Inhibitory Activity of Plant Stilbene Oligomers against DNA Topoisomerase II

Masashi YAMADA,^a Ken-ichiro HAYASHI,^b Shogo IKEDA,^b Ken TSUTSUI,^c Kimiko TSUTSUI,^c Tetsuro ITO,^d Munekazu INUMA,^e and Hiroshi NOZAKI^{*,b}

^aMeiji Dairies Corporation; 1–2–10 Shinsuna, Koto-ku, Tokyo 136–8908, Japan; ^bDepartment of Biological Chemistry, Faculty of Science, Okayama University of Science; 1–1 Ridai-cho, Okayama 700–0005, Japan; ^cGraduate School of Medicine, Dentistry and Pharmaceutical Science, Okayama University; 2–5–1 Shikatacho, Okayama 700–8558, Japan; ^dGifu Prefectural Institute of Health and Environmental Sciences; 1–1 Naka-Fudogaoka, Kakamigahara, Gifu 504–0838, Japan; and ^eDepartment of Pharmacognosy, Gifu Pharmaceutical University; 5–6–1 Mitahora-higashi, Gifu 502–8585, Japan. Received March 9, 2006; accepted April 5, 2006; published online April 18, 2006

The inhibitory activity of 40 stilbene oligomers isolated from six plant species against topoisomerase II was evaluated, of which nine compounds showed a potent inhibitory effect, stronger than daunorubicin, a topoisomerase II inhibitor, used as an anti-cancer drug. The specificity of active stilbene oligomers on topoisomerase II was assessed by their effect on DNA restriction enzyme. In particular, specific inhibitory activity was observed in α -viniferin 13-O- β -glucopyranoside (2) and hemsleyanol C (13).

Key words stilbene; topoisomerase; inhibitor

Type II DNA topoisomerases (topo II) catalyze topological changes in the DNA molecule through transient breakage and rejoining of double-stranded DNA. These reactions relax supercoiled DNA and catenate or decatenate covalently closed circular DNA.¹⁾ Topo II are essential cellular enzymes required for cell proliferation and, therefore, are clinical targets for anti-cancer drugs. Although inhibitors against topo II have been developed and applied clinically, their severe side-effects remain a serious problem. In the development of new inhibitors, natural products from plant sources can be valuable when used directly and may also serve as a suitable lead for the production of semi-synthetic active agents. In the continuous search for topo II inhibitors in plant extracts, we have found that stilbene oligomers, nepalensinols from *Kobresia nepalensis*, showed significant inhibitory activity against topo II.^{2,3)} This finding prompts us to investigate topo II inhibitory activity of other stilbene oligomers. We herein report the inhibitory effect of 40 stilbene oligomers from six species of plant and their structure–activity correlation.

The inhibitory activity of stilbene oligomers **1**–**40** against topo II was evaluated by their inhibitory effect against the decatenation activity of topo II on kinetoplast DNA. Initial screening for inhibition was performed at concentrations of 10, 100 and 500 μ g/ml. The 35 stilbenes exhibiting inhibitory activity at less than 500 μ g/ml were further analysed to determine their IC₅₀ values against topo II and DNA restriction enzyme, *Hind*III. IC₅₀ values, as determined from three individual experiments with three replicates for each concentration, are listed in Table 1. Twelve compounds (**2**, **6**, **7**, **8**, **9**, **13**, **14**, **15**, **21**, **22**, **28**, **36**) showed IC₅₀ values ranging from 0.9 to 7.2 μ M, which were stronger than the positive control, daunorubicin (8.5 μ M), a topo II inhibitor. In particular, compound **13** exhibited the most potent activity, which was eight times stronger than daunorubicin.

Compounds **1** and **2** are resveratrol trimers with a nine-membered ring, and **2** is the glucoside of **1** at the C-13b position. The inhibitory activity of **2** was stronger than that of **1**–substitution of glucose resulting in increased activity in this type of stilbene. Resveratrol dimers **3**–**5**, and trimer **6**,

have the same partial structure of a seven-membered ring. Compound **5**, an epimer at C-7b of **4**, was less active than **4**. In addition, compound **3** is a stereoisomer at C-7b and C-8b of **4**, and it displayed less activity than **5**. On the other hand, compound **6**, a resveratrol trimer, showed the most potent activity. On the basis of these findings, the stereochemistry (7b- β and 8b- α hydrogen) at C-7b and C-8b, and the addition of a resveratrol unit, appears to be essential for inhibitory activity in this type of stilbene. Compounds **7**–**10** are resveratrol tetramers composed of two dimers with a seven-membered ring, and stereoisomers to each other. Different levels of inhibitory activity were seen in these compounds, *i.e.* compounds **7**–**9** showed significantly strong activity, while **10** was considerably less active. Their structural differences are in a symmetrical pattern, *i.e.* molecular structures in **7**–**9** are symmetrical and compound **10** is asymmetrical. Therefore, in this type of stilbene, an asymmetric pattern results in decreased inhibitory activity. This can be seen clearly where compounds **11** and **12** are inactive over 50 μ M. Compounds **13**–**15** have the same partial structure with five- and seven-membered rings. All three compounds showed significant activity higher than daunorubicin and compound **13** was the most potent of the stilbene oligomers examined in this assay. The slight difference of activity between these compounds seems to originate in the configuration pattern at 7b, 8b, 7c and 8c, and the addition of another resveratrol unit. Although compounds **16**–**21** also have the same partial structure, the linkage style with the third resveratrol unit is different from compounds **13**, **14** and **15**. Among compounds **16**–**21**, only compound **21**, a highly condensed resveratrol hexamer, showed comparatively high activity. However, the structure–activity correlation in this type of stilbene is unclear. Compound **22**, a resveratrol trimer, was active at 5.3 μ M and more potent than **23** or **24**. The addition of a resveratrol unit to **23** seems to produce an increase in activity. Compounds **25**–**27** have same partial stereostructure, but compound **27** was inactive. The C2-asymmetrical nature and addition of a glucose moiety at C-12' and C-12'' in **26** caused a remarkable diminution in inhibitory activity.

* To whom correspondence should be addressed. e-mail: nozaki@dbc.ous.ac.jp

Compounds 7-10 are giastereomeric

7: symmetrical; (H-7a, H-8a)=(β , α); (H-7b, H-8b) = (β , α)

8: symmetrical; (H-7a, H-8a)=(α , β); (H-7b, H-8b) = (β , α)

9: symmetrical; (H-7a, H-8a)=(β , α); (H-7b, H-8b) = (α , α)

10: asymmetrical; (H-7a, H-8a)=(α , β); (H-7b, H-8b) = (β , α)
 (H-7c, H-8c)=(β , α); (H-7d, H-8d) = (β , α)

15: giastereomer of **14**
 H-8b, H-7c and H-8c = (β , β , α)

18: giastereomer of **16**
 H-7b, H-7c and H-8c = (β , α , β)

Table 1. Inhibitory Activity of Stilbene Oligomers against Topo II and *HindIII*

Compounds	Topo II ^{a)}	<i>HindIII</i> ^{a)}	Specificity ^{b)}
Daunorubicin	8.5	103.9	12.3
1	27	68	3
2	4	279	67
3	145	268	2
4	11	355	33
5	47	523	11
6	5	36	8
7	6	33	6
8	6	n.d.	n.d.
9	4	78	22
10	76	73	1
11	>50	n.d.	n.d.
12	>50	n.d.	n.d.
13	1	69	80
14	3	n.d.	n.d.
15	6	143	23
16	65	253	4
17	77	594	8
18	82	90	1
19	70	97	1
20	85	187	2
21	5	12	2
22	5	60	11
23	>500	415	1
24	63	812	13
25	11	n.d.	n.d.
26	11	40	4
27	483	483	1
28	7	n.d.	n.d.
29	>500	617	1
30	21	307	15
31	262	500	2
32	197	800	4
33	>500	>1000	n.d.
34	>500	>1000	n.d.
35	>500	>1000	n.d.
36	7	104	14
37	104	429	4
38	88	594	7
39	11	796	71
40	26	148	6

a) IC₅₀ values (μM). b) Specificity values are indicated as the ratio of IC₅₀ of *HindIII* to topo II. n.d.: not determined.

To evaluate the specificity of active stilbenes on topo II inhibition, the effect of these compounds on the activity of a *HindIII* restriction enzyme was examined. *HindIII* enzyme recognizes DNA as a substrate and catalyzes the cleavage of DNA at a specific sequence. Compounds **2**, **9**, **13** and **15** displayed higher activity and specificity for topo II than daunorubicin. In particular, the specificity value (ratio of IC₅₀ of *HindIII* to topo II) of **2** and **13** was over 60. These compounds, therefore, could be suitable candidates as specific inhibitors of topo II.

Some topoisomerase inhibitors such as doxorubicin, am-sacrine and ellipticine are DNA intercalators. In order to determine the DNA binding activity of several stilbene oligomers isolated in this study, ethidium bromide competition assay was carried out using salmon sperm DNA. Two compounds (**5**, **21**) competed with ethidium bromide for DNA, indicating that the stilbenes have some ability to intercalate into DNA strand (data not shown). Further analysis would clarify the inhibitory mechanism of the stilbenes on

topo II.

MATERIALS AND METHODS

Compounds Stilbene oligomers were isolated from *Shorea hemsleyana*^{4–7)} (compounds **2**, **3**, **6**, **9**, **13**, **15**, **24**, **27**, **29**, **30**, **39**), *Vatica rassak*^{8–10)} (**16**–**21**, **23**, **31**, **32**, **36**–**38**), *Vatica indica*¹¹⁾ (**4**, **10**, **26**), *Hopea utilis*¹²⁾ (**5**, **40**), *Gnetum parvifolium*¹³⁾ (**33**, **34**, **35**), *Kobresia nepalensis*^{2,3,23)} (**8**, **11**, **12**, **14**, **22**, **25**, **28**), and two stilbenoids (**1**, **7**) from *Shorea hemsleyana*⁴⁾ and *Kobresia nepalensis*, respectively.

Plant Materials *Shorea hemsleyana* and *Vatica rassak* were collected in Indonesia in October 1997, *Vateria india* and *Hopea utilis* in India in August 1999, *Gnetum parvifolium* in the Botanical Garden of the University of Tokyo in April 1999, and *Kobresia nepalensis* in Himalaya, Nepal in August 1995.

Topoisomerase II Assay Purified human topoisomerase II (topo II) was purchased from TopoGen, Inc. (U.S.A.) and kinetoplast DNA was purified from *Crithidia fasciculata* with cesium chloride using step-gradient centrifugation, as described in a previous report.¹⁵⁾ Topo II activity was assessed by a decatenation reaction of kinetoplast DNA.¹⁶⁾ The assay was performed in a reaction mixture (20 μl) containing 50 mM Tris-HCl (pH 7.9), 120 mM KCl, 10 mM MgCl₂, 0.5 mM dithiothreitol, 0.5 mM EDTA (pH 8.0), 0.5 mM ATP, 30 μg/ml bovine serum albumin and 0.25 μg/ml of kinetoplast DNA, as described previously.¹⁷⁾ A DMSO solution of the stilbenoids was added to the reaction mixture to give the desired concentrations. The reaction was initiated by adding 1 μl of topo II (0.75 U). After incubation at 30 °C for 30 min, the reaction was terminated by addition of 4 μl of the solution (0.66% SDS and 0.33 mg/ml proteinase K) prior to analysis of DNA products by 0.8% agarose gel electrophoresis. Gels were stained with ethidium bromide and photographed under UV light. Monomer minicircles released from the kinetoplast DNA were quantified by NIH image software (NIH, Bethesda, MD, U.S.A.).

HindIII Assay *HindIII* activity was assessed by conversion of supercoiled plasmid DNA to the linear form. The assay was performed in a reaction mixture (20 μl) containing 10 mM Tris-HCl (pH 7.9), 50 mM NaCl, 10 mM MgCl₂, and 200 ng of pBluescript SK(–) DNA with or without test compounds. The reaction was started by adding 1 μl of *HindIII* (1.25 U; New England Biolab., U.S.A.). After incubation at 37 °C for 60 min, the plasmids were separated by agarose gel electrophoresis in the presence of ethidium bromide. The linear form DNAs generated by *HindIII* reaction were quantified by densitometry.

REFERENCES

- 1) D'Arpa P., Liu L. F., *Biochim. Biophys. Acta*, **989**, 163–177 (1989).
- 2) Yamada M., Hayashi K., Hayashi H., Ikeda S., Hoshino T., Tsutsui Ke., Tsutsui Ki., Iinuma M., Nozaki H., *Phytochemistry*, **67**, 307–313 (2006).
- 3) Yamada M., Hayashi K., Hayashi H., Tsuji R., Kakumoto K., Ikeda S., Hoshino T., Tsutsui Ke., Tsutsui Ki., Ito T., Iinuma M., Nozaki H., *Chem. Pharm. Bull.*, **54**, 354–358 (2006).
- 4) Ito T., Tanaka T., Ido Y., Nakaya K., Iinuma M., Riswan S., *Chem. Pharm. Bull.*, **48**, 1001–1005 (2000).
- 5) Ito T., Tanaka T., Iinuma M., Nakaya K., Takahashi Y., Nakamura H., Nakagawa H., Riswan S., *Helv. Chim. Acta*, **86**, 3394–3401 (2003).

- 6) Tanaka T., Ito T., Nakaya K., Iinuma M., Takahashi Y., Naganawa H., Riswan S., *Heterocycles*, **55**, 729—740 (2001).
- 7) Ito T., Tanaka T., Ido Y., Nakaya K., Iinuma M., Riswan S., *Chem. Pharm. Bull.*, **48**, 1959—1963 (2000).
- 8) Tanaka T., Ito T., Nakaya K., Iinuma M., Riswan S., *Phytochemistry*, **54**, 63—69 (2000).
- 9) Ito T., Tanaka T., Nakaya K., Iinuma M., Takahashi Y., Naganawa H., Ohyama M., Nakanishi Y., Bastow K. F., Lee K. H., *Tetrahedron*, **57**, 7309—7321 (2001).
- 10) Ito T., Tanaka T., Ido Y., Nakaya K., Iinuma M., Takahashi Y., Naganawa H., Riswan S., *Heterocycles*, **55**, 557—567 (2001).
- 11) Ito T., Tanaka T., Iinuma M., Nakaya K., Takahashi Y., Sawa R., Naganawa H., Chelladurai V., *Tetrahedron*, **59**, 1255—1264 (2003).
- 12) Tanaka T., Ito T., Ido Y., Nakaya K., Iinuma M., Chelladurai V., *Chem. Pharm. Bull.*, **49**, 785—787 (2001).
- 13) Tanaka T., Iliya I., Ito T., Furusawa M., Nakaya K., Iinuma M., Shirataki Y., Matsuura N., Ubukata M., Murai J., Shimozone F., Hirai K., *Chem. Pharm. Bull.*, **49**, 858—862 (2001).
- 14) Ohyama M., Tanaka T., Iinuma M., Burandt C. L., *Chem. Pharm. Bull.*, **46**, 663—668 (1998).
- 15) Englund P. T., *J. Biol. Chem.*, **254**, 4895—4900 (1979).
- 16) Miller K. G., Liu L. F., Englund P. T., *J. Biol. Chem.*, **256**, 9334—9339 (1981).
- 17) Tsutsui K. E., Tsutsui K. I., Sakurai H., Shohmori T., Oda T., *Biochem. Biophys. Res. Commun.*, **138**, 1116—1122 (1986).