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Bioorganic & Medicinal Chemistry 13 (2005) 1403–1408
Bioorganic & Medicinal Chemistry

Cancer preventive agents. Part 1: Chemopreventive potential of cimigenol, cimigenol-3,15-dione, and related compounds

Nobuko Sakurai,[a] Mutsuo Kozuka,[a] Harukuni Tokuda,[b] Teruo Mukainaka,[b] Fumio Enjo,[b] Hoyoku Nishino,[b] Masahiro Nagai,[c] Yojiro Sakurai[a] and Kuo-Hsiung Lee[a,*]

[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]*Hoshi University, Ebara, Shinagawa-ku, Tokyo 142-8501, Japan*

Received 8 December 2003; accepted 20 October 2004
Available online 8 December 2004

Abstract—In continuation of our previous report, cimigenol (**1**) and 15 related compounds were screened as potential antitumor promoters by using the in vitro short-term 12-*O*-tetradecanoylphorbol-13-acetate (TPA)—induced Epstein-Barr virus early antigen (EBV-EA) activation assay. Cimigenol-3,15-dione (**2**) displayed the greatest potency (100% inhibition at 1000 mol ratio/TPA) and consequently was further examined for antitumor-promoting activity in a two-stage carcinogenesis assay of mouse skin tumors (DMBA/TPA). In this assay, compound **2** showed significant activity, reducing the number of papillomas per mouse to 48% of the control group at 20 weeks. In addition, compounds **1** and **2** were examined for antitumor-initiating activity in a two-stage carcinogenesis assay of mouse skin tumors induced by peroxynitrite as an initiator and TPA as a promoter. Results showed that these two triterpenoids were almost equipotent with epigallocatechin gallate (EGCG) and slightly more potent than tocinol (group V), the positive controls. Thus, compounds **1** and **2** exhibited not only strong antitumor-promoting activity but also significant antitumor-initiating effect on mouse skin. These data suggest that both compounds might be valuable chemopreventors.

1. Introduction

We have been interested in the use of natural products for cancer chemoprevention. In our studies, we first carry out a primary in vitro assay indicated by the inhibitory effects on EBV-EA activation by TPA and, subsequently, evaluate promising compounds for antitumor-initiating and antitumor-promoting activity in two-stage carcinogenesis assays of mouse skin using peroxynitrite as an initiator and TPA as a promotor.[1]

The rhizome of *Cimicifuga* species is an important medicinal herb. In China and Japan, *C. dahurica*, *C. heracleifolia*, and *C. foetida* have been used as antipyretic and analgesic agents. In Europe and the United States, *C. racemosa* has been used to reduce the frequency and intensity of hot flashes. Each *Cimicifuga* species contains many different triterpenoid glycosides.[2] Cimigenol (23*R*,24*S*)-16,23;16,24-diepoxy-9,19-cyclolanostan-3β,15α,25-triol (**1**), an acid- and base-stable triterpenoid found in *C. racemosa*, *C. dahurica*, *C. japonica*, and other species,[3] and 39 related compounds were previously screened by us[4] as antitumor promoters by using the in vitro short-term 12-*O*-tetradecanoylphorbol-13-acetate (TPA)–induced Epstein-Barr virus early antigen (EBV-EA) activation assay. Among them, **1** and some cimigenol derivatives showed significant activity, and **1** also exhibited strong inhibitory effects on mouse skin tumor promotion induced by TPA in a two-stage carcinogenesis test.

In continuation of the previous report,[4] 15 additional compounds [9,19-cyclolanostane triterpenoid aglycones (**7**, **9**, **11**, **13**, **15**, **17**, **19**, **21**) and glycosides (**5**, **8**, **14**, **18**), isoferulic acid (**22**), cimifugin (**23**), and fulinoic acid (**24**)] were assayed as antitumor promoters by using the in vitro short-term TPA-induced EBV-EA activation assay. Cimigenol-3,15-dione (**2**) was also examined for antitumor-promoting activity in a two-stage carcinogenesis assay with TPA as inducer. In addition, **1** and **2** were

Keywords: Cimigenol; Cimigenol-3,15-dione; Antitumor-initiating effect; Antitumor-promoting effect; Epstein-Barr virus; Two-stage carcinogenesis; 9,19-Cyclolanosterol.
* Corresponding author. Tel.: +1 919 962 0006; fax: +1 919 966 3893; e-mail: khlee@unc.edu

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doi:10.1016/j.bmc.2004.10.062

examined for antitumor-initiating activity in a two-stage carcinogenesis assay of mouse skin tumors induced by peroxynitrite as an initiator and TPA as a promoter. These results should help in understanding the physiological functions and biochemical mechanism of cancer chemopreventive activity of both **1** and **2**.

2. Materials and methods

2.1. Test compounds

The chemical structures are shown in Figure 1. Cimigenol (**1**), cimigenol xyloside (**4**), and 24-*O*-acetylhydroshengmanol xyloside (**10**) were isolated from *C. dahurica*.[3] Cimicifugosides H-1 (**16**), H-2 (**18**), and H-4 (**20**), actein (**12**), and 23-*epi*-26-deoxyactein (**14**) were isolated from commercially available *Cimicifuga* plants.[5] Each aglycone was hydrolyzed from the corresponding glycosides using molsin (crude glucosidase, from *Aspergillus saitoi*).[3] Compounds **2**, **3**, **5**, **7**, and **8** were prepared by oxidation or esterification of **1** or **6**.[3] The structures of all compounds were identified from their NMR and MS data compared with those of literature compounds.[2,3]

2.2. 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). Peroxynitrite was obtained from Dojindo Laboratories Co. (Kumamoto, Japan). EBV-EA positive serum from a patient with nasopharyngeal carcinoma (NPC)

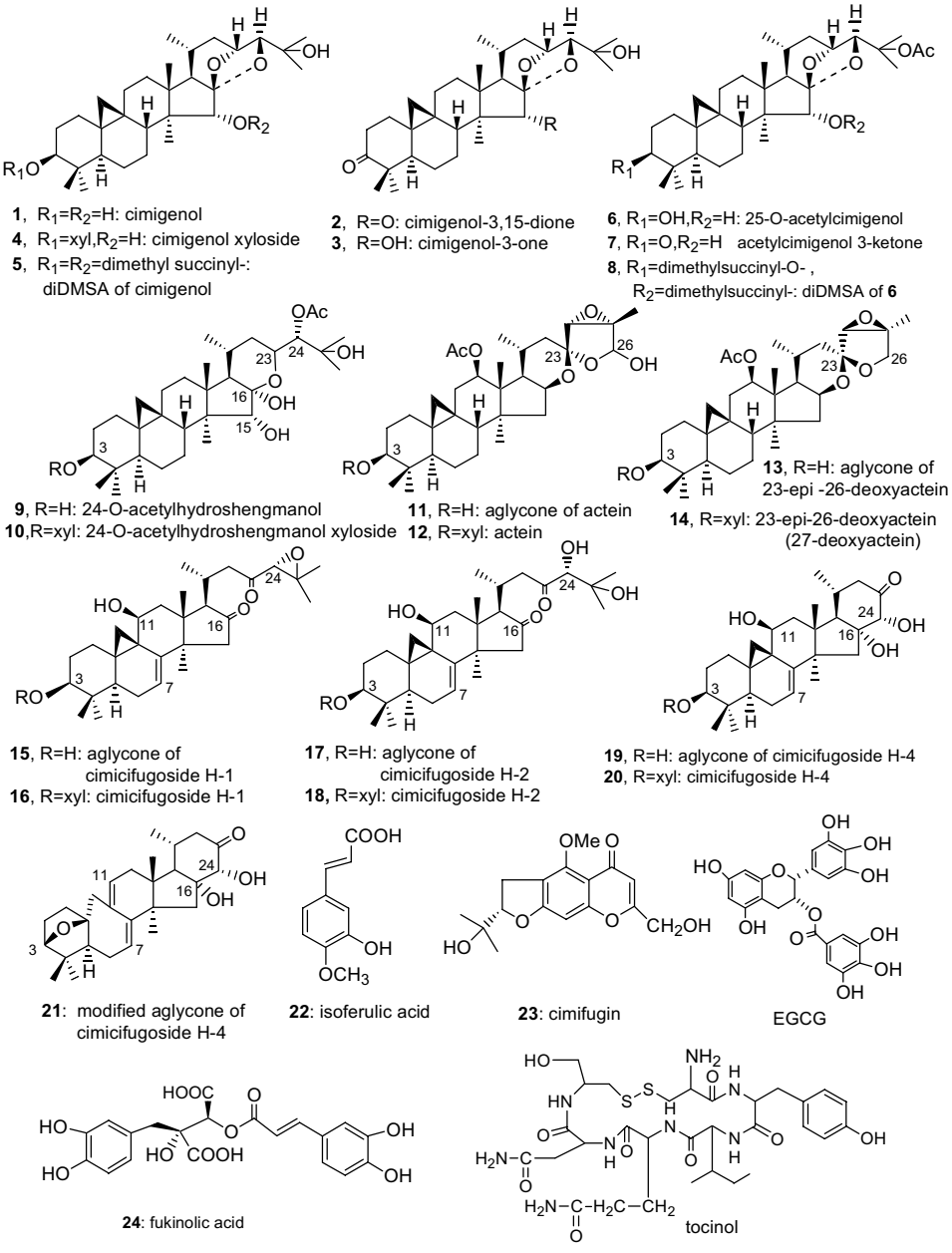

Figure 1. Structures of compounds **1**–**24**.

was a gift from Professor H. Hattori, Department of Otorhinolaryngology, Kobe University.

2.3. Cells

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) under the conditions described previously.[6] Spontaneous activation of EBV-EA in our subline of Raji cells was less than 0.1%.

2.4. Animals

Specific pathogen-free (SPF) female ICR (6 weeks old) and female SENCAR (6 weeks old) mice were obtained from Japan SLC, Inc. (Hamamatsu, Japan), and maintained under SPF conditions in Animal Center of Kyoto Prefectural University of Medicine. The mice were housed five per polycarbonate cage in a temperature-controlled room at 24 ± 2 °C and were fed food (Oriental MF, Oriental Yeast Co., Tokyo, Japan) and water ad libitum during the experiments.

2.5. Inhibition of EBV-EA activation assay

The inhibition of EBV-EA activation was assayed using Raji cells (virus nonproducer type), an EBV genome-carrying human lymphoblastoid cell, as described previously.[1,6] The indicator cells (1×10^6/mL) were incubated at 37 °C for 48 h in medium (1 mL) containing *n*-butyric acid (4 mM) as trigger, and TPA [32 pM = 20 ng in 2 μL of dimethylsulfoxide (DMSO)] as inducer, 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 with high titer EBV-EA positive serum from NPC patients and detected by an indirect immunofluorescence technique.[7] 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 data point. The average EBV-EA induction of the test compound was expressed as a relative ratio to the positive control experiment (100%), which was carried out with *n*-butyric acid (4 mM) plus TPA (32 pM). In the experiments, the EBV-EA induction was ordinarily around 35%, and this value was taken as the positive control (100%). *n*-Butyric acid (4 mM) alone induced 0.1% EA-positive cells. The viability of treated Raji cells was assayed by the Trypan blue staining method. The cell viability of the TPA positive control was greater than 80%. Therefore, only the compounds that induced less than 80% (% of control) of the EBV-activated cells (those with a cell viability of more than 60%) were considered able to inhibit the activation caused by promoter substances. Student's *t*-test was used for all statistical analyses.

2.6. In vivo two-stage carcinogenesis test on mouse skin papillomas induced by DMBA/TPA[8]

The animals (SPF female ICR mice, 6 weeks old) 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) as an initiation treatment. For group I (positive control group), one week after the initiation with DMBA, 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 II received a topical application of cimigenol-3,15-dione (**2**) (85 nmol) in acetone (0.1 mL) 1 h before the promotion treatment. The incidence of papilloma bearers and numbers of papillomas per mouse were observed weekly for 20 weeks. A pathologist checked the type of tumors in this experiment by histological examination. Statistical significance was determined using Student's *t*-test.

2.7. In vivo two-stage carcinogenesis test on mouse skin papillomas initiated by peroxynitrite[9,10]

The animals (SPF female SENCAR mice, 6 weeks old) were divided into five experimental groups of 15 mice each. The back of each mouse was shaved with surgical clippers, and the mice were treated topically with acetone (0.1 mL) and after 10 s, peroxynitrite (33.1 μg, 390 nmol in 0.1 mL of 1 mM NaOH) as an initiation treatment. For group I (positive control group), one week after the initiation, papilloma formation was promoted by the twice weekly application of TPA (1 μg, 1.7 nmol) in acetone (0.1 mL) on the skin (no papilloma formation was seen with topical application of the acetone solvent alone). For groups II, III, IV, and V, test samples, cimigenol (**1**), cimigenol-3,15-dione (**2**), epigallocatechin gallate (EGCG), and tocinol (each 0.0025% in drinking water) were orally administered for 2 weeks before the promotion treatment (1 week both before and after the initiation). Subsequently, the each group was promoted by the twice a week application with TPA (1 μg, 1.7 nmol) in acetone (0.1 mL). The incidence of papilloma bearers and numbers of papillomas per mouse were detected weekly for 20 weeks. Student's *t*-test was used for statistical analyses of the numbers of papillomas per mouse. The animal weights were not statistically different between any of the groups in all in vivo assays.

3. 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**–**24** on the 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 vivo assay). As shown in Table 1, cimigenol (**1**), cimigenol-3,15-dione (**2**), cimigen-3-one (**3**), and isoferulic acid (**22**) exhibited significant inhibitory effects (100%, 70–85%, and 30–40% at 1000, 500, and 100 mol ratio/TPA, respectively). Compounds **6**

Table 1. Relative ratio[a] of EBV-EA activation with respect to positive control (100%) in the presence of cimigenol-related compounds (**1**–**24**)

Compound	Percentage EBV-EA positive cells			
	Compound concentration (mol ratio/TPA[b])			
	1000	500	100	10
1[d]	0 ± 0.5 (70)[c]	13.8 ± 0.7 (>80)	56.9 ± 2.0 (>80)	86.0 ± 1.0 (>80)
2[d]	0 ± 0.4 (70)	20.4 ± 1.5 (>80)	70.3 ± 1.8 (>80)	92.1 ± 0.4 (>80)
3[d]	0 ± 0.3 (70)	17.7 ± 1.0 (>80)	68.7 ± 1.8 (>80)	89.4 ± 1.1 (>80)
4[d]	10.4 ± 0.7 (70)	27.2 ± 1.5 (>80)	73.6 ± 2.3 (>80)	100 ± 0.4 (>80)
5	10.8 ± 0.5 (60)	25.0 ± 1.3 (>80)	73.5 ± 1.3 (>80)	100 ± 0.5 (>80)
6[d]	7.8 ± 0.6 (70)	23.8 ± 1.4 (>80)	71.9 ± 2.1 (>80)	96.7 ± 0.3 (>80)
7	17.0 ± 0.6 (70)	32.6 ± 1.6 (>80)	80.4 ± 2.0 (>80)	100 ± 0.4 (>80)
8	18.2 ± 0.6 (60)	33.9 ± 1.3 (>80)	81.6 ± 1.4 (>80)	100 ± 0.3 (>80)
9	12.7 ± 0.6 (60)	27.5 ± 1.3 (>80)	76.7 ± 1.8 (>80)	100 ± 0.6 (>80)
10[d]	11.5 ± 0.6 (70)	27.6 ± 1.4 (>80)	75.2 ± 1.6 (>80)	100 ± 0.5 (>80)
11	14.9 ± 0.7 (70)	31.3 ± 1.4 (>80)	82.6 ± 2.1 (>80)	100 ± 0.4 (>80)
12[d]	13.8 ± 0.9 (70)	29.6 ± 2.0 (>80)	79.5 ± 2.3 (>80)	100 ± 0.2 (>80)
13	18.4 ± 0.6 (70)	34.8 ± 1.2 (>80)	83.1 ± 2.1 (>80)	100 ± 0.3 (>80)
14	13.4 ± 1.7 (70)	29.0 ± 1.4 (>80)	79.0 ± 1.3 (>80)	100 ± 0.5 (>80)
15	15.5 ± 0.8 (70)	28.6 ± 1.3 (>80)	76.9 ± 1.9 (>80)	100 ± 0.5 (>80)
16[d]	12.5 ± 0.6 (70)	26.4 ± 1.6 (>80)	75.2 ± 2.1 (>80)	100 ± 0.5 (>80)
17	11.7 ± 0.4 (70)	25.9 ± 1.0 (>80)	74.9 ± 2.2 (>80)	100 ± 0.4 (>80)
18	9.9 ± 0.4 (70)	24.9 ± 1.1 (>80)	71.0 ± 1.7 (>80)	97.5 ± 1.2 (>80)
19	9.3 ± 0.4 (70)	26.0 ± 1.5 (>80)	78.4 ± 2.2 (>80)	100 ± 0.5 (>80)
20[d]	8.9 ± 0.7 (70)	25.7 ± 1.3 (>80)	76.5 ± 1.9 (>80)	100 ± 0.3 (>80)
21	11.4 ± 0.5 (70)	26.7 ± 1.5 (>80)	75.3 ± 2.1 (>80)	100 ± 0.5 (>80)
22	0 ± 0.2 (60)	22.8 ± 1.1 (>80)	68.0 ± 1.9 (>80)	92.7 ± 0.5 (>80)
23	25.8 ± 1.1 (60)	41.4 ± 1.5 (>80)	86.9 ± 1.8 (>80)	100 ± 0.4 (>80)
24	10.8 ± 0.4 (70)	25.7 ± 1.1 (>80)	79.3 ± 1.6 (>80)	100 ± 0.6 (>80)

[a] Values represent relative percentages to the positive control value (100%).
[b] TPA concentration = 20 ng/mL (32 pmol/mL).
[c] Values in parentheses are viability percentages of Raji cells.
[d] Data were previously described in Ref. 4.

and **18**–**20** showed moderate inhibitory effects (>90%, 75%, and 20% at 1000, 500, and 100 mol ratio/TPA, respectively). The inhibitory activities of these compounds were stronger than that of glycyrrhetic acid,[11] which is known as a strong antitumor promoter.

The 9,19-cycloartane triterpenes (**1**–**21**) in this study fall into four general structural series: cimigenol (**1**–**8**), hydroshengmanol (**9**, **10**), actein/deoxyactein (**11**–**14**), and cimicifugoside (**15**–**21**) compounds. The actein/deoxyactein (**11**–**14**) and hydroshengmanol (**9**, **10**) triterpenes generally were less active than the other two classes. Among the cimicifugosides, the H-4 and H-2 compounds (**17**–**20**) were more active than the H-1 compounds (**15**, **16**). Thus, the structure of the triterpene skeleton, particularly the D ring side chain, influenced activity.

Among the cimigenol-type compounds, cimigenol (**1**) and acetylcimigenol (**6**), with a C-3 OH group, were more active than the analogous compounds (**3** and **7**, respectively), with a C-3 ketone group. In addition, the inhibitory effects of EBV-EA activation of cimigenol (**1**) and its oxidative metabolites (**3** and **2**) decreased slightly in that order. Thus, the greater number of hydroxyl groups enhanced the inhibitory activity on EBV-EA activation. The metabolic fates of the compounds in this series could possibly account for some of the observed activities in the in vitro assay, and in the in vivo assays discussed below.

Among the 24 compounds tested, the glycosides (xylosides) showed slightly increased or mostly equivalent activity compared to corresponding aglycones except in the case of cimigenol (**1**) and its xyloside (**4**), where the latter compound showed decreased potency. Cimigenol (**1**) and cimigenol-3-one (**3**) were also more active than their esters (**5**, **7**, and **8**).

In our past work, inhibitory effects on EBV-EA induction by TPA have correlated well with antitumor-promoting activity in vivo. In the previous paper,[4] we reported that cimigenol (**1**) exhibited a significant inhibitory effect on the tumor-promotion induced by DMBA and TPA. In this paper, on the basis of the results of the in vitro assay, the effects of cimigenol-3,15-dione (**2**) 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 Figures 2A and B, respectively.

As shown in Figure 2A, in group I (positive control), the first tumor appeared after 6 weeks, and 100% of the mice bore papillomas after 10 weeks of promotion. Further, averages of 5.4 and 9.3 papillomas were formed per mouse at 10 and 20 weeks of promotion, respectively, as shown in Figure 2B. On the other hand, when cimigenol-3,15-dione (**2**) was applied before TPA treatment (group II), it delayed and reduced the formation of

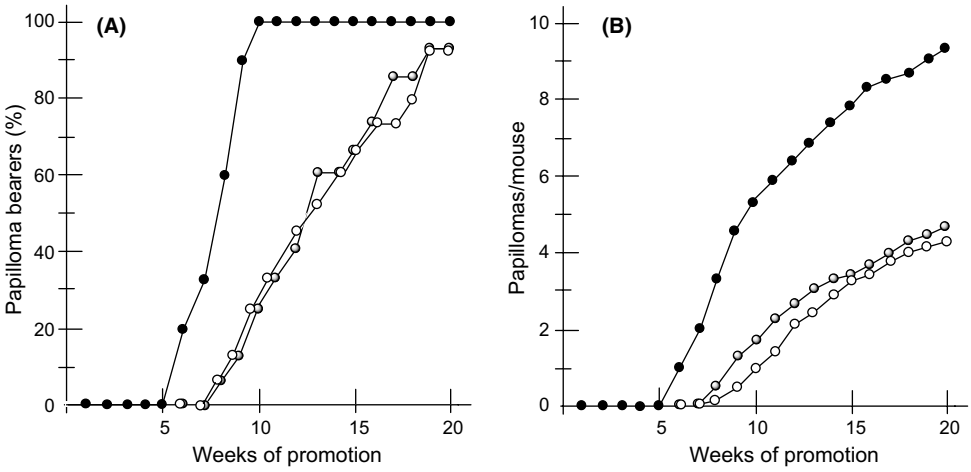

Figure 2. Inhibition of DMBA/TPA-induced tumor promotion by multiple application of cimigenol (**1**) and cimigenol-3,15-dione (**2**) (topical administration). All mice were carcinogenically initiated with DMBA (390 nmol) and promoted with 1.7 nmol of TIPA given twice weekly starting 1 week after initiation. (A) Percentage of mice bearing papillomas; (B) average number of papillomas per mouse; (-●-): positive control, TPA alone: group I; (-○-): TPA + 85 nmol of cimigenol (**1**): group II; (-◐-): TPA + 85 nmol of cimigenol 3,15-dione (**2**): group III. At 20 weeks of promotion, group II and group III were significantly different from group I ($p < 0.05$, using Student's *t*-test) on papillomas per mouse.

papillomas as follows: the first tumor appeared after 8 weeks and the incidence of papilloma-bearing mice was reduced to 27% and 93% at 10 and 20 weeks of promotion, respectively (Fig. 2A). Also, only 1.8 and 4.8 papillomas per mouse were recognized at 10 and 20 weeks of promotion. The latter number corresponded to 48% of the control group as shown in Figure 2B. These results were almost the same as those of the group III (treatment with **1**).

From the above results, cimigenol (**1**) and cimigenol-3,15-dione (**2**) exhibited the highest antitumor-promoting potency among the tested compounds. Further, their antitumor-initiating effect on the two-stage carcinogenesis of mouse skin initiated by peroxynitrite and promoted by TPA was investigated.

Figure 3A shows the time course of the tumor formation in five groups [incidence (%) of papilloma bearers]. In group I (positive control, treated with peroxynitrite/TPA), the first tumor appeared after 7 weeks and the incidence of the tumor bearing mice was 100% after 11 weeks. Whereas, in groups II and III (treated with **1** and peroxynitrite/TPA, treated with **2** and peroxynitrite/TPA, respectively), the first tumor appeared after 9 weeks, and the incidence of the tumor bearer was approximately 27% and 80% (group II), and 33% and 87% (group III) after 11 and 20 weeks of promotion,

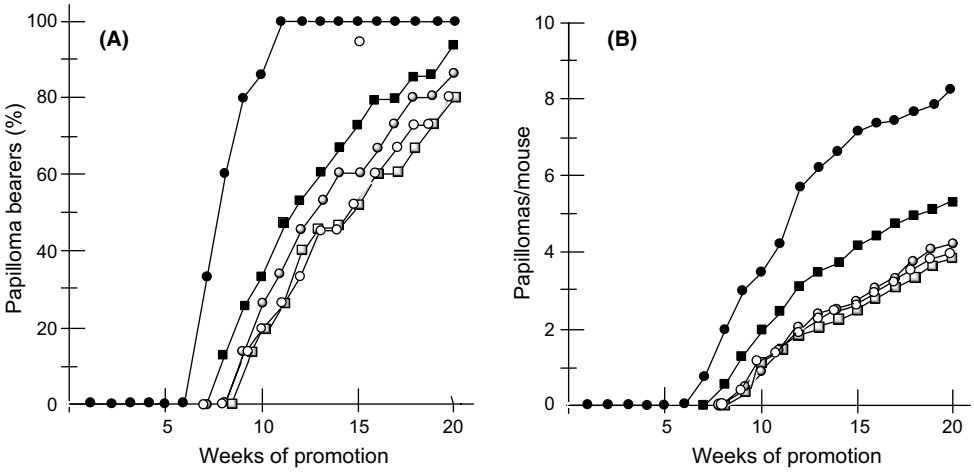

Figure 3. Inhibition of peroxynitrite/TPA-induced tumor promotion by multiple application of cimigenol (**1**), cimigenol-3,15-dione (**2**), EGCG, and tocinol (topical administration). All mice were carcinogenically initiated with peroxynitrite (390 nmol) and promoted with 1.7 nmol of T1PA given twice weekly starting 1 week after initiation. (A) Percentage of mice bearing papillomas; (B) average number of papillomas per mouse; (-●-): positive control, peroxynitrite (390 nmol) + TPA (1.7 nmol): group I; (-○-): peroxynitrite (390 nmol) + 0.0025% of cimigenol (2 weeks) + TPA (1.7 nmol): group II; (-◐-): peroxynitrite (390 nmol) + 0.0025% of cimigenol-3,15-dione (2 weeks) + TPA (1.7 nmol): group III; (-□-): peroxynitrite (390 nmol) + 0.0025% of ECGC (2 weeks) + TPA (1.7 nmol): group IV; (-■-): peroxynitrite (390 nmol) + 0.0025% of Tocinol (2 weeks) + TPA (1.7 nmol): group V. At 20 weeks of promotion, groups II, III, IV, and V were significantly different from group I ($p < 0.05$) on papillomas per mouse.

respectively. In the average number of papillomas per mouse (Fig. 3B), **1** and **2** reduced the number of the tumors compared to the control group. In group I, approximately eight tumors per mouse were observed after 20 weeks of promotion, whereas, groups II and III reduced to papillomas by approximately one-half over the same period. Compounds **1** and **2** were almost equipotent with EGCG (group IV) and slightly more potent than tocinol (group V) against tumor formation as shown in Figure 3.

From the results obtained in this study, the following conclusions can be drawn. At 1000 mol ratio, cimigenol (**1**), cimigenol-3,15-dione (**2**), and cimigenol-3-one (**3**) exhibited strong inhibitory effects on EBV-EA activity. Cimigenol xyloside (**4**) was less active than its aglycone **1**, and likewise, the dimethylsuccinyl diesters (**5** and **8**) were less active than the parent alcohols (**1** and **6**, respectively). Meanwhile, glycosides **10**, **12**, **14**, **16**, **18**, and **20** showed almost the same activity as their corresponding aglycones (**9**, **11**, **13**, **15**, **17**, and **19**, respectively). Cimigenol (**1**) and cimigenol-3,15-dione (**2**) displayed useful physiological activities, including both antitumor-initiating activity and antitumor-promoting activity in two-stage carcinogenesis assays of mouse skin tumors. Both compounds delayed the formation of papillomas compared to the control group.

4. Conclusion

These investigations suggested that certain cimigenol-related compounds including cimigenol (**1**) and cimigenol-3,15-dione (**2**) could be valuable as chemopreventors or as lead compounds for new chemopreventive agents.

Acknowledgements

This investigation was supported in part by a Grant from the National Cancer Institute (CA 17625) awarded to K. H. Lee, and by a Grant from the Ministry of Education, Culture, Sports, Science, and Technology, and Ministry of Health, Labor, and Welfare, Japan awarded to H. Nishino. We thank Dr. K. Ohgi of Hoshi University for her generous supply of molsin.

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