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Inhibition of Human Cytomegalovirus IE Gene Expression by Dihydro-β-agarofuran Sesquiterpenes Isolated from *Euonymus* Species

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Abstract. *The development of strategies intended to inhibit human cytomegalovirus (HCMV) immediate-early (IE) antigen expression is an important goal in research designed to prevent and treat certain forms of cancer. The aim of this study was to identify potent IE antigen-targeting natural compounds as antitumor promoters in an in vitro model of tumor promotion. Nineteen dihydro-β-agarofuran sesquiterpenes isolated from Euonymus species were evaluated for their ability to inhibit HCMV IE antigen expression in human lung adenocarcinoma (A549) cells. Five esters of penta- and hexahydroxydihydro-β-agarofuran proved to be active components in these Euonymus species, inhibiting the IE antigen expression of HCMV. The highest activity was displayed by 2β,6α,15-triacetoxy-1β-benzoyloxy-9α-nicotinoyloxydihydro-β-agarofuran. These effective compounds may be regarded as prototypes of antitumor promoters, as secondary chemopreventive agents which can modify or halt tumor promotion in general.*

Sesquiterpene esters have attracted great interest because of their immunosuppressive, cytotoxic, antiviral and anticarcinogenic activities. Compounds isolated from *Tripterygium, Celastrus, Euonymus* and *Maytenus* species (Celastraceae) are known as promising cancer-preventive agents (1). Many sesquiterpenes exert inhibitory effects on the Epstein-Barr virus early antigen, induced by the tumor promoter 12-*O*-tetradecanoyl-phorbol-13-acetate in Raji cells (2, 3). Besides the *in vitro* findings, studies on a two-stage *in vivo* carcinogenesis model have revealed that some compounds inhibit tumor promotion on mouse skin (4).

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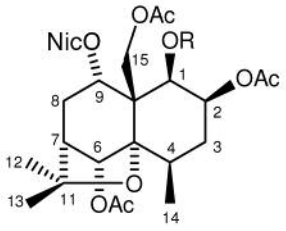
Key Words: Antitumor promotion, *Euonymus* species, dihydro-β-agarofuran, sesquiterpene, cytomegalovirus, IE antigen expression.

Sesquiterpene alkaloids from *Tripterygium* species possess considerable anti-HIV activity and inhibit HIV replication in H9 lymphocytes (5, 6). Triptofordin C-2, a sesquiterpene tetraester of *Tripterygium wilfordii* var. *regelii*, has been reported to display moderate virucidal activity against several enveloped viruses, including herpes simplex virus type 1 (HSV-1), human cytomegalovirus (HCMV), measles virus and influenza A virus; it suppresses the viral protein synthesis of infected cells when added during the early steps of HSV-1 replication, and it inhibits translation of the transcripts of the immediate-early (IE) genes (7).

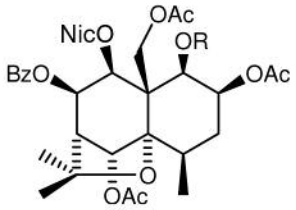
Recent pathological investigations demonstrated that HCMV can be found with high frequency in Epstein-Barr virus-negative Hodgkin's disease, colorectal cancer, malignant glioma and prostate cancer cells (8-11). HCMV infection fails to transform susceptible normal human cells, but it modulates the malignant properties of cancer cells through its ability to interfere with a variety of cellular signal transduction processes, leading to accelerated cell proliferation, enhanced survival, angiogenesis, cell motility and adhesion (12). HCMV infection promotes tumor cell survival by inhibiting apoptosis, interfering with both the intrinsic and the extrinsic cellular apoptosis pathways (13). These oncomodulatory effects are mediated mainly by the activity of HCMV regulatory proteins and rely on the persistence of the viral infection in the malignant cells. On the basis of the appearance of the respective mRNA or protein, the sequential expression of the HCMV genome has been divided into three phases: IE, early and late (14). Both latent infection and reactivation are determined by the activity of IE gene products; these accumulate in infected cells and may lead to tumor promotion and progression.

In the present study, the effects of 19 structurally related dihydro-β-agarofuran esters, obtained from *Euonymus* species, on the HCMV IE antigen expression in A549 (human lung adenocarcinoma) cells were investigated with the aim of identifying potent IE antigen-targeting natural compounds as antitumor promoters.

Sesquiterpenes of *Euonymus verrucosus*

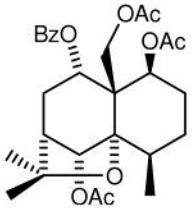


	R
1	MeBu
2	Bz



	R
3	Ac
4	Bz

Sesquiterpenes of *E. japonicus*



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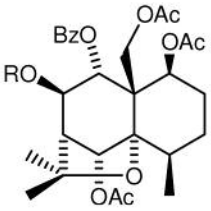
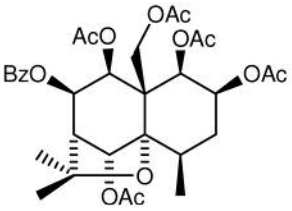


	R
6	Ac
7	H



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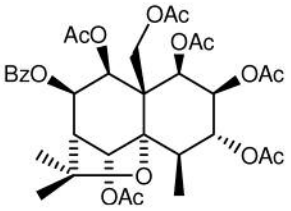

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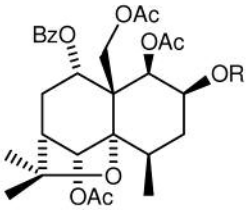
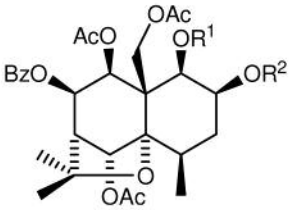
Sesquiterpenes of *E. sachalinensis*



	R
10	Ac
11	Bz



	R^1	R^2
12	Bz	Bz
13	Bz	H
14	H	Bz

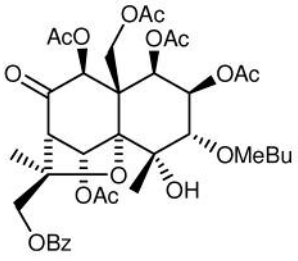


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Figure 1. *continued*

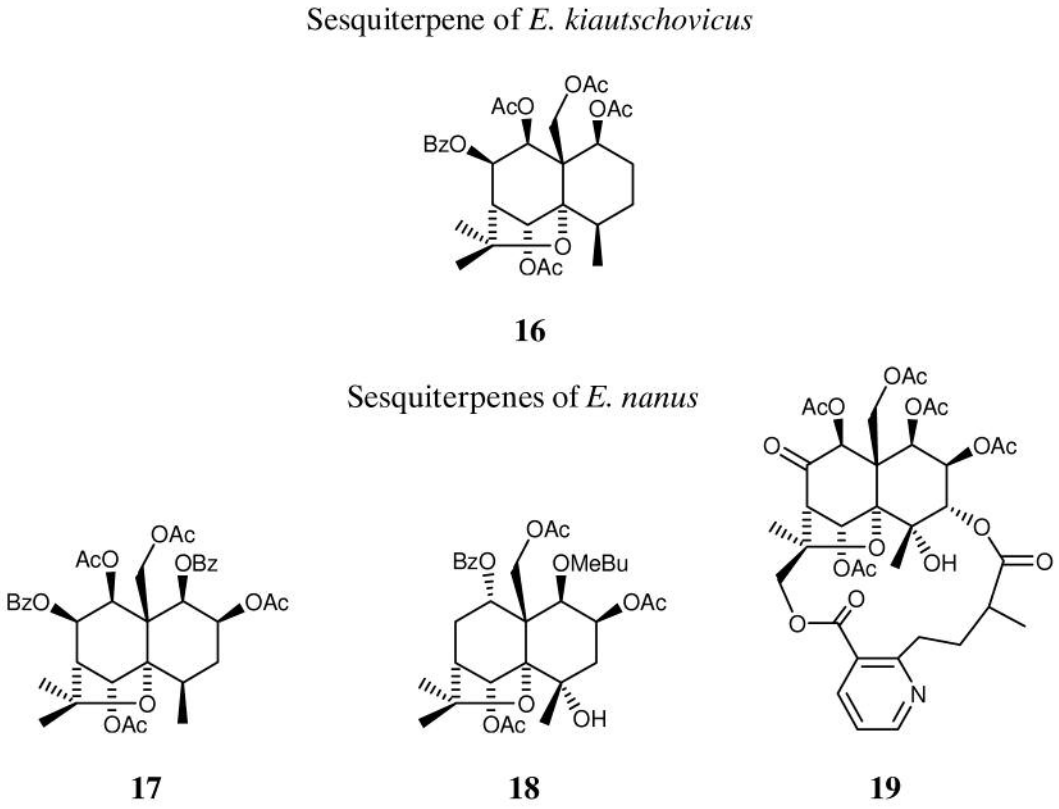

Figure 1. *Chemical structures of the investigated sesquiterpenes. Ac=acetyl, MeBu=2-methylbutanoyl, Nic=nicotinoyl, Bz=benzoyl.*

Materials and Methods

Investigated compounds. The compounds shown in Figure 1 were isolated from the fresh fruits of *Euonymus verrucosus* (**1-4**) (15), *E. japonicus* (**5-9**) (16), *E. sachalinensis* (**8, 10-15**) (17, 18)], *E. kiautschovicus* (**16**) (19) and *E. nanus* (**17-19**) (17, 20).

Sample preparation. Compounds **1, 5-9** and **12-19** were dissolved in dimethyl sulfoxide (DMSO) at a final concentration of 10.0 mg/ml, and **2-4, 10** and **11** at a final concentration of 1 mg/ml, further dilutions being made in the appropriate medium used for cell culturing.

Cell culture. A549 cells were cultivated in Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS); for immunofluorescence studies, cells were grown on glass coverslips in 24-well plates containing 2×10^5 cells/well.

Virus. The stock of HCMV laboratory-adapted strain Towne (American Type Culture Collection VR-977) was propagated in confluent MRC-5 cells grown in RPMI medium supplemented with 10% FCS and antibiotics. The infectivity titer was determined by plaque assay with the inoculation of confluent MRC-5 in 24-well plates.

Assays for cytotoxic effect. The cytotoxicity of each of the listed compounds on A549 cells was tested by a method reported elsewhere (21). The effects of increasing concentrations of the compounds on cell growth were tested in 96-well flat-bottomed microtiter plates. The compounds were diluted in a volume of 50 μl, and 1×10^4 cells in 0.1 ml of medium were then added to each well, with the exception of the medium-containing control wells. The culture plates were incubated at 37˚C for 48 h, at the end of which 15 μl of MTT (methyltetrazolium salt) solution (from a 5 mg/ml stock) were added to each well. After incubation at 37˚C for 4 h, 100 μl of sodium dodecyl-sulfate solution (10%) were measured into each well and the plates were further incubated at 37˚C overnight. The cell growth was determined by measuring the optical density (OD) at 550 nm (ref. 630 nm) with a Dynatech MRX vertical beam ELISA reader (Labsystems, Helsinki, Finland). The extent of inhibition of cell growth (as a percentage) was determined *via* the following formula, in which OD cell control relates to the OD of untreated cells:

$$100 - \left[\frac{OD\,sample - OD\,medium\,control}{OD\,cell\,control - OD\,medium\,control}\right]\times 100$$

Immunofluorescence assay for HCMV IE gene expression. The immofluorescence assay was described elsewhere (21). One-day-old A549 cell cultures on coverslips were infected with the Towne strain of HCMV at a multiplicity of infection of 2.4. The infected A549 cultures were next centrifuged for 60 min at 1200 rpm in a Heraeus Megafuge 1.0 (Osterode, Germany) at room temperature and then incubated for 1 h at 37˚C. The unabsorbed virus was removed and the cells were

washed three times with serum-free medium. After washing, the cells were overlayed with MEM with 1% FCS and antibiotics containing the appropriate concentration of a compound or DMSO. After incubation for 48 h, the cells were washed twice with cold phosphate-buffered saline and fixed with a cold acetone:ethanol 1:1 mixture for 20 min at –20˚C. The fixed cells were stored at –20˚C until immunofluorescence assays were performed.

Human CMV IE antigen was detected in the nuclei of infected cells by immunostaining using monoclonal antibody (MAB810) (Chemicon International Inc., Temecula, CA, USA) and fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG (Sigma, Budapest, Hungary).

Statistical analysis. The mean number ±SD of IE antigen-positive cells per 30 microscopic fields containing 400 cells each was calculated. The frequencies of IE antigen-expressing cells in the treated cultures are reported as percentages of that of the control.

Results

The inhibitory activities of dihydro-β-agarofuran sesquiterpenes (**1-19**) isolated from *E. verrucosus*, *E. japonicus*, *E. sachalinensis*, *E. kiautschovicus* and *E. nanus* against human CMV IE gene expression were investigated on A549 lung cancer cells. On the basis of the inhibition dose 50 (ID_{50}) values, the compounds and the pure solvent DMSO were tested at non-cytotoxic concentrations, ID_1 and ID_{10}, for modification of the HCMV IE antigen expression in the different dihydro-β-agarofuran sesquiterpene-treated cells. The degree of inhibition of the IE antigen expression of HCMV in the presence of nontoxic doses of these dihydro-β-agarofuran sesquiterpenes was evaluated as an antipromoting effect that can reflect the chemopreventive activity of a compound.

The results are summarized in Table I. The measured biological effects allow the studied compounds to be categorized into four groups, as follows: Compounds which markedly inhibited the HCMV IE gene expression (**1-3, 5** and **11**); compounds which did not cause any dose-dependent inhibition and were considered inactive (**8, 9, 13** and **14**); compounds which exhibited a biphasic effect, with inhibition at low concentrations, in contrast to an enhanced IE expression observed at the highest concentrations applied (**4, 6, 7, 10, 16** and **17**); and compounds which slightly increased the IE antigen expression to above 100% , which theoretically means promotion of expression (**12, 15, 18** and **19**).

Table I. *The effects of dihydro-β-agarofuran esters (**1-19**) on the human cytomegalovirus IE gene expression in A549 cells.*

Compound	ID_{50} (μg/ml)	ID	IE antigen-expressing cells (% of control)
1	1.0	10	60
		1	94
2	20.01	10	37
		1	61
3	25.42	10	59
		1	73
4	30.60	10	80
		1	69
5	10.00	10	83
		1	92
6	10.00	10	84
		1	74
7	10.00	10	85
		1	75
8	49.11	10	93
		1	92
9	10.00	10	92
		1	92
10	37.49	10	64
		1	57
11	29.15	10	50
		1	81
12	27.93	10	114
		1	87
13	44.95	10	96
		1	98
14	37.76	10	73
		1	78
15	41.44	10	127
		1	79
16	39.60	10	82
		1	65
17	38.75	10	94
		1	84
18	40.23	10	119
		1	81
19	37.74	10	106
		1	118

ID: inhibitory dose for A549 cells; ID_{50}: ID causing 50% inhibition.

Discussion

Since some of these sesquiterpenes were able to reduce the IE antigen expression in HCMV-infected lung cancer cells, we presume that these inhibitors may act as antipromoters by acting in the promotion and progression stages through one of the main mechanisms, such as the inhibition of the arachidonic acid and ornithine decarboxylase activity or the induction of differentiation (22-24).

The ability of HCMV to preferentially infect tumor tissues suggests a unique character of mutual interaction between the mechanisms of tumor cells and HCMV (8-10). IE gene product of the virus accumulates in the infected cells causing disturbances of host cell functions. The oncomodulatory effects of the HCMV infection may lead to a shift to a more malignant phenotype of the tumor cells contributing to tumor progression (25). This study has revealed the dose-dependent inhibitory effect of certain dihydro-β-agarofuran polyesters (**1-3, 5** and **11**) isolated from *Euonymus* species on the expression of the IE antigen.

As far as the structure-activity relationships are concerned, it may be stated that the most effective compounds were the penta- and hexasubstituted derivatives of dihydro-β-agarofuran (**1-4, 6-8, 10-14** and **16-18**). The tetrasubstituted compound **5** exhibited only moderate activity and the esters of hepta- and octahydroxydihydro-β-agarofurans (**9, 15** and **19**) were merely weakly effective or inactive. Among the pentasubstituted sesquiterpenes, better activities were recorded for the 1β,2β,6α,9α,15-pentahydroxydihydro-β-agarofuran esters (**1, 2, 10** and **11**) than for the 1β,6α,8β,9α/β,15-pentahydroxydihydro-β-agarofuran esters (**6, 7** and **16**). 2β,6α,15-Triacetoxy-1β-benzoyloxy-9α-nicotinoyloxydihydro-β-agarofuran (**2**) was the most effective inhibitor of the HCMV virus IE gene expression. Compounds **3, 4** and **14**, with a 1β,2β,6α,8β,9β,15-hexahydroxydihydro-β-agarofuran polyol nucleus, also exhibited significant activity. As regards the nature of the ester groups, the most active compounds (**2, 3** and **11**) proved to be those substituted with two aromatic (nicotinoyl or benzoyl) substituents.

Considering the results of the present study and the need for the development of IE gene-targeting compounds, the precise mechanism underlying the inhibitory action of the effective sesquiterpenes needs to be determined.

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