Pergamon

SCIENCE DIRECT

Bioorganic & Medicinal Chemistry 11 (2003) 1137–1140

BIOORGANIC & MEDICINAL CHEMISTRY

Antitumor Agents 220. Antitumor-Promoting Effects of Cimigenol and Related Compounds on Epstein–Barr Virus Activation and Two-Stage Mouse Skin Carcinogenesis

Nobuko Sakurai,[a] Mutsuo Kozuka,[a] Harukuni Tokuda,[b] Yoshitaka Nobukuni,[b] Junko Takayasu,[b] Hoyoku Nishino,[b] Akiko Kusano,[c] Genjiro Kusano,[c] Masahiro Nagai,[d] Yojiro Sakurai[a] and Kuo-Hsiung Lee[a,e,*]

[a]*Natural Products Laboratory, School of Pharmacy, University of North Carolina, Chapel Hill, NC 27599-7360, USA*
[b]*Department of Biochemistry, Kyoto Prefectural University of Medicine, Kamigyo-ku, Kyoto 602-0841, Japan*
[c]*Osaka University of Pharmaceutical Science, Nasahara, Takatsuki 569-1094, Japan*
[d]*Hoshi University, Ebara, Shinagawa-ku, Tokyo 142-8501, Japan*
[e]*Plantaceutica, Inc., 99 Alexander Drive, Research Triangle Park, NC 27709-2060, USA*

Received 25 June 2002; accepted 7 August 2002

Abstract—Cimigenol (**1**) and 39 related compounds were screened as potential antitumor promoters by examining the ability of the compounds to inhibit Epstein–Barr virus early antigen (EBV-EA) activation (induced by 12-*O*-tetradecanoylphorbol-13-acetate) in Raji cells. Structure–activity relationship analysis indicated that compound **1** showed the highest activity and also exhibited significant inhibitory effects on mouse skin tumor promotion in an in vivo two-stage carcinogenesis test. These data suggest that **1** and the related compounds might be valuable anti-tumor promoters.

Introduction

Cimigenol (23*R*,24*S*)-16,23;16,24-diepoxy-9,19-cyclolanostan-3β,15α,25-triol (**1**) was isolated as an aglycone of cimigenol xyloside (**2**), obtained from *Cimicifuga racemosa* (Ranunculaceae).[1] Recently, other cimigenol glycosides were reported from the same plant.[2] We further reported that cimigenol xyloside (**2**) was a transformation product of acetylshengmanol xyloside (**22**) and 24-*O*-acetylshengmanol xyloside (**19**), which are main components of *C. dahurica*.[3] *C. racemosa*, commonly known as black cohosh, has been used for relief of menopausal symptoms in America and Europe.[4] In Japan and China, some *Cimicifuga* spp. are used as antipyretic and analgesic agents in traditional Chinese medicine.[5]

We have been extremely interested in the chemoprevention of cancer by natural products. We found that cucurbitane triterpenoids exhibited strong inhibitory effects on EBV-EA activation induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in Raji cells.[6] Further, many compounds that inhibit EBV-EA induction by tumor promoters have been shown to act as inhibitors of tumor promotion in vivo.[7]

In this study, we report the results of primary screening for the inhibitory effects of 40 cimigenol related compounds (**1**–**40**) on EBV-EA activation. We also report the results of an in vivo two-stage carcinogenesis test on mouse skin tumor promotion with cimigenol (**1**), the most potent compound in the EBV-EA assay.

Materials and Methods

Test products

The chemical structures are shown in Figure 1. Cimigenol (**1**), cimigenol xyloside (**2**), acetylshengmanol xyloside (**19**), and 24-*O*-acetylhydroshengmanol xyloside (**22**) were isolated from *C. japonica*.[5] Cimicifugosides H-1

*Corresponding author. Tel.: +1-919-962-0066; fax: +1-919-966-3893; e-mail: khlee@unc.edu

0968-0896/03/$ - see front matter © 2002 Elsevier Science Ltd. All rights reserved.
PII: S0968-0896(02)00432-7

(**24**), H-4 (**25**), and actein (**26**) were isolated from commercially available *Cimicifuga* plants.[8] Compound **27** was obtained as an aglycone of cimicifugoside from *C. simplex*.[9] The remaining compounds have been reported in the literature.[5,10]

Chemicals

The cell culture reagents, *n*-butyric acid, and other reagents were purchased from Nacalai Tesque Inc. (Japan). TPA and 7,12-dimethylbenz[*a*]anthracene (DMBA) were purchased from Sigma Chemical Co. (St. Louis, MO).

In vitro EBV-EA activation experiments

EBV-EA positive serum from a patient with nasopharyngeal carcinoma (NPC) was a gift from Professor H. Hattori, Department of Otorhinolaryngology, Kobe University. The EBV genome carrying lymphoblastoid cells (Raji cells derived from Burkitt's lymphoma) were cultured in 10% fetal bovine serum (FBS) in RPMI-1640 medium (Nissui, Japan). Spontaneous activation of EBV-EA in our subline of Raji cells was less than 0.1%. The inhibition of EBV-EA activation was assayed using Raji cells (virus non-producer type) as described previously.[11] The cells were incubated at 37 °C for 48 h

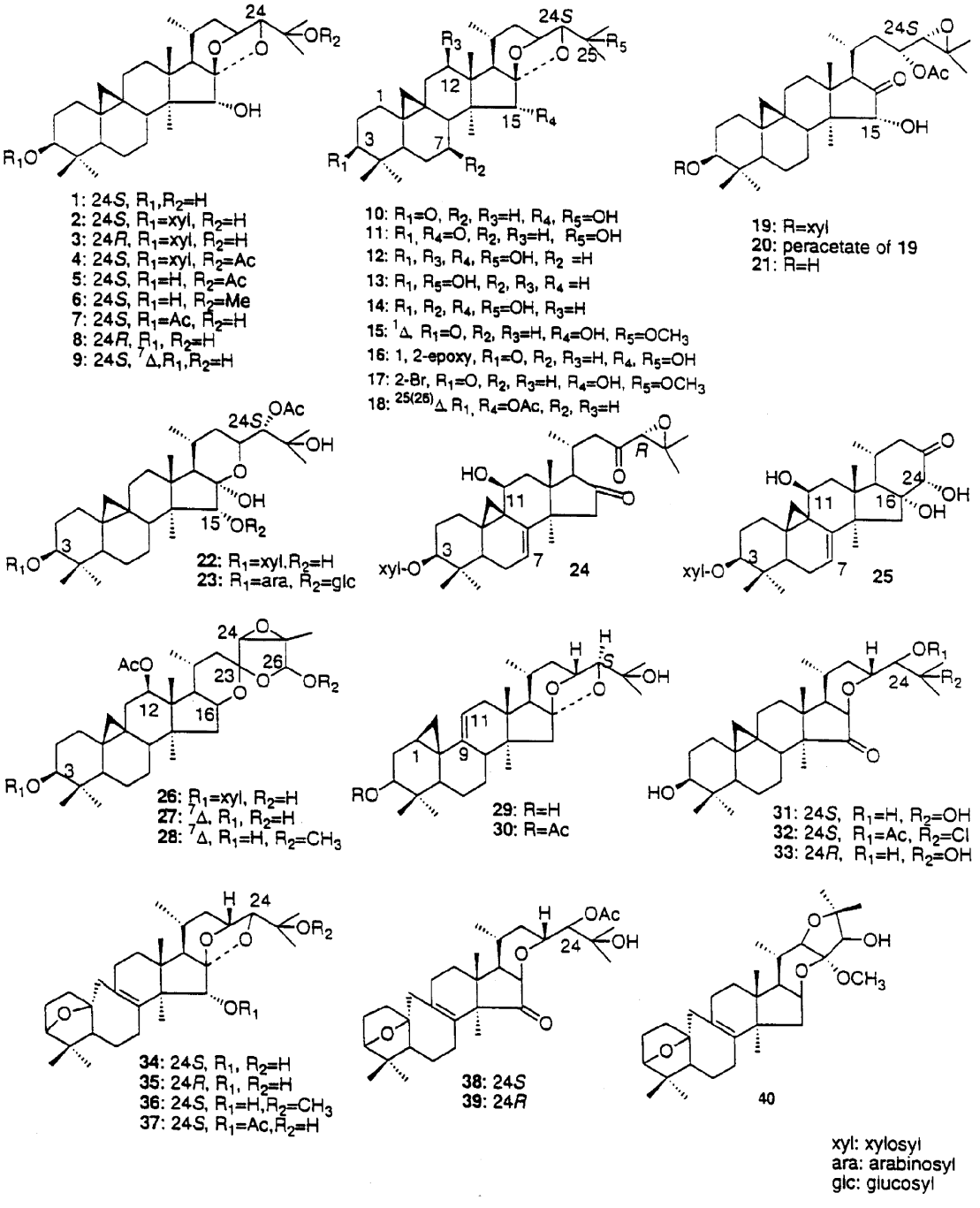

Figure 1. Structures of compounds **1**–**40**.

in medium containing *n*-butyric acid (4 mM), TPA [(32 pM = 20 ng in dimethyl-sulfoxide (DMSO), 2 μL)] as inducer in 5 mL DMSO, and various amounts of the test compounds dissolved in 5 μL of DMSO. Smears were made from the cell suspension. The EBV-EA inducing cells were stained by the means of an indirect immunofluorescence technique.[12] In each assay, at least 500 cells were counted, and the number of stained cells (positive cells) was recorded. Triplicate assays were performed for each compound. The average EBV-EA induction of the test compound was expressed as a ratio relative to the control experiment (100%), which was carried out with *n*-butyric acid (4 mM) plus TPA (32 pM). EBV-EA induction was ordinarily around 35%. The viability of treated Raji cells was assayed by the Trypan blue staining method.

In vivo two-stage carcinogenesis test on mouse skin papillomas

Specific pathogen-free female ICR mice (6 weeks old) were obtained from Japan SLC, Inc. (Hamamatsu, Japan), and were housed five per polycarbonate cage in a temperature-controlled room. All mice were fed oriental MF (Oriental Yeast Co., Tokyo, Japan) and water ad libitum during the experiment. The animals were divided into two experimental groups of 15 mice each. The back of each mouse was shaved with surgical clippers, and the mice were treated topically with DMBA (100 μg, 390 nmol) in acetone (0.1 mL). One week after the initiation, papilloma formation was promoted twice a week by the application of TPA (1 μg, 1.7 nmol) in acetone (0.1 mL) on the skin. Group I received TPA treatment alone and group II received TPA and cimigenol (**1**) (85 nmol) 1 h before each TPA treatment. The incidence and numbers of papillomas were detected weekly for 20 weeks.

Results and Discussion

The primary screening test was carried out using a short-term in vitro synergistic assay on EBV-EA activation. Table 1 lists inhibitory effects of compounds (**1**–**40**) on EBV-EA activation induced by TPA and the associated viability of Raji cells.

In this assay, all compounds tested showed inhibitory effects on EBV-EA activation without cytotoxicity on Raji cells (a high viability of Raji cells is necessary for in vitro assay using an indirect immunofluorescence technique by antigen–antibody reaction and is beneficial for the subsequent in vitro assay). As shown in Table 1, cimigenol (**1**), cimigen-3-one (**10**), cimigen-3,15-dione (**11**), 12β-hydroxycimigenol (**12**), 15-deoxycimigenol (**13**), and 7β-hydroxycimigenol (**14**) exhibited significant inhibitory effects (100% inhibition of activation at 1×10^3 mol ratio/TPA, 70–85% inhibition at 500 mol ratio/TPA, and 30–40% inhibition of activation even at 100 mol ratio/TPA). The inhibitory activities of these compounds were stronger than that of glycyrrhetic acid,[6] which is known as a strong anti-tumorpromoter. Because many natural products that strongly inhibit EBV-EA activation induced by the tumor promoter TPA have also been shown to act as inhibitors of tumor promotion in vivo,[6,7] these in vitro results with cimigenol related compounds strongly suggested that these triterpenoids might be valuable anti-tumor promoters as well.

The effects of cimigenol (**1**) on the two-stage carcinogenesis test of mouse skin papillomas were investigated using DMBA as an initiator and TPA as a promoter. The incidence (%) of papilloma-bearing mice and the average numbers of papillomas per mouse are presented in Figure 2A and B, respectively. As shown in Figure 2A, in the positive control, 100% of the mice bore papillomas as early as 10 weeks of promotion. Further, averages of 4.8 and 9.1 papillomas were formed per mouse at 10 and 20 weeks of promotion, respectively, as shown in Figure 2B. On the other hand, when cimigenol (**1**) was

Table 1. Relative ratio[a] of EBV-EA activation with respect to positive control (100%) in presence of cimigenol related compounds (**1**–**40**)

Compound	Percentage EBV-EA positive cells			
	Concentration (mol ratio/TPA)[b]			
	1000	500	100	10
1	0±0.5 (70)[c]	13.8±0.7	56.9 ±2.0	86.0±1.5
2	10.4±0.7 (70)	27.2±1.5	73.6±2.3	100±0.4
3	12.6±0.8 (70)	27.9±1.7	74.8±2.1	100±0.5
4	16.3±0.5 (70)	30.2±1.9	81.4±1.9	100±0
5	7.8±0.6 (70)	23.8±1.4	71.9±2.1	96.7±0.3
6	2.1±0.3 (70)	20.7±1.3	59.5±1.8	90.3±0.5
7	2.1±0.4 (70)	20.3±1.4	68.8±1.7	92.4±0.3
8	9.4±0.7 (70)	24.7±1.6	73.2±2.1	100±0.2
9	5.1±0.5 (70)	21.9±1.0	79.5±2.3	96.8±0.3
10	0±0.3 (70)	17.7±1.0	68.7±1.8	89.4±1.1
11	0±0.4 (70)	20.4±1.5	70.3±1.8	92.1±0.4
12	0±0.3 (70)	19.5±0.9	66.4±1.5	90.2±0.5
13	0±0.4 (70)	19.9±1.0	67.0±1.7	91.6±0.4
14	0±0.5 (70)	21.7±1.2	69.6±1.9	95.0±0.3
15	13.7±0.7 (70)	29.9±1.5	78.0±2.2	100±0.4
16	10.3±0.8 (60)	25.8±1.4	74.6±2.1	100±0.5
17	17.5±0.7 (70)	32.5±2.1	80.2±2.5	100±0.2
18	13.4±0.5 (70)	30.0±1.5	78.2±1.9	100±0.4
19	9.6±0.6 (70)	26.7±1.3	71.5±1.7	100±0.6
20	18.5±1.2 (60)	34.2±1.7	83.0±2.2.	100±0.3
21	10.3±0.7 (70)	26.4±1.4	76.0±2.1	100±0.5
22	11.5±0.6 (70)	27.6±1.4	75.2±1.6	100±0.5
23	20.7±1.1 (70)	36.9±1.7	86.0±2.3	100±0.1
24	12.5±0.6 (70)	24.6±1.6	75.2±2.1	100±0.5
25	8.9±0.7 (70)	25.7±1.3	76.5±1.9	100±0.3
26	13.8±0.9 (70)	29.6±2.0	79.5±2.3	100±0.2
27	15.7±1.1 (70)	29.8±1.8	80.3±2.5	100±0.1
28	7.9±0.5 (70)	22.5±1.1	73.5±1.7	100±0.5
29	15.6±0.8 (70)	30.7±1.6	79.6±2.4	100±0.3
30	13.2±0.9 (70)	29.2±1.5	79.0±2.4	100±0.2
31	8.4±0.7 (70)	25.8±1.3	74.2±1.9	100±0.5
32	18.7±1.2 (70)	35.6±1.6	85.1±2.2	100±0.1
33	9.9±0.8 (70)	27.2±1.3	77.0±1.9	100±0.6
34	8.7±0.5 (70)	23.5±1.5	72.1±1.5	100±0.5
35	8.3±0.6 (70)	24.1±1.6	71.4±1.3	100±0.5
36	6.1±0.5 (70)	22.6±1.1	70.7±1.1	96.8±0.7
37	4.6±0.3 (70)	20.8±1.3	69.2±1.4	94.2±0.8
38	10.2±0.7 (70)	24.6±1.4	78.2±1.7	100±0.5
39	11.3±0.8 (70)	26.9±1.7	79.7±1.8	100±0.6
40	13.5±0.8 (70)	28.5±1.5	79.4±1.9	100±0.6

[a]Values represent percentages relative to the positive control value (100%).
[b]TPA concentration is 20 ng/mL (32 pmol/mL).
[c]Values in parentheses are viability percentages of Raji cells.

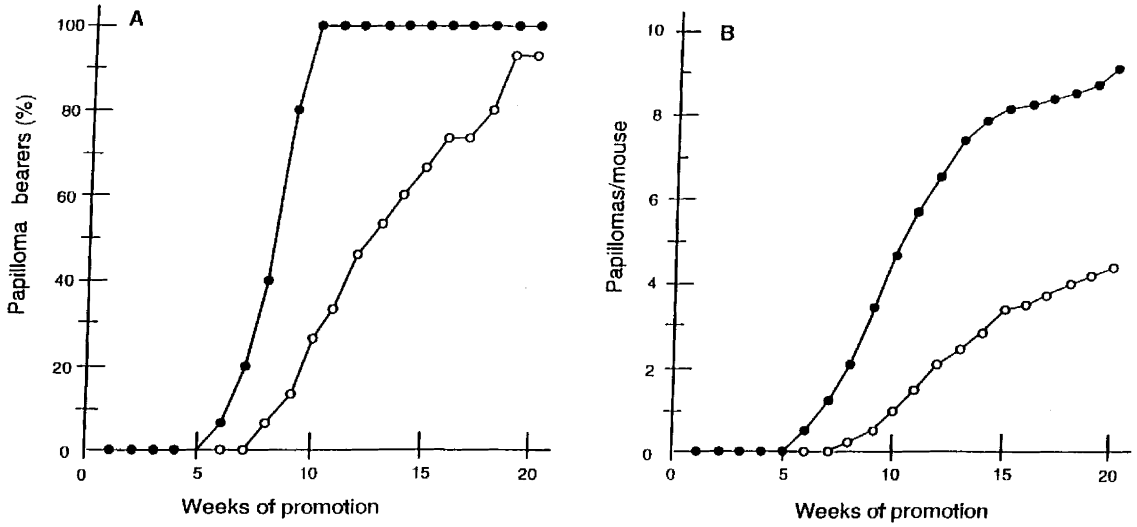

Figure 2. Inhibition of TPA-induced tumor promotion by multiple application of cimigenol. All mice were carcinogenically initiated with DMBA (390 nmol) and promoted with 1.7 nmol of TPA given twice weekly starting 1 week after initiation. A: percentage of mice bearing papillomas; B: average number of papillomas per mouse. ●, control TPA alone (group I); ○, TPA + cimigenol (average dose per mouse per day: 85 μg) (group II). At 20 weeks of promotion, group II was different from group I ($P < 0.05$) in numbers of papillomas per mouse.

applied before each TPA treatment, it delayed the formation of papillomas as follows. In the group treated with cimigenol (**1**), the numbers of papilloma-bearing mice were remarkably reduced to only 27 and 93% of the positive control at 10 and 20 weeks, respectively (Fig. 2A). Also, only 1 papilloma per mouse was recognized at 10 weeks and 4.3 papillomas per mouse at 20 weeks of promotion. The latter number corresponded to 53% of the control group as shown in Figure 2B.

From the results obtained in the present study, the following conclusions can be drawn. Cimigenol (**1**), cimigen-3-one (**10**), cimigen-3,15-dione (**11**), 12β-hydroxycimigenol (**12**), 15-deoxycimigenol (**13**), and 7β-hydroxycimigenol (**14**) exhibited strong inhibitory effects on EBV-EA activity at 1000 mol ratio/TPA. In general, adding sugars or an acetyl group to the 3- or 25-hydroxyl groups tended to decrease the inhibitory activity. Glycosides **2**, **3**, and **4** were less active than the aglycones **1**, **8**, and **5**, respectively. The acetates **5** and **7** and the methyl ether **6** showed reduced activity compared with cimigenol (**1**). Skeleton modified cyclolanostane compounds **29**, **30**, and **34–40** were also less active than **1**. Likewise, the 1,2-epoxy compound **16**, the Δ^7-compound **9**, and the 16,24-epoxy ring opened compounds **31**, **32**, and **33** showed reduced potency compared with **1**. Cimigol, which is 24-epicimigenol (**8**), was also less active than cimigenol (**1**). However, compounds **2**, **34**, and **38**, which are the corresponding 24-epimers of **3**, **35** and **39**, respectively, were equipotent with their epimers. The above results suggested that the presence of 16,23; 16,24-diepoxy and hydroxyl or carbonyl groups at positions 3 and 25 in a 9,19-cyclolanostane skeleton seem to be important for anti-tumor promotion activity.

These investigations suggested that certain cimigenol related compounds including cimigenol (**1**) could be valuable as anti-tumor promoters or as lead compounds for new anti-cancer drug development.

Acknowledgements

This investigation was supported in part by a grant from the National Cancer Institute (CA 17625) awarded to K. H. Lee. This study was supported in part by a grant from the Ministry of Education, Sciences, Sports and Cultures, and Ministry of Health and Welfare, Japan (Kyoto).

References and Notes

1. Piancatelli, G. *Chim. Ital.* **1971**, *101*, 139.
2. Shao, Y.; Harris, A.; Wang, M.; Zhang, H.; Cordell, G. A.; Bowman, M.; Lemmo, E. *J. Nat. Prod.* **2000**, *63*, 905.
3. Sakurai, N.; Kimura, O.; Inoue, T.; Nagai, M. *Chem. Pharm. Bull.* **1981**, *29*, 955.
4. McKenna, D. J.; Jones, K.; Humphrey, S.; Hughes, K. *Alternative Therapies* **2001**, *7*, 93.
5. Sakurai, N.; Nagai, M. *Yakugaku Zasshi* **1996**, *116*, 850.
6. Konoshima, T.; Takasaki, M.; Tatsumoto, T.; Kozuka, M.; Kasai, R.; Tanaka, O.; Nie, R. L.; Tokuda, H.; Nishino, H.; Iwashima, A. *Biol. Pharm. Bull.* **1994**, *17*, 668.
7. Ishida, J.; Kozuka, M; Wang, H. K.; Konoshima, T.; Tokuda, H.; Okuda, M.; Mou, X. Y.; Nishino, H.; Sakurai, N.; Lee, K. H.; Nagai, M. *Cancer Lett.* **2000**, *159*, 135.
8. Sakurai, N.; Koeda, M.; Aoki, Y.; Nagai, M. *Chem. Pharm. Bull.* **1995**, *43*, 1475.
9. Kusano, G.; Hojo, S.; Kondo, Y.; Takemoto, T. *Chem. Pharm. Bull.* **1977**, *25*, 3182.
10. Kusano, A.; Shibano, M.; Kusano, G. *Chem. Pharm. Bull.* **1995**, *43*, 1167.
11. Takasaki, M.; Konoshima, T.; Fujitani, K.; Yoshida, S.; Nishimura, H.; Tokuda, H.; Nishino, H.; Iwashima, A.; Kozuka, M. *Chem. Pharm. Bull.* **1990**, *38*, 2737.
12. Henle, G.; Henle, W. *J. Bacteriol.* **1966**, *91*, 1248.