treatment on the proliferation of cells isolated from the *Xenopus* blastula

	50 μg/ml		10 μg/ml		2 μg/ml		0.5 μg/ml		0.1 μg/ml	
	Inh.	Gro.	Inh.	Gro.	Inh.	Gro.	Inh.	Gro.	Inh.	Gro.
Inhibition of cell proliferation										
20-*O*-(2′*E*,4′*E*)-ingenol (**1**)	115	6	151	11	121	8	102	32	29	85
Percentage of inhibited cells	93		93		94		76		25	
20-*O*-(2′*E*,4′*Z*)-ingenol (**2**)	105	5	164	7	104	23	104	23	40	74
Percentage of inhibited cells	95		96		82		82		35	
3-*O*-(2′*E*,4′*Z*)-ingenol (**3**)	103	4	168	4	129	2	142	15	41	86
Percentage of inhibited cells	97		97		98		90		32	
3-*O*-(2′*E*,4′*E*)-ingenol (**4**)	112	5	149	12	113	9	106	17	41	101
Percentage of inhibited cells	96		92		93		86		29	
3-*O*-(2′*E*,4′*Z*)-5-acetate ingenol (**5**)	78	0	115	12	27	122	—	—	—	—
Percentage of inhibited cells	100		91		18					
3-*O*-(2′*E*,4′*E*)-20-acetate ingenol (**6**)	91	1	86	32	45	125	—	—	—	—
Percentage of inhibited cells	99		93		26					
ingenol (**7**)	85	0	98	26	24	111	—	—	—	—
Percentage of inhibited cells	100		79		18					
20-*O*-(decanoyl)-ingenol (**8**)	97	3	143	4	106	20	129	25	89	59
Percentage of inhibited cells	97		97		86		84		58	
5-*O*-(2′*E*, 4′*E*)-ingenol (**9**)	95	1	123	45	131	21	120	43	43	135
Percentage of inhibited cells	99		73		86		74		24	
5-*O*-benzoyl-20-deoxyingenol (**10**)	96	0	124	0	118	32	96	25	51	139
Percentage of inhibited cells	100		100		79		79		27	
3-*O*-benzoyl-20-deoxyingenol (**11**)	99	0	123	1	126	33	113	45	38	126
Percentage of inhibited cells	100		99		79		72		23	
3-*O*-(2′*E*,4′*E*-decadienoyl)-20-deoxyingenol (**12**)	79	35	71	63	48	60	78	75	—	—
Percentage of inhibited cells	69		53		44		51			

Isolated cells from *Xenopus* blastulae were cultured in a Terasaki plate containing a salt solution, to which diterpenes had been added. After 16 h, the blastomeres were examined for cleavage under a microscope. Abbreviations: Inh, Inhibiting cell of proliferation; Gro, growing cell.

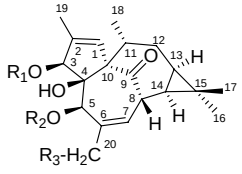
of these compounds inhibited cell growth in a dose-dependent manner. Compounds **1**, **2**, **3**, **4**, **8**, **9**, and **10** (Fig. 1) inhibited cell growth even at concentration as low as 0.5 μg/ml, and compounds **5**, **6**, **7**, and **12** inhibited cell growth at a higher concentration of 10 μg/ml or more. The acetyl ingenol compounds (**5**, **6**, and **7**) showed weaker inhibitory activity against cell growth than the other ingenol compounds (**1**, **2**, **3**, **4**, **8**, and **9**).

2.2. Inhibition of topoisomerase II

The ability of compounds (**1**–**12**) (Fig. 1) to inhibit topoisomerase II activity was quantified by measuring their actions on the supercoiled pBR322 DNA substrate as a function of increasing concentration of terpenes, using agarose gel electrophoresis (Fig. 2). The inhibitory activity of 20 and 100 μg/ml of all the compounds on the activity of topoisomerase II was assayed. For compounds which showed no inhibitory activity against topoisomerase II activity even at 20 μg/ml, the assay at 2 μg/ml was omitted, and for compounds showing no inhibitory activity at 100 μg/ml the assay was carried out at 500 μg/ml of the drug. Compounds **1**, **2**, **3**, **5**, **6**, **7**, **8**, **9**, and **12** inhibited the relaxation of pBR 322 DNA, while compounds **4**, **10**, and **11** did not inhibit the relaxation of pBR322 DNA at any concentration between 2 and 500 μg/ml. Compounds **6** and **9** exerted the most potent topoisomerase II inhibition. Compounds **2**, **5**, and **7** exerted more potent activity than compounds **1**, **8**, and **12**.

2.3. Relationship of inhibition of cell proliferation and topoisomerase II

In this study, the terpene compounds exerted more potent inhibitory activity against cellular proliferation (in vivo) than against human topoisomerase II activity



1 R^1, R^2 = H, R^3 = -O-CO-(CH=CH)$_2$-$(CH_2)_4$-CH_3 (E/E)

2 R^1, R^2 = H, R^3 = -O-CO-(CH=CH)$_2$-$(CH_2)_4$-CH_3 (E/Z)

3 R^1 = CO-(CH=CH)$_2$-$(CH_2)_4$-CH_3, R^2, R^3 = OH (E/Z)

4 R^1 = CO-(CH=CH)$_2$-$(CH_2)_4$-CH_3, R^2, R^3 = OH (E/E)

5 R^1 = CO-(CH=CH)$_2$-$(CH_2)_4$-CH_3, R^2 = COMe, R^3 = OH (E/Z)

6 R^1 = CO-(CH=CH)$_2$-$(CH_2)_4$-CH_3, R^2 = H, R^3 = -O-COMe (E/Z)

7 R^1 = CO-(CH=CH)$_2$-$(CH_2)_4$-CH_3, R^2 = H, R^3 = -O-COMe (E/E)

8 R^1, R^2 = H, R^3 = -O-CO-$(CH_2)_8$-CH_3

9 R^1 = H, R^2 = CO-(CH=CH)$_2$-$(CH_2)_4$-CH_3, R^3 = OH (E/E)

10 R^1=H, R^2=benzoyl, R^3=H

11 R^1=benzoyl, R^2=H, R^3=H

12 R^1=CO-(CH=CH)$_2$-$(CH_2)_4$-CH_3, R^2=H,R^3=H (E/E)

Figure 1. Structures of the diterpene compounds.

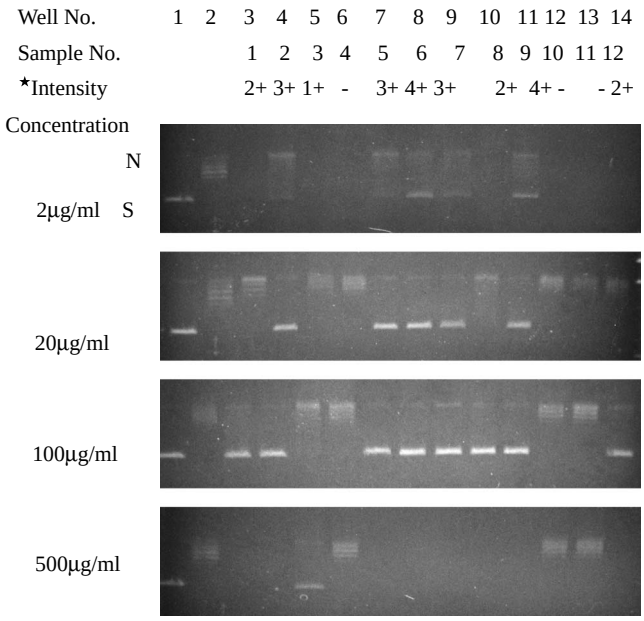


Figure 2. Inhibition of human topoisomerase II by diterpenes (**1**–**12**). Each reaction mixture (20 μl) contained 30 ng pBR322 plasmid DNA, 0.2 U of human topoisomerase IIα, and 2, 20, 100, or 500 μg/ml diterpenes. After incubation for 1 h at 37 °C, the mixture was loaded onto a sample well for agarose gel electrophoresis. Lanes: 1, no enzyme; 2, plus enzyme, 3, plus 20-*O*-(2′*E*,4′*E*-decadienoyl)-ingenol (**1**), 20-*O*-(2′*E*,4′*Z*-decadienoyl)-ingenol (**2**), 3-*O*-(2′*E*,4′*Z*-decadienoyl)-ingenol (**3**), 3-*O*-(2′*E*,4′*E*-decadienoyl)-ingenol (**4**), 3-*O*-(2′*E*,4′*Z*-decadienoyl)-5-*O*-acetyl-ingenol (**5**), 3-*O*-(2′*E*,4′*Z*-decadienoyl)-20-*O*-acetyl-ingenol (**6**), 3-*O*-(2′*E*,4′*E*-decadienoyl)-20-*O*-acetyl-ingenol (**7**), 20-*O*-(decanoyl)-ingenol (**8**), 5-*O*-(2′*E*,4′*E*-decadienoyl)-ingenol (**9**), 5-*O*-benzoyl-**20**-deoxyingenol (**10**), 3-*O*-benzoyl-20-deoxyingenol (**11**), and 3-*O*-(2′*E*,4′*E*-decadienoyl)-20-deoxyingenol (**12**). Empty wells: 2 μg/ml, well Nos. 3, 5, 6, 10, 12, 13, and 14; 500 μg/ml, well Nos. 3, 4, 7, 8, 9, 10, 11, and 14. Abbreviations: *Intensity, the intensities of inhibition of topoisomerase II activity are shown. N, nicked DNA; S, supercoiled DNA.

(in vitro). We compared the sensitivity of the cellular proliferative activity and topoisomerase II activity to inhibition by ICRF 193; while inhibition of cellular proliferative activity was observed at a concentration of 50 μg/ml, inhibition of topoisomerase II activity was observed only at a concentration of 250 μg/ml or more (unpublished data). We, therefore, concluded that cellular proliferative activity is more sensitive than topoisomerase II activity to inhibition by ICRF 193.

The cell viability of the embryonic cells was checked by trypan blue exclusion. The viability of the embryonic cells remained at 100% following treatment with any of compounds **1**–**12** at any concentration in the range of 0.1–250 μg/ml. We have previously reported that adriamycin kills almost 100% of the cells at 0.1 μg/ml.[1] All anti-topoisomerase II agents are able to interfere with at least one step of the catalytic cycle.[7–9] Agents able to stabilize the covalent DNA topoisomerase II complex are called topoisomerase II poisons (adriamycin, etoposide), while agents acting on other steps of the catalytic cycle are called catalytic inhibitors (ICRF-193, merbarone); it has also been shown that catalytic inhibitors show weaker cytotoxicity than topoisomerase poisons.[10,14,15] We have shown in this study that terpene compounds can inhibit both cellular proliferation and topoisomerase II activity and still show no cytotoxic effects.

Although ingenol compound **3** faintly inhibited topoisomerase II activity (500 μg/ml), its inhibition of embryonic cell growth was more pronounced, and the compound inhibited cellular proliferation at even very low concentrations (0.5 μg/ml). Compound **6** also significantly inhibited topoisomerase II activity (2 μg/ml) and in the presence of a high concentration (10 μg/ml), this compound also inhibited cellular proliferation activity. The intensity of inhibition of cellular proliferation by terpenes (in vitro) was not correlated with the intensity of inhibition of topoisomerase II activity by these compounds (in vitro). It appears likely that the cellular uptake may play an important role in determining the intensity of inhibition of cellular proliferation. The stability was also different among the terpene compounds. Inhibition of cellular proliferation by compounds **4**, **10**, and **11** may be mediated by their effects on any other cellular enzyme. Thus, in this study, we isolated terpene compounds having varying capacities for inhibition of cellular proliferation and topoisomerase II activity.

In conclusion, we isolated many terpene compounds from the dried roots of the plant *kansui* and selected anti-proliferative compounds using our isolated *Xenopus* embryonic cell assay system. To understand how the diterpene compounds inhibit the cell cycle, we studied effects of the compounds on topoisomerase II activity. Anti-proliferative compounds showed different capacities for inhibition of cellular proliferation and topoisomerase II activity.

3. Experimental

3.1. Embryos

Xenopus laevis eggs were obtained by natural amplexus of the males and females after injection of human chorionic gonadotropin (200 IU) and de-jellied with 2.5% cysteine. The embryos were then allowed to develop to stage 8 at room temperature.

3.2. Cell culture

Twelve ingenol-type diterpenes have been isolated from the root of *E. kansui*: 20-*O*-(2′*E*,4′*E*-decadienoyl)-ingenol (**1**), 20-*O*-(2′*E*,4′*Z*-decadienoyl)-ingenol (**2**), 3-*O*-(2′*E*,4′*Z*-decadienoyl)-ingenol (**3**), 3-*O*-(2′*E*,4′*E*-decadienoyl)-ingenol (**4**), 3-*O*-(2′*E*,4′*Z*-decadienoyl)-5-*O*-acetyl-ingenol (**5**), 3-*O*-(2′*E*,4′*Z*-decadienoyl)-20-*O*-acetyl-ingenol (**6**), 3-*O*-(2′*E*,4′*E*-decadienoyl)-20-*O*-acetyl-ingenol (**7**), 20-*O*-(decanoyl)-ingenol (**8**), 5-*O*-(2′*E*,4′*E*-decadienoyl)-ingenol (**9**), 5-*O*-benzoyl-20-deoxyingenol (**10**), 3-*O*-benzoyl-20-deoxyingenol (**11**), and 3-*O*-(2′*E*,4′*E*-decadienoyl)-20-deoxyingenol (**12**) (Fig. 1). Compounds **1**, **2**, **5**, **9**, and **12** are new compounds, compound **4** has a new geometric configuration, and compound **8** is the first to be described as a natural compound. The diterpene compounds were dissolved

in dimethylsulfoxide (DMSO), and after appropriate dilution, 1 μl of each of these compounds was added to 200 μl of 2 mg/ml γ-globulin in a simple salt solution (NAM/2: 55 mM NaCl, 1 mM KCl, 0.5 mM $Ca(NO_3)_2$, 0.5 mM $MgSO_4$, 0.05 mM EDTA, 0.5 mM $NaHCO_3$, and 1 mM sodium phosphate buffer, pH 7.5). As a control solution, 1 μl of the same dilution of DMSO without the drug was added to 200 μl of the medium. Animal cap pieces were dissected from stage 8 blastulae. Single cells from the inner surface of the pieces were separated by directing a gentle stream of a calcium and magnesium-free medium (50 mM phosphate buffer containing 35 mM NaCl and 1 mM KCl, pH 7.0). Two or three cells were transplanted into a well of a Terasaki microculture plate containing 10 μl medium and cultured in the presence or absence of an anti-cancer drug or anti-cancer drug candidate. After incubation for 16 h at 25 °C, the cells were counted under a binocular microscope.

3.3. Assay of topoisomerase II activity

The activity of the compounds on the relaxation of DNA topoisomerase IIα (human recombinant in *E. coli*, USB Corporation, Ohio) was determined by measuring the conversion of supercoiled PBR 322 plasmid DNA to its relaxed form. The reaction mixture contained 10 mM Tris–HCl (pH 7.9, 50 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 15 μg/ml BSA, 1 mM ATP, 30 ng PBR 322, 0.2 U enzyme, and different concentrations of the drugs in a total volume of 20 μl). After incubation for 1 h at 37 °C, the mixtures were subjected to electrophoresis on 0.7% agarose gel. After electrophoresis, the gels were stained with ethidium bromide and photographed under UV light.

Acknowledgments

This work was financially supported in part by a Grant-in-Aid for Scientific Research (No.14572017) from the Ministry of Education, Science, Sports and Culture of Japan, by a grant from the Ministry of Education, Culture, Sports, Science and Technology to promote multidisciplinary research projects, and by a Grant-in-Aid for Research on Eye and Ear Science, Immunology, Allergy and Organ Transplantation from the Health Sciences Research Grants, for Ministry of Health Labour and Welfare.

References and notes

1. Miyata, S.; Wang, L. Y.; Wang, L. Y.; Yao, X. S.; Kitanaka, S. *Cell Biol. Int.* **2004**, *28*, 179.
2. Wang, L. Y.; Wang, N. L.; Yao, X. S.; Miyata, S.; Kitanaka, S. *J. Nat. Prod.* **2003**, *66*, 630.
3. Wang, L. Y.; Wang, N. L.; Yao, X. S.; Miyata, S.; Kitanaka, S. *J. Nat. Prod.* **2002**, *65*, 1246.
4. Okouchi, T.; Abe, T.; Araki, S.; Arai, S.; Iida, T.; Wang, L. Y.; Kitanaka, S.; Miyata, S. *Bioorg. Med. Chem.* **2005**, *13*, 3847.
5. Ishida, R.; Sato, M.; Narita, T.; Utsumi, K. R.; Nishimoto, T.; Morita, T.; Nagata, H.; Andoh, T. *J. Cell Biol.* **1994**, *126*, 1341.
6. Anderson, H.; Roberge, M. *Cell Growth Differ.* **1996**, *7*, 83.
7. Wang, J. C. *Annu. Rev. Biochem.* **1985**, *54*, 665.
8. Gellert, M. *Annu. Rev. Biochem.* **1981**, *50*, 879.
9. Liu, L. F. *Annu. Rev. Biochem.* **1989**, *58*, 351.
10. Andoh, T.; Ishida, R. *Biochim. Biophys. Acta* **1998**, *1400*, 1155.
11. Larsen, A. K.; Escargueil, A. E.; Skladanowski, A. *Prog. Cell Cycle Res.* **2003**, *5*, 295.
12. Hande, K. R. *Biochim. Biophys. Acta* **1998**, *1400*, 173.
13. Kaufmann, S. H. *Biochim. Biophys. Acta* **1998**, *1400*, 195.
14. Perez, C.; Vilaboa, N. E.; Garcia-Bermejo, L.; de Blas, E.; Creighton, A. M.; Aller, P. *J. Cell Sci.* **1997**, *110*, 337.
15. Jensen, L. H.; Nitiss, K. C.; Rose, A.; Dong, J.; Zhou, J.; Hu, T.; Osheroff, N.; Jensen, P. B.; Sehested, M.; Nitiss, J. L. *J. Biol. Chem.* **2000**, *275*, 2137.