Ophiobolin A, a sesterterpenoid fungal phytotoxin, displays higher *in vitro* growth-inhibitory effects in mammalian than in plant cells and displays *in vivo* antitumor activity

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Abstract. Ophiobolin A, a sesterterpenoid produced by plant pathogenic fungi, was purified from the culture extract of *Drechslera gigantea* and tested for its growth-inhibitory activity in both plant and mammalian cells. Ophiobolin A induced cell death in *Nicotiana tabacum* L. cv. Bright Yellow 2 (TBY-2) cells at concentrations ≥10 μM, with the TBY-2 cells showing typical features of apoptosis-like cell death. At a concentration of 5 μM, ophiobolin A did not affect plant cell viability but prevented cell proliferation. When tested on eight cancer cell lines, concentrations <1 μM of ophiobolin A inhibited growth by 50% after 3 days of culture irrespective of their multidrug resistance (MDR) phenotypes and their resistance levels to pro-apoptotic stimuli. It is, thus, unlikely that ophiobolin A exerts these *in vitro* growth-inhibitory effects in cancer cells by activating pro-apoptotic processes. Highly proliferative human keratinocytes appeared more sensitive to the growth-inhibitory effects of ophiobolin A than slowly proliferating ones. Ophiobolin A also displayed significant antitumor activity at the level of mouse survival when assayed at 10 mg/kg in the B16F10 mouse melanoma model with lung pseudometastases. Ophiobolin A could, thus, represent a novel scaffold to combat cancer types that display various levels of resistance to pro-apoptotic stimuli and/or various MDR phenotypes.

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Abbreviations: CTAB, hexadecyltrimethylammonium bromide; DHR, dihydrorhodamine; EI-MS, electron ionization mass spectrometry; ESI-MS, electrospray ionization mass spectrometry; HPLC, high-performance liquid chromatography; HR, hypersensitive response; MDR, multidrug resistance; LC-MS, liquid chromatography-mass spectroscopy; MEM, minimal essential media; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NMR, nuclear magnetic resonance; NSCLC, non-small cell lung cancer; OD, optical density; PCD, programmed cell death; PCV, package cell volume; SD, standard deviation; TBY-2, *Nicotiana tabacum* L. cv. Bright-Yellow 2; TLC, thin layer chromatography

Key words: ophiobolin A, plant cells, mammalian cells, cytotoxicity, *in vivo* antitumor activity

Introduction

Ophiobolins are secondary metabolites belonging to the family of sesterterpenoid compounds and are produced by phytopathogenic fungi, mainly of the genus *Bipolaris*. Their discovery filled the gap between diterpenes (C20), which have four isoprene units and triterpenes (C30), which have six isoprene units (1). Ophiobolin A was the first member of the group to be isolated and characterized in the mid-1960s

(2,3). Currently, 25 biogenic ophiobolins have been identified and marine-derived fungal ophiobolins were recently identified and demonstrated to inhibit the biofilm formation of *Mycobacterium* species (4).

Ophiobolin-producing terrestrial fungi attack several crops, such as rice, maize and sorghum, by causing brown spot lesions on the leaves. These fungi mainly attack monocotyledons, but they can also attack various herbaceous dicotyledon species, although grass weeds proved to be more sensitive to the phytotoxins (5). The interest in *Bipolaris* spp. and their bioactive metabolites derives from their previous implication in two devastating plant disease epidemics: the Bengal rice famine in India in 1943 and the Southern corn leaf blight epidemic in the USA in 1972 (1).

Ophiobolins can lead to cell death in plants through multiple mechanisms of action, including inhibition of root and coleoptile growth in wheat seedlings, inhibition of seed germination, changes in cell membrane permeability, stimulation of β-cyanin leakage, releases of electrolytes and glucose from the roots and decreases in photosynthetic CO_2-fixation, which cause respiratory changes and enhance stomatal opening (reviewed in ref. 1). Ophiobolin A is able to inhibit protein and nucleic acid synthesis or act as an inhibitor of β-1,3-glucan synthetase in plant cells (1).

While a body of information on the deleterious effects of ophiobolin A in plants is already available, only a few of these reports mention the anticancer effects of ophiobolin A and these reports are limited to *in vitro* studies. Cytotoxic effects were reported for ophiobolin A (6,7) and ophiobolin O (8) but not for ophiobolin I (6) in various cancer cell lines.

The present study further aims to characterize ophiobolin A-mediated effects on cell proliferation versus cell death in normal plant (tobacco) versus normal mammalian cells and then in mammalian cancer cells.

Materials and methods

Ophiobolin A production and stability

Fungus. A strain of *Drechslera gigantea* (Heald & Wolf) was used to produce ophiobolin A. This fungus is stored in the Fungal Collection at the Institute of Sciences of Food Production in Bari, Italy (# ITEM 7004) and it was previously reported to produce ophiobolin A (5). The fungus was grown and maintained on Petri dishes containing PDA (potato-dextrose-agar, Oxoid, UK).

Production, extraction and purification of ophiobolin A. Ophiobolin A was produced by growing the fungus, extracted from the fungal culture, purified and its identity confirmed as described previously (5). The ophiobolin A purity (>95%) was confirmed by RP-HPLC-UV. HPLC analyses were performed on an Agilent 1100 series HPLC system (Agilent, Diegem, Belgium). The chromatographic system was a C8 SunFire 150x4.6-mm I.D., 3.5-μm particle size (Waters, Milford, MA, USA). The solvents were CH_3OH:TFA 0.1% in water (65:35), with a flow rate of 0.75 ml/min and λ = 240 nm.

Physicochemical stability measurements for ophiobolin A. The stability of ophiobolin A was assayed by using the same conditions used for the growth of both mammalian and plant cells. A 10^{-3} M solution of ophiobolin A was prepared from a stock solution in DMSO (10^{-2} M) diluted in minimal essential cell culture medium (MEM) (Invitrogen, Merelbeke, Belgium) and used for growing mammalian cells. After 7 days of incubation at 37˚C, the solution was diluted with the appropriate solvent (see below) to a final concentration of 10^{-5} M; it was analyzed by LC-MS and compared to freshly prepared ophiobolin A (10^{-5} M) and 3-anhydro-6-*epi*-ophiobolin (10^{-5} M) solutions, this latter being a degradation product of the main metabolite. The LC-MS analyses were performed using an Agilent RRLC-UV-VIS 1200 series coupled to a quadrupole time-of-flight mass spectrometer (Q-TOF) 6520 (Palo Alto, CA, USA). The LC conditions were the same as for the purity test described above. ESI-Q-TOF parameters were as follows: positive mode; high resolution acquisition mode (4 GHz); gas temperature of 350˚C; drying gas flow rate of 11 l/min; nebulizer pressure of 50 psig; capillary voltage of 4500 V; skimmer voltage of 150 V; MS scan range and rate, 50-1,700 at 2 spectra/s. The data were acquired and analyzed using the Mass Hunter Acquisition® and Qualitative Analysis® software, respectively (Agilent Technologies).

The ophiobolin A stability in tobacco cell culture media was monitored for 4 days. Solutions of $5x10^{-6}$ M and 10^{-5} M ophiobolin A were incubated in the same conditions as the plant cell culture (modified Linsmaier and Skoog media on a rotary shaker at 130 rpm at 27˚C in the dark; see below for details). At one-day intervals, the stability of ophiobolin A and the generation of degradation products were assayed as described below. The chromatograms were recorded on Agilent Technologies 1200 series UV-VIS detector. The column used was C18 HD 250x4.6-mm I.D. Nucleosil 100-5 (Macherey-Nagel, GmbM & Co. KG, Duren, Germany). The solvents were CH_3OH:H_2O (8:2), with a flow rate of 0.5 ml/min and λ = 240 nm.

Ophiobolin A and plant cells

Growing conditions, mitotic index and viability of plant cells. A suspension of tobacco (*Nicotiana tabacum* L. cv Bright-Yellow 2) cells, hereafter referred to as TBY-2 cells, was routinely propagated and cultured at 27˚C, according to Nagata *et al* (9). A stationary culture was diluted 4:100 (v:v) and cultured for 3 days as described by Vacca *et al* (10). An ethanolic solution of ophiobolin A was used for the treatments. The final concentration of ethanol in the media never exceeded 0.2%.

Cell growth was evaluated by optical density at 600 nm (11) and by package cell volume (PCV), which is the ratio between the cell volume after centrifugation at 250 x g for 6 min and the total suspension volume (12).

TBY-2 cell viability was measured using trypan blue staining and cell morphology was investigated by means of a phase contrast light microscope, as described previously (13).

The mitotic index was calculated as the percentage of cells undergoing mitosis. For this assay, TBY-2 cells were stained with Hoechst 33258, as reported in Houot *et al* (14) and visualized using a fluorescence microscope (DMLS, Leica, Wetzlar, Germany) with an excitation filter of 340-380 nm and a barrier filter of 410 nm.

Nuclear morphology and DNA fragmentation analysis. The nuclear morphology of the TBY-2 cells was analyzed

by staining the cells with Hoechst 33258 and visualized as previously described (14). The DNA from the TBY-2 cells was isolated according to the CTAB method described by Edwards *et al* (15). DNA fragments were separated by electrophoresis on a 1.5% (w/v) agarose gel and then visualized by staining with ethidium bromide as detailed previously (16).

H_2O_2 production in plant cells. The extracellular release of hydrogen peroxide (H_2O_2) was determined by measuring the absorbance at 560 nm of the Fe^{3+}-xylenol orange complex, according to Locato *et al* (13). The intracellular production of H_2O_2 was also observed by staining the cells with the fluorescent probe dihydrorhodamine (DHR) 123 (17) and visualized using a fluorescence microscope with an excitation filter of 450-490 nm and a barrier filter of 510 nm.

Ophiobolin A and mammalian cells

Determination of in vitro growth-inhibitory activity in normal human cells. Normal human adult keratinocytes were isolated and grown as detailed previously (18). At ~40% culture confluence, the keratinocytes were grown under autocrine conditions by excluding all of the growth factors from the culture media; 2 days later, hyperproliferating undifferentiated cultures were analyzed while still subconfluent (SC). After four additional days of incubation, the cultures had become confluent (C) and were analyzed as differentiating cultures; in these culture conditions, confluent cultures are growth-arrested and express suprabasal markers of epidermal differentiation (19,20).

The *in vitro* global growth levels of SC versus C keratinocytes that were left untreated (control) or treated with ophiobolin A were determined by means of the MTT colorimetric assay, as detailed below.

Determination of the in vitro growth-inhibitory activity in mouse and human cancer cells. Three human and one mouse cancer cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). The three human cancer cell lines were the A549 non-small cell lung cancer cell line (NSCLC; DSMZ code ACC107), the SKMEL-28 melanoma cell line (ATCC code HTB-72) and the Hs683 oligodendroglioma (ATCC code HTB-138) cell line; the mouse cancer cell line used was the B16F10 melanoma (ATCC code CRL-6475) model, which was also assayed *in vivo* as detailed below. The cells were cultured in RPMI (Invitrogen) media supplemented with 10% heat-inactivated fetal calf serum (Invitrogen), 4 mM glutamine, 100 μg/ml gentamicin and penicillin-streptomycin (200 U/ml and 200 μg/ml; Invitrogen). The overall growth level of each cell line was determined using the colorimetric MTT (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide, Sigma, Belgium) assay as previously described (18,21). The data are presented as the means ± SD values obtained from three experiments, each of which was performed on six replicates.

Multidrug resistant (MDR) cancer cell cultures. Human cell lines and their chemoresistant sub-lines used in this study were obtained as described below. The pro-myelocytic leukemia HL60 cell line and its P-glycoprotein overexpressing sub-lines that have been rendered resistant to adriamycin (HL60/adr), vincristine (HL60/vinc) or mitoxantrone (HL60/mx) (22,23) were generously donated by Dr M. Center (Kansas State University, Manhattan, KS, USA). The A2780 ovarian carcinoma cell line and its variant that is resistant to cisplatin were obtained from Sigma-Aldrich. The small cell lung carcinoma cell line GLC4 and its MRP1- and LRP-overexpressing adriamycin-resistant sub-line GLC4/ADR (24,25) were generously donated by Dr E.G. deVries (Groningen, The Netherlands). The human colon cancer HCT116 p53 wild-type cell line and its p53 (-/-) delete clone (26) were generously donated by Dr B. Vogelstein (John Hopkins University, Baltimore, MD, USA). Immunoblotting validation of overexpressed ABC transporters and p53 deletion are available upon request. All cell lines were cultured in RPMI-1640 media supplemented with 10% fetal bovine serum, with the exception of HCT116 cells, which were grown in McCoy's media. In case of the resistant sub-lines, the respective selective drug was added as detailed previously (23,25).

For the cell viability assays, $2x10^3$ cells in 100 μl were plated into individual wells in 96-well plates and allowed to attach for 24 h. Appropriate concentrations of ophiobolin A were added to the wells in another 100 μl of growth media for 72 h. The proportion of viable cells at the end of the treatment was then determined by means of the MTT assay colorimetric assay, as in the previous section.

Computer-assisted phase-contrast microscopy analyses. Direct visualization of ophiobolin A-induced effects on the cell proliferation and morphology of human Hs683 glioma cells was performed by means of time-lapse computer-assisted phase contrast microscopy, i.e., quantitative videomicroscopy, as detailed previously (27,28).

Characterization of in vivo ophiobolin A-mediated anticancer activity. B16F10 melanoma pulmonary pseudometastases were obtained by i.v. (lateral tail vein) injection of $2.5x10^5$ B16F10 cells (200 μl), as we detailed previously (29). The B16F10 melanoma cells used for these *in vivo* experiments were also assayed *in vitro* with respect to ophiobolin A-mediated growth-inhibitory effects (Table II). As we only had limited amounts of ophiobolin A available, we referred to the published data to select the doses to be administered to the mice. The LD_{50} doses of ophiobolin A for mice are 238 mg/kg when administered subcutaneously, 21 mg/kg when administered intraperitoneally, 12 mg/kg when administered intravenously and 73 mg/kg when administered orally (1). A suspension of microcrystalline ophiobolin A in 0.9% NaCl was intraperitoneally administered at 5 and 10 mg/kg to the B16F10 melanoma-bearing mice three times a week (Monday, Wednesday, Friday) for three consecutive weeks. Treatments began on the 5th day after the tumor grafting procedure. Each experimental group included 10 mice. Mouse survival was checked daily, while mouse weight was recorded three times per week (Monday, Wednesday, Friday). Each B16F10 melanoma-bearing mouse was sacrificed either when it had lost 20% of its weight compared to its weight at the time of the tumor graft or if it was suffocating. The animal experiment used 6-week-old female B6D2F1 mice (18-22 g; Charles Rivers, Arbresle, France) and was performed on the basis of authorization no. LA1230568

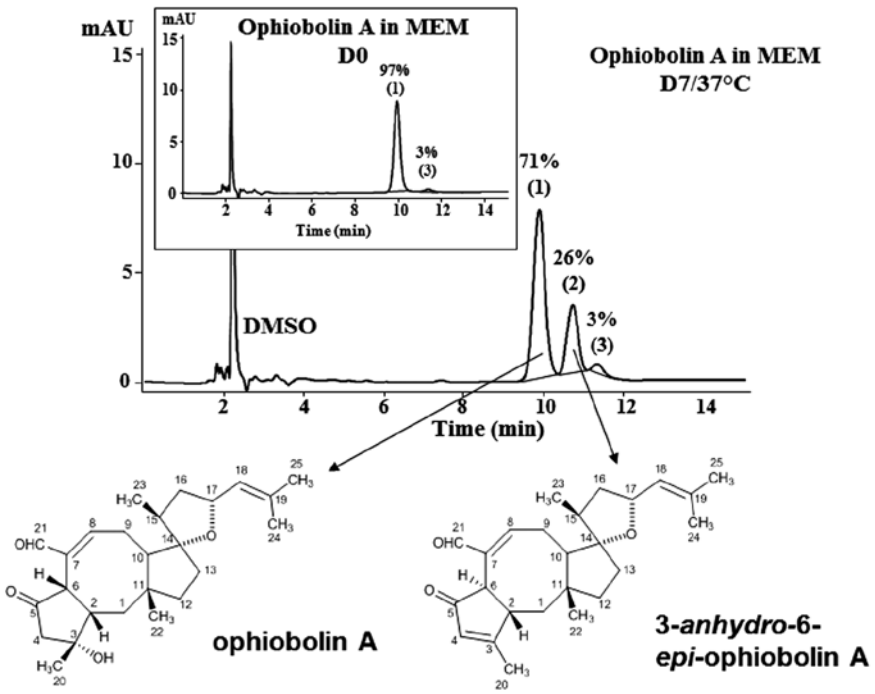

Figure 1. The stability of ophiobolin A in MEM culture media. Ophiobolin A was present at 97% at day 0 (D0). After 7 days of incubation at 37˚C in MEM, some of the ophiobolin A was degraded to 3-anhydro-6-*epi*-ophiobolin A.

Table I. Ophiobolin A stability in TBY-2 plant cell culture media over time.[a]

	Starting solution 5 μM		Starting solution 10 μM	
Time (days)	Ophiobolin A	3-anhydro-6-*epi*-ophi-A	Ophiobolin A	3-anhydro-6-*epi*-ophi-A
1	93±1	7±1	92±1	7±2
2	78±2	22±1	76±1	24±1
3	72±1	27±1	71±2	29±2
4	59±2	41±2	57±1	43±1

[a]The production of 3-anhydro-6-*epi*-ophiobolin A (3-anhydro-6-*epi*-ophi-A) by ophiobolin A degradation was measured over time by incubating ophiobolin A at two different concentrations in TBY-2 culture media and in the same cell growth conditions (at 27˚C in the dark on a rotary shaker at 130 rpm). The amounts of ophiobolin A and 3-anhydro-6-*epi*-ophiobolin A were assayed over time as described in Materials and methods. The results are expressed as the percentage of ophiobolin A present at time 0 and are the mean values ± SD of three different experiments.

from the Animal Ethics Committee of the Federal Department of Health, Nutritional Safety and the Environment (Belgium).

Statistical analyses. Survival analyses were carried out by means of Kaplan-Meier curves, which were compared with the log-rank test. All the statistical analyses were performed using Statistica (StatSoft, Tulsa, OK, USA). The data from the *in vitro* experiments were statistically analyzed via the one-way ANOVA test using the Sigma Plot software (Systat Software Inc., San Jose, CA, USA).

Results

Characterization of ophiobolin A stability. The physico-chemical stability of ophiobolin A was analyzed in the two culture conditions in order to establish whether the different sensitivities of plant and animal cells to ophiobolin A were due to the compound itself or to its degradation product(s) in the culture media used for animal and plant cell growth. The data show that ophiobolin A can be almost completely recovered immediately after its dissolution in MEM as a major peak corresponding to 97% of the sesterterpenoid (peak 1; Fig. 1). One major degradation product was obtained when ophiobolin A was maintained for 7 days at 37˚C in MEM culture media (peak 2; Fig. 1); this product has been identified as 3-anhydro-6-*epi*-ophiobolin A (Fig. 1) by means of TLC (with comparisons to the available standards), ESI-MS, EI-MS and NMR analyses, according to the previously reported spectroscopic data (5). HPLC analyses revealed that 71% of ophiobolin A remained after one week (D7) at 37˚C in

Table II. Characterization of the *in vitro* growth-inhibitory activity (MTT colorimetric assay) of ophiobolin A and 3-anhydro-6-*epi* ophiobolin A on four cancer cell lines.[a]

	Mean IC_{50} *in vitro* growth inhibitory concentration (μM) ± SD				
	Cancer cells displaying various levels of resistance to pro-apoptotic stimuli		Cancer cells displaying actual sensitivity to pro-apoptotic stimuli		
Compound	A549	SKMEL28	Hs683	B16F10	Global mean IC_{50} ± SD
Ophiobolin A	0.42±0.01	0.37±0.03	0.62±0.04	0.29±0.05	0.4±0.1
3-anhydro-6-*epi* ophiobolin A	30±1	27.0±0.4	30±3	22±3	27±4

[a]Treatments were performed for 72 h. The data are presented as the mean ± SD from three experiments, with each experiment having six replicates.

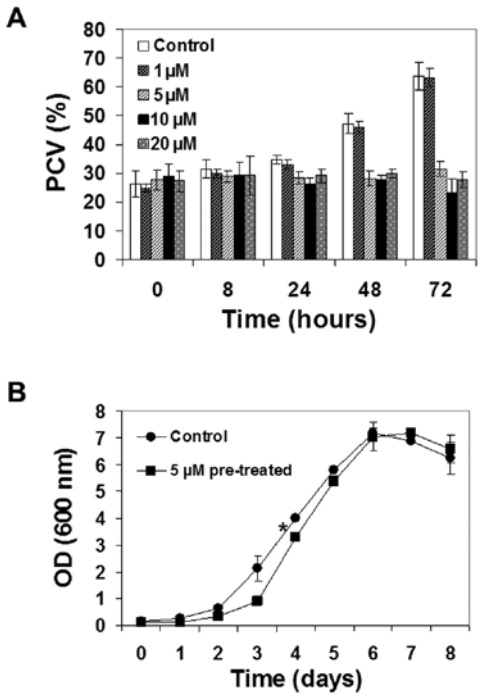

Figure 2. The effects of ophiobolin A on TBY-2 cell growth. TBY-2 cells in exponential phases were incubated with different concentrations of ophiobolin A. (A) PCV, an indicator of the increase in cell number and volume, was measured. (B) Time course of the growth rate of the TBY-2 control cells and the ophiobolin A-treated cells; the latter were washed and re-cultured in fresh ophiobolin A-free media after 24 h of treatment with 5 μM ophiobolin A. These values are the mean of three independent experiments ± SD.

MEM (Fig. 1). A minor peak of 3% was observed at D0 and remained stable up to 7 days. This minor peak corresponds to an unidentified compound; however, the ophiobolin A used in the present study was 97% pure.

The degradation of ophiobolin A was faster in TBY-2 plant cell culture media (Table I) than in mammalian MEM (Fig. 1). Indeed, after 4 days of incubation, ~40% of the ophiobolin A was degraded to 3-anhydro-6-*epi*-ophiobolin A (Table I).

Plant cell sensitivity to ophiobolin A. Ophiobolin A affected plant cell growth, with 5 μM being the lowest concentration required for altering culture growth (Fig. 2A). At this concentration, ophiobolin A completely blocked the growth of TBY-2 cells, as the package cell volume (PCV) remained unchanged over time, while lower ophiobolin A concentrations did not alter TBY-2 growth (Fig. 2A). Similar results were obtained by measuring cell culture growth via monitoring the increase in optical density (OD) at 600 nm of the cell suspension (data not shown). Analysis of the mitotic index confirmed that 5 μM ophiobolin A blocked cell division, reducing the number of cells undergoing mitosis by 69±5% 24 h post-treatment.

TBY-2 cells regained their proliferative ability when they were washed and cultured in ophiobolin A-free media after 24 h of 5 μM ophiobolin A treatment, even if a weak but nevertheless statistically significant delay in reaching the exponential phase was observed ($^{*}p<0.05$; Fig. 2B). Higher concentrations of ophiobolin A (10 and 20 μM) showed similar effects on cell growth when compared to treatment with 5 μM ophiobolin A but also caused cell death, as detailed below.

The effects of ophiobolin A and its degradation product 3-anhydro-6-*epi*-ophiobolin A on TBY-2 plant cell viability were analyzed using the trypan blue assay, as the trypan blue dye selectively enters dead cells. The data from this analysis revealed that ophiobolin A was lethal for TBY-2 cells at concentrations ≥10 μM (Fig. 3A). Indeed, TBY-2 cell viability was almost negligible 24 h after treatments with 10 and 20 μM ophiobolin A, whereas treatment with 1 or 5 μM ophiobolin A did not induce any decrease in cell viability until 72 h after the treatments (Fig. 3A). Furthermore, 3-anhydro-6-*epi*-ophiobolin had no effect on the TBY-2 cells; neither the growth nor the viability of the cells were altered, even after 72 h of treatment and at concentrations ≤10 μM of 3-anhydro-6-*epi*-ophiobolin (data not shown).

Cytoplasm shrinkage has been recognized as a useful hallmark to distinguish programmed cell death (PCD) from the necrotic processes in plant cell cultures (30). Fig. 3B shows that treatment with 10 μM ophiobolin A induced cytoplasm shrinkage in nearly all trypan blue-positive cells, suggesting that the compound induced apoptosis-like cell death in the TBY-2 plant cells. No cellular shrinkage was detected in the control cells or in cells treated with 5 μM ophiobolin A (Fig. 3B).

The analysis of the nuclear morphology of the cells further supports the activation of PCD in cells treated with 10 μM

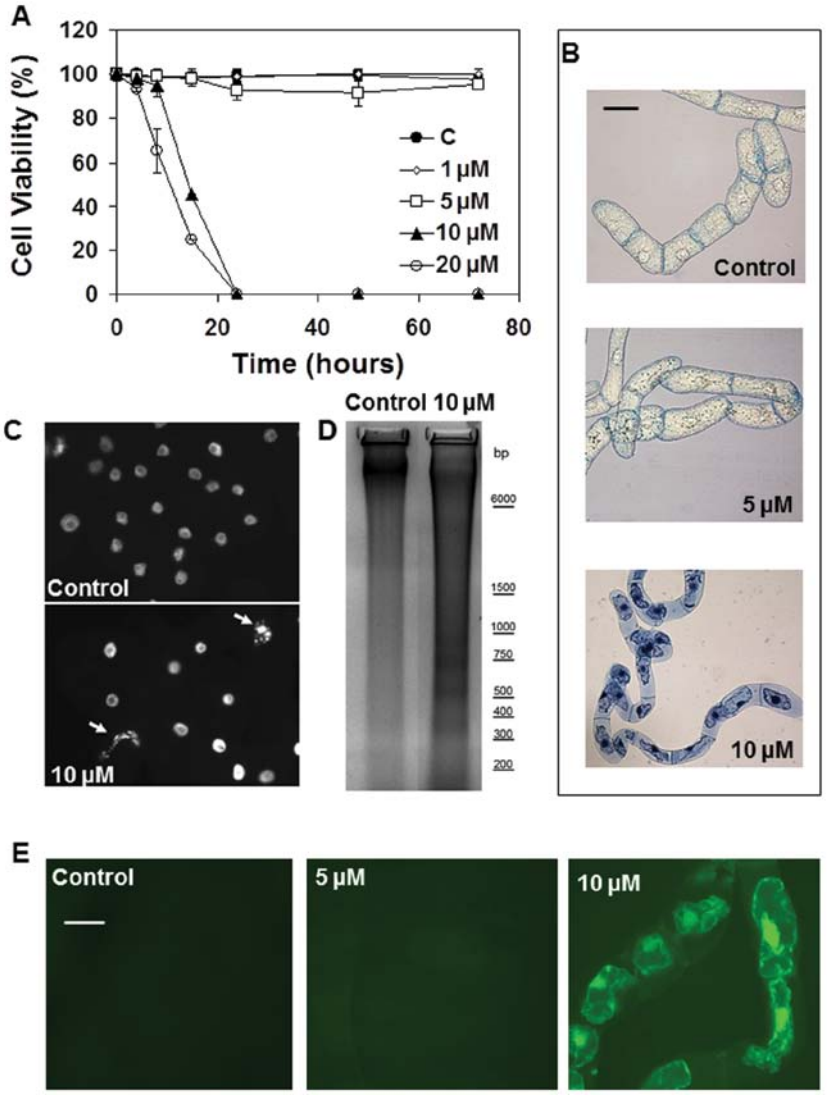

Figure 3. The effect of ophiobolin A on TBY-2 cell viability. (A) Viability of the TBY-2 cells was followed over time in the presence of different ophiobolin A concentrations. The values are the means of three independent experiments ± SD. (B) Cellular morphology of the control cells and the ophiobolin A-treated cells stained with trypan blue after 24 h of treatment. Cytoplasmic shrinkage of dying cells was evident only in cells treated with 10 μM ophiobolin A. (C) Nuclear morphology of the control cells and the ophiobolin A-treated cells. The cells were stained with Hoechst 33258 to visualize the nuclear morphology under fluorescence microscopy. The images show cell nuclei 24 h after treatment. Bar, 20 μm. (D) Analyses of DNA laddering in the control cells and the ophiobolin A-treated cells. The cells were collected after 72 h of treatment. The image shows a representative electrophoretic run; 100 μg of DNA were loaded in each line. (E) Intracellular production of H_2O_2 in TBY-2 cells under all the analyzed experimental conditions. The TBY-2 cells were stained with DHR 123 and visualized under fluorescence microscopy. The images show the control cells and the ophiobolin A-treated cells 24 h after treatment. Bar, 20 μm.

ophiobolin A because the micronuclei formation that typically occurs in apoptotic-like plant cell death was observed in TBY-2 cells undergoing ophiobolin A-dependent cell death (Fig. 3C). In contrast, nuclei from TBY-2 cells treated with 5 μM ophiobolin A had the same morphology as those from the control cells (data not shown). DNA laddering induced by the activation of endonucleases during the PCD process was also evident in TBY-2 cells treated with 10 μM ophiobolin A but was not apparent in either cells subjected to 5 μM ophiobolin treatment (data not shown) or the control cells (Fig. 3D).

It is well known that early H_2O_2 production acts as a signal to activate PCD-dependent defense mechanisms in plant cells (30). Under ophiobolin A treatment, H_2O_2 production only occurred in tobacco cells when PCD had already been activated (Table III). Indeed, H_2O_2 accumulation was observed only in dying cells (Fig. 3E). H_2O_2 production was not detectable in either the control cells or the cells treated with 5 μM ophiobolin A over all the analyzed periods (Fig. 3E).

Effects of ophiobolin A and its degradation product on mammalian cells. Three human cancer cell lines and one mouse cancer cell line were used to determine the *in vitro* growth-inhibitory effects induced by ophiobolin A and its degradation product 3-anhydro-6-*epi*-ophiobolin A (Table II). The data obtained revealed that the mean IC_{50} growth inhibitory concentrations for 3-anhydro-6-*epi*-ophiobolin A were approximately 60 times higher than those for ophiobolin A (Table II). We thus pursued our investigations with ophiobolin A only.

Ophiobolin A delays cell proliferation in both normal and cancer mammalian cells. Treatment with 1 μM ophiobolin A

Table III. The extracellular release of H_2O_2 by TBY-2 cells after 10 μM ophiobolin A treatment.[a]

Time after treatment (h)	Cell viability (%)	H_2O_2 (μM)
0	100.0±0.1	ND
4	98±2	ND
8	95±5	0.3±0.2
15	42±10	9±2

[a]The production of H_2O_2 by tobacco cells was detectable only as cultured cells started dying (see also Fig. 3E). The data are presented as the mean ± SD of three independent experiments; ND, not detectable.

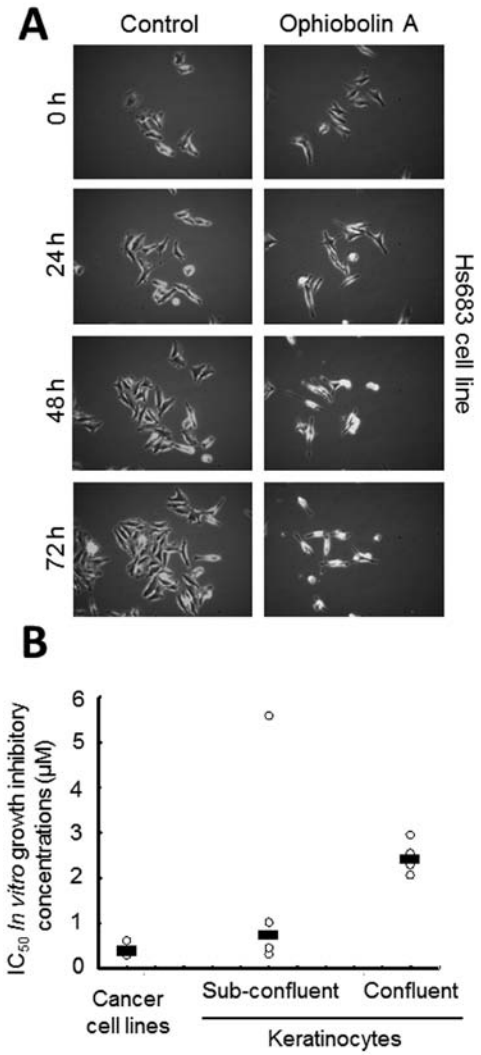

Figure 4. (A) Computer-assisted phase-contrast microscopy (quantitative videomicroscopy) characterization of ophiobolin A-induced cytotoxic effects. Human Hs683 oligodendroglioma cells were maintained in the presence of 1 μM ophiobolin A for 72 h. (B) Determination of the growth-inhibitory effects induced by ophiobolin A by means of the MTT colorimetric assay in four cancer cell lines (Table II) and four normal cell lines, which included four keratinocyte primocultures. We used proliferating subconfluent keratinocytes versus non-proliferating confluent keratinocytes for each of the four keratinocyte primocultures. The effects of ophiobolin A are illustrated as IC_{50} growth-inhibitory concentrations (calculated from three replicates at least for each cell line; the open dots) and their median values (the horizontal black bars).

was found to decrease the cell proliferation of human Hs683 oligodendroglioma cells during the first 72 h of treatment, as revealed by quantitative videomicroscopy analyses (Fig. 4A). However, in contrast to the TBY-2 plant cells, the human Hs683 cancer cells did not regain growth potential when the cancer cells were cultured for 24 h with 1 μM ophiobolin A and then washed and cultured in ophiobolin A-free media (data not shown).

The data shown in Fig. 4A suggest that ophiobolin A might exert cytostatic effects, such as delaying cell proliferation, rather than cytotoxic effects, such as directly killing cells, during the first 24 h of contact with Hs683 cancer cells. We thus investigated whether the global growth rates of mammalian cell populations could influence the growth-inhibitory effects of ophiobolin A, at least *in vitro*. We used four primocultures of keratinocytes and each primoculture was analyzed both while highly proliferating (in a subconfluent stage) and while weakly or non-proliferating (in a confluent stage), as detailed in Materials and Methods. The data revealed that the non-proliferating keratinocytes displayed 5-10 times less sensitivity to the ophiobolin A-induced growth-inhibitory effects than the cancer cells, while the subconfluent keratinocytes displayed intermediate sensitivity (Fig. 4B).

Ophiobolin A is active in vitro against MDR cancer cells. While ophiobolin A induces growth-inhibitory effects in submolar concentrations in various cancer cell lines (Table II), it is not a substrate for MDR-related efflux pumps. Indeed, the data obtained clearly show that ophiobolin A displays similar *in vitro* growth-inhibitory activity in ovarian cancer cells that are either sensitive or resistant to cisplatin (Fig. 5A), in GLC-4 small cell lung cancer cells that are either sensitive or resistant to adriamycin (Fig. 5B) and in leukemia cells that are either sensitive or resistant to mitoxantrone, vincristine or adriamycin (Fig. 5C). These cell lines are known to overexpress also other resistance mechanism key ABC transporter proteins important for MDR such as ABCB1 [HL60/vinc (22,23)], ABCCs [HL60/adr (22,23)], GLC4/ADR (24,25) and ABCG2 [HL60/mx (22,23)]. The efficiency of ophiobolin A in terms of growth-inhibitory activity also remained similar in HCT-116 colon cancer cells that displayed either functional or deficient p53 (26) (Fig. 5D).

Ophiobolin A displays in vivo anticancer activity. At first glance, the data illustrated in Fig. 4B might seem to suggest that ophiobolin A could be weakly bioselective and thus toxic, between normal mammalian cells and cancer cells. We thus transplanted *in vivo* the B16F10 melanoma cell line that displayed actual *in vitro* sensitivity to the growth-inhibitory effects of ophiobolin A (Table II) to determine whether ophiobolin A could represent a potential anticancer agent; i.v. injection of the B16F10 melanoma cells into the tail vein of mice leads to the development of highly aggressive lung pseudometastases in 100% of the injected mice (29).

As detailed in Materials and methods, the *in vivo* dosages used for ophiobolin A (5 and 10 mg/kg chronically administered intraperitoneally three times a week over three consecutive weeks) induced no apparent toxicity as assessed by monitoring the behavior (daily) and weight (twice a week) of the mice (data not shown). In contrast, we observed a

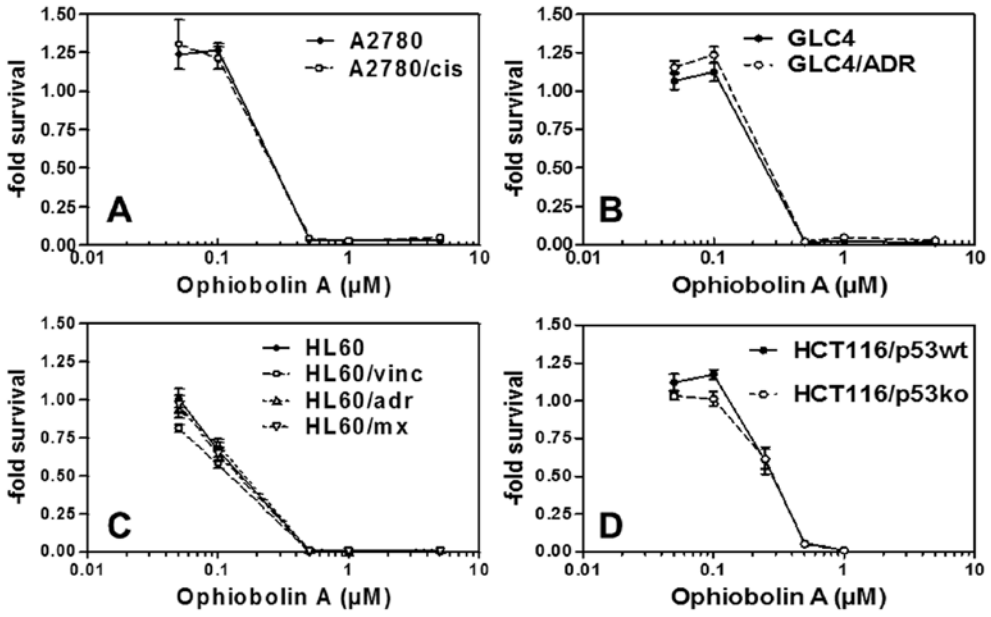

Figure 5. Characterization of the *in vitro* anticancer activity of ophiobolin A in various human MDR cancer cell lines. The anticancer activity of ophiobolin A was assayed in cisplatin-sensitive versus cisplatin-resistant A2780 ovarian cells (A), in adriamycin-sensitive versus adriamycin-resistant small-cell lung cancer cells (B) and in sensitive (HL60) versus adriamycin- (HL60/adr), vincristine- (HL60/vinc) and mitoxantrone- (HL60/mx) resistant pro-myelocytic leukemia HL60 cells (C). Wild-type (wt) versus p53 knock-out (ko) HCT-116 colon cancer cells were also treated with ophiobolin A (D). The charts of the positive controls are available upon request.

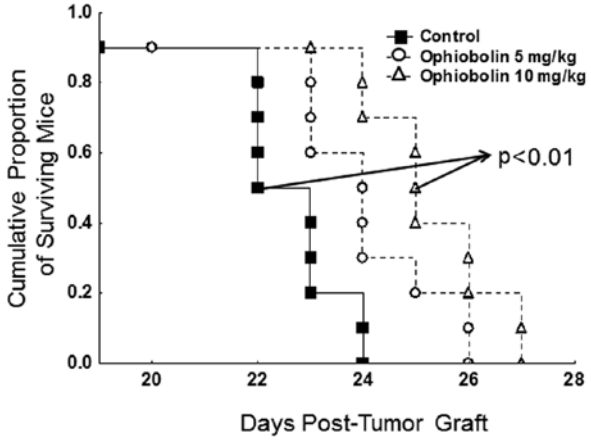

Figure 6. The effects of ophiobolin A on the survival of mice bearing B16F10 melanoma pulmonary pseudometastases obtained by the i.v. administration of $2.5x10^5$ B16F10 cells. Treatments began 5 days after the grafting procedure. Ophiobolin A (5 versus 10 mg/kg) was administered intraperitoneally three times a week for three consecutive weeks.

significant ($p<0.01$) increase in the survival periods of the B16F10 melanoma-bearing mice that were chronically treated with 10 mg/kg (but not with 5 mg/kg) ophiobolin A (Fig. 6).

Discussion

The data from the present study reveal that plant cells are less sensitive to ophiobolin A-induced growth-inhibitory effects *in vitro* than mammalian cells and that slowly proliferating mammalian cells are less sensitive to these ophiobolin A-induced growth-inhibitory effects than highly proliferative ones.

Morphological effects in terms of coleoptile growth and the inhibition of seed germination have been reported in monocot-susceptible plants (1). The data obtained in this study suggest that this inhibitory effect could be correlated with the ability of ophiobolin A to inhibit cell division. At a threshold concentration (5 μM in TBY-2 cells, as shown in the present study), ophiobolin A is able to block cell proliferation without affecting viability. At higher concentrations (>10 μM), ophiobolin A induces cell death and displays PCD hallmarks, suggesting that the compound is able to activate an apoptosis-like process, depending on the applied dose. In plants, PCD is activated in specific cells during normal developmental processes or as a consequence of stress (13,31). Interestingly, PCD is also part of the hypersensitive response (HR), a defense response activated against pathogens by resistant plants to block the penetration of pathogens by surrounding them with a barrier of dead tissue (32). The activation of PCD in tobacco cells by ophiobolin A suggests the ability to activate defense responses against ophiobolin-producing fungi, which is consistent with the fact that dicot plants have been reported to be more resistant than monocots to *Bipolaris* spp. (5). In addition, the plant cell wall is endowed with a plethora of enzymatic systems aimed at degrading pathogen-produced molecules and using them as signal molecules for activating HR or other defense responses (33). During biotic stress, the production of reactive oxygen species (ROS) occurs via the activation of a plasma membrane NADPH oxidase and/or cell wall enzymes. This ROS overproduction is used to promote pathogen killing, to strengthen the cell wall by means of peroxidative crosslinking reactions and to induce the PCD signaling pathway (30). Among ROS, H_2O_2 has been reported as the pivotal signal molecule because of its ability to cross cell membranes and its long half-life relative to those of the

radical ROS (34). Consistently, a biphasic production of H_2O_2 characterizes HR, with the first peak occurring very early after infection, before any morphological symptoms of cell death are evident (35). Surprisingly, the ophiobolin A-dependent PCD was not preceded by any H_2O_2 production; only a late H_2O_2 increase was observed after the death process was already well evident. Recently, an uncommon model of PCD has been demonstrated to be induced by fumonisin B1 and AAL toxin, which are mycotoxins produced by phytopathogenic fungi; in this case, it has been demonstrated that PCD activation was not dependent on H_2O_2 production (36). As these molecules are not chemically related to ophiobolin A and because they are both produced by phytopathogenic fungi, our data suggest that H_2O_2-independent PCD could be a plant response to pathogens that occur with a certain frequency. Further studies are required to identify the signaling pathway leading to this H_2O_2-independent PCD.

The fact that plant cells are less sensitive than mammalian cells to the growth-inhibitory effects of ophiobolin A, at least *in vitro*, could be related to two facts: i) when their viability is not affected, plant cells appear to be able to degrade ophiobolin A, while mammalian cells cannot, and ii) plant cells have been reported to be more resistant to apoptosis-inducing stimuli than mammalian cells (37).

Ophiobolin A was described as an apoptosis-inducer in leukemia cells (38) and more recently, this feature was observed for ophiobolin O in MCF-7 mammary cancer cells (8). Both leukemia and MCF-7 mammary cancer cells are highly sensitive to pro-apoptotic stimuli. In addition, in the four cancer cell lines examined here, the IC_{50} growth inhibitory concentrations were in the same range as those reported in the literature for the ovarian cancer cell line OVCAR3 (6). An interesting feature revealed by the present study is that, of the four cancer cell lines analyzed, the A549 NSCLC and the SKMEL-28 melanoma cell lines displayed various levels of resistance to pro-apoptotic stimuli (39), while the human Hs683 oligodendroglioma (40) and the mouse B16F10 melanoma (41) cell lines displayed actual sensitivity to pro-apoptotic stimuli. No differences in the sensitivity to ophiobolin A were observed between these four cancer cell lines in the MTT assay colorimetric (Table II); it is therefore unlikely that ophiobolin A would mainly exert its growth-inhibitory effects through pro-apoptotic signals in all of these cancer cell lines. Accordingly, we performed flow cytometry analyses of ophiobolin A-induced apoptosis in human cancer cell lines and we indeed observed that ophiobolin A at IC_{50} concentrations induced only weak, if any, pro-apoptotic signals (data not shown). Furthermore, the efficiency of ophiobolin A in terms of growth-inhibitory activity remained similar to that of HCT-116 colon cancer cells that display either functional or deficient p53 (Fig. 5D).

While ophiobolin A induces cytotoxic effects in cancer cells after 72 h (Fig. 4A), it is not a substrate for MDR-related efflux pumps. We used a panel of MDR cancer cell lines exhibiting numerous MDR phenotypes, including the resistant HL60 leukemia cell line that is associated with the ABCC1, ABCB1 and ABCG2 MDR phenotypes (23,25) and the resistant GLC-4 cell line associated with the ABBC1 (MRP1) and LRP MDR phenotypes (25). Ophiobolin A has already been shown to exert weak but nevertheless significant inhibitory effects on P-glycoprotein-mediated transports of drugs (42).

An *in vitro* interaction of ophiobolin A with maize calmodulin has been reported, which supports the hypothesis that the latter could be the target for ophiobolin A in plant cells (43,44). Calmodulin, which acts as a regulator of cell cycle progression, is the principal Ca^{2+} sensor in eukaryotes and is essential for the activation of the cell cycle machinery involved in cell cycle progression (45). The concentration of ophiobolin A required to reach the half-maximal inhibition of calmodulin-dependent cyclic nucleotide phosphodiesterase activity is ~10 μM (43), which is a lethal dose for the plant cells used in the present study. In addition, the concentration required for *in vitro* experiments to reach 50% of the maximum inhibition of the calmodulin-dependent pathways (10 μM) (43) is much higher than either the IC_{50} growth inhibitory concentrations observed in cancer cells (0.3-0.6 μM) or the concentration that completely blocks plant cell growth (<5 μM). More detailed studies are required to determine whether the responses triggered by ophiobolin A in mammalian and plant cells could be closely related to the inhibitory activity of the toxin toward calmodulin.

The fact that ophiobolin A is not a simple poisonous fungal metabolite is evidenced by its ability to increase the survival of mice bearing very aggressive lung pseudometastases, even though the experimental schedule and dosing used to assay the ophiobolin A-mediated *in vivo* antitumor effects were not optimized because of the limited amounts of the compound that were available. These data are encouraging because the B16F10 melanoma model displays a limited response (e.g., no single mouse can be cured) to potent anticancer agents, including temozolomide, cisplatin, adriamycin, irinotecan and taxol (29).

In conclusion, ophiobolin A is a fungal metabolite that has been considered to be a phytotoxin for decades. The present study indeed shows that ophiobolin A causes tobacco plant cell death at certain concentrations but decreases only tobacco plant cell proliferation, not viability, at lower concentrations. The capacity of ophiobolin A to affect either plant cell proliferation or plant viability, according to its concentration, makes it an interesting tool for the study of the different responses of the plant-microorganism interaction. Moreover, this study reports additional evidence supporting the hypothesis that H_2O_2 production might not be implicated in all types of plant PCD.

Mammalian cancer cells appear to be more sensitive to the ophiobolin A-induced growth-inhibitory effects *in vitro* and this fungal metabolite displays antitumor activity *in vivo* for mice bearing lung pseudometastases. While the growth-inhibitory effects mediated by ophiobolin A in plant cells seem related to the activation of apoptotic processes, in mammalian cancer cells, ophiobolin A treatment causes similar activity in both apoptosis-sensitive and apoptosis-resistant cancer cells and also in cancer cells displaying various MDR phenotypes. Current investigations aimed at deciphering the mechanisms of action through which ophiobolin A exerts its anticancer effects are ongoing.

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