Abstract. Human cytomegalovirus (CMV) preferentially infects tumor tissues and the accumulated CMV immediate-early (IE) antigen may lead to tumor promotion and progression. The development of strategies to inhibit human CMV IE antigen expression and/or function is an important goal to prevent and treat certain forms of cancers associated with human CMV. The aim of this study was to search for antitumor promoters from plant sources. The effect of six macrocyclic lathyrane-type diterpenoids, latilagascenes A-E (1-5) and jolkinol B (6), isolated from the methanol extract of Euphorbia lagascae, on the expression of IE antigen in lung cancer cells (A549) infected by CMV was studied. All the compounds, except latilagascene D (4), decreased IE antigen expression of CMV.

Euphorbia species (Euphorbiaceae) have been used in traditional medicine for treatment of cancer, tumors and warts for hundreds of years (1). Their use has been limited due to the occurrence of skin irritant and often tumor-promoting phorboids that also characterize these plants (2). Besides the presence of phorboid compounds, Euphorbia species have also provided a wide range of structurally unique polyoxygenated macrocyclic diterpenes, such as lathyranes, jatrophanes and their polycyclic derivatives. Recent investigations have revealed that those diterpenes are promising modulators of multidrug resistance (MDR) in tumor cells (3-6). A few jatrophanes have also been considered as new microtubule-interacting agents (7). Furthermore, a rearranged jatrophane derivative, isolated from Euphorbia peplus, has shown in vivo anti-inflammatory activity (8).

Euphorbia lagascae Spreng is a herb that has been cultivated for the production of vernolic acid, an epoxidated fatty acid that is found in high levels in the seeds of this species and has high potential industrial value (9). The stilbene piceatannol, a potent protein-tyrosine kinase inhibitor, is an antileukemogenic agent, which was first isolated from the seeds of Euphorbia lagascae (10, 11). Previous studies on this species have also demonstrated lathyrane-type diterpenoids, latilagascenes A-C (1-3), as powerful Pgp inhibitors (6). As a continuation of our studies in the search for new bioactive compounds from this species, the new macrocyclic lathyrane diterpenes 4 and 5, as well as the known compound 6 have been isolated and characterized by spectroscopic means. Their isolation and structural characterization will be reported elsewhere.

A high frequency of the human cytomegalovirus (CMV) genome and antigens in tumor samples of patients with different malignancies, such as Epstein-Barr virus (EBV), negative Hodgkin’s disease, colon cancer, and malignant neuroblastoma, is well documented (12-14). CMV infection can modulate multiple cellular regulatory and signaling pathways in a manner similar to that of the oncoproteins of small DNA tumor viruses, such as human papilloma virus or adenoviruses. However, in contrast to these DNA tumor viruses, CMV infections fail to transform susceptible normal human cells. CMV infection modulates properties of tumor cells, such as growth, apoptosis, production of angiogenic factors, cell invasion and immunogenic properties. These oncomodulatory effects are mediated mainly by the activity of CMV regulatory proteins and rely on the persistence of the viral infection in the malignant cells. The sequential expression of the CMV genome has been divided into three phases: immediate-early (IE), early (E) and late (L) based on the appearance of the respective mRNA or protein (15). Both latent infection and reactivation are determined by the activity of the IE gene products. The IE gene products accumulate in infected cells causing disturbance of host cell functions.

The main goal of the present study was to examine the effects of six macrocyclic lathyrane-type diterpenes on CMV IE antigen expression in lung cancer cells.

Materials and Methods

Plant material. Euphorbia lagascae Spreng. (Euphorbiaceae) was collected in Cova da Beira, Coimbra, Portugal and identified by Dr.
Teresa Vasconcelos of the Instituto Superior de Agronomia (ISA), University of Lisbon. A voucher specimen (n° 323) has been deposited at the herbarium of ISA.


Compounds 4-6 were isolated from the methanol extract of Euphorbia lagascae and identified by spectroscopic methods. Their isolation and identification will be reported elsewhere. Compounds 1-3 were also isolated from the methanol extract of Euphorbia lagascae, as described previously (6). Compounds were dissolved in dimethylsulfoxide (DMSO) at 2.0 mg final concentration, and further dilution was made in the appropriate culture media used for cell culture.

Cell culture. A549 cells (human lung alveolar epithelial cells) were cultivated in Eagle’s MEM supplemented with 10% fetal calf serum (FCS) and for immunofluorescence studies cells were grown on glass coverslips in 24-well plates containing 2x10⁵ cells/well. Virus. The stock of human CMV laboratory-adapted strain Towne was propagated in confluent MRC-5 cells grown in RPMI medium supplemented with 10% FCS and antibiotics. The infectivity titre was determined by plaque assay with the inoculation of confluent MRC-5 in 24-well plates.

Assay for cytotoxic effect. The effects of increasing concentrations of compounds on cell growth were tested in 96-well flat-bottomed microtitre plates. The compounds were diluted in a volume of 50 µL. Then, 1x10⁴ cells in 0.1 mL of medium were added to each well, with the exception of the medium control wells. The culture plates were further incubated at 37°C for 48 h; at the end of the incubation period, 15 µL of MTT (methyl-terasolium salt) solution (from a 5 mg/mL stock) was added to each well. After incubation at 37°C for 4 h, 100 µL of SDS (sodium dodecyl-sulphate) solution (10%) was 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). Inhibition of cell growth (as a percentage) was determined according to the formula in which ODcell control means the untreated cells:

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\text{Inhibition of cell growth (as a percentage)} = \left(1 - \frac{\text{OD}_{\text{sample}} - \text{OD}_{\text{medium control}}}{\text{OD}_{\text{cell control}} - \text{OD}_{\text{medium control}}}\right) \times 100
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Assay for CMV immediate-early (IE) gene expression by immunofluorescence. One-day-old A549 cultures on the coverslips
Based on the ID$_{50}$ values, ID 1 and ID 10 inhibitory doses were measured in the first stage of the study (Table I). Cytotoxicity of lathyranes on the lung cancer cells (A549), between appears to be important in explaining the activity difference saturation region concerning the biological effect. This idea antigen. It is assumed that both doses might be in the modified

**Results**

In the present work, the human CMV was used in a modified in vitro model for characterizing lathyrane compounds with antipromotion effect. To develop an objective method for studying the antipromotion effect, the compounds had to be applied in non-toxic doses. Therefore, the inhibitory dose fifty (ID$_{50}$) values, representing cytotoxicity of lathyranes on the lung cancer cells (A549), were measured in the first stage of the study (Table I). Based on the ID$_{50}$ values, ID$_1$ and ID$_{10}$ inhibitory doses (considered as non-toxic doses) were tested for modification of CMV IE antigen expression in the different lathyrane-treated cells. The inhibition of IE antigen expression of CMV in the presence of non-toxic doses of lathyranes was evaluated as an antipromoting effect that can reflect the chemopreventive activity of a compound.

The results are summarized in Table II. Latilagascenes B-C (2-3), E (5) and jolkinin B (6) showed inhibitory activity in a dose dependent manner against IE antigen expression of CMV. Latilagascene A (1) in dose ID$_1$ was more effective than ID$_{10}$ in inhibition of production of IE antigen. It is assumed that both doses might be in the saturation region concerning the biological effect. This idea is supported by the ID$_{50}$ value, which was lower for the latilagascene A (1). Latilagascene D (4) was inactive. As can be observed in Figure 1, the structures of the six compounds are very similar. They differ in the substitution pattern of ring A with compound 5 also having a different substitution at C-20 where the methyl group is oxidized, having been replaced by a –CH$_2$OH. This structural feature appears to be important in explaining the activity difference between latilagascene D (4) and latilagascene E (5) which showed the highest activity. The comparison of the activity of compounds 4 and 1, whose structures differ only at the benzoyl moiety in the former has a negative action on the inhibitory effect.

**Discussion**

Carcinogenesis is a multistage process by which a normal cell is transformed into a cancerous cell. Transformation involves initiation, usually from DNA damaging agents, promotion, during which cell proliferation is increased, and progression, involving additional genetic alterations (16). Cancer chemoprevention is defined as the use of natural, synthetic, or biological agents to prevent, suppress, or reverse either the initiation phase of carcinogenesis or the carcinogenic progression (17). Recently, cancer prevention by natural products has been the object of considerable attention and great importance has been given to dietary intake of fruits and vegetables, which have been shown to reduce the risk of developing cancer. Furthermore, a number of active compounds, isolated from non-edible plants, have also been reported (16, 18-20). Consequently, several plant-derived compounds, working by various mechanisms of action targeting initiation, promotion and progression of carcinogenesis, have been used in clinical trials (16, 21).

Cancer chemopreventive agents have been classified, as blocking or suppressing agents, according to the stage of carcinogenesis in which they have shown activity. Blocking agents prevent the initiation stage and the suppressing agents act at the promotion and progression stages preventing the evolution of the neoplastic process. Some compounds have both effects (22). The mechanism of action of chemopreventive compounds acting as antitumor promoters is not well known. However, a significant number of them have been considered to inhibit the tumor promotion stage by interacting with the protein kinase C which plays a crucial role in the regulation of cell growth (23, 24).

Short-term in vitro models have been developed for the identification of antitumor-promoting agents. EBV is known to be activated by tumor-promoters to produce early antigens (EA). Thus, evaluation of the EBV-EA inhibition has been used as a primary screening in the search for effective antitumor promoting agents (23). Other in vitro models, such as the one that uses human adenovirus type 12 have been reported (25-27).

The ability of human CMV to preferentially infect tumor tissues suggests a unique character of mutual interaction between the mechanisms of tumor cells and human CMV (12-14). IE gene products of the virus accumulate in the infected cells causing disturbances of host cell functions. The oncomodulatory effects of the human CMV infection
may lead to a shift to a more malignant phenotype of the tumor cells contributing to tumor progression (28). This study revealed the dose dependent inhibitory effect of certain macrocyclic lathyrane-type diterpenoids on the expression of the IE antigen.

Considering the result of the present study and the need for the development of IE antigen targeting compounds, the precise mechanism underlying the inhibitory action of the effective lathyrane diterpenes needs to be determined.

Acknowledgements

This work was supported by FCT, Portugal (POCTI, Quadro Comunitário de Apoio III) and by the Foundation for Cancer Research Szeged, Szeged, Hungary. The authors thank Dr. Teresa Vasconcelos (ISA, University of Lisbon, Portugal) for identification of the plant and Katalin Hegedüs for the excellent technical work.

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Received August 2, 2006
Revised October 16, 2006
Accepted October 20, 2006