Investigations of Fungal Secondary Metabolites with Potential Anticancer Activity

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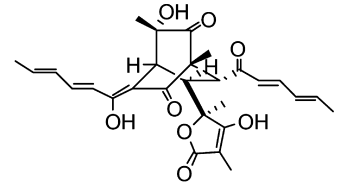
Fourteen metabolites, isolated from phytopathogenic and toxigenic fungi, were evaluated for their in vitro antigrowth activity for six distinct cancer cell lines, using the MTT colorimetric assay. Bislongiquinolide (**1**) and dihydrotrichodimerol (**5**), which belong to the bisorbicillinoid structural class, displayed significant growth inhibitory activity against the six cancer cell lines studied, while the remaining compounds displayed weak or no activity. The data show that **1** and **5** have similar growth inhibitory activities with respect to those cancer cell lines that display certain levels of resistance to pro-apoptotic stimuli or those that are sensitive to apoptosis. Quantitative videomicroscopy analysis revealed that **1** and **5** exert their antiproliferative effect through cytostatic and not cytotoxic activity. The preliminary results from the current study have stimulated further structure−activity investigations with respect to the growth inhibitory activity of compounds belonging to the bisorbicillinoid group.

Purified bioactive compounds derived from medicinal mushrooms may be an important group of new anticancer agents.[1,2] Indeed, many patented anticancer compounds have already been identified in terrestrial fungi, such as polypores, which belong to the phylum Basidiomycota (basidiomycetes).[3] Apart from small cytotoxic compounds, high molecular weight polysaccharides isolated from the polypore cell walls also display direct anticancer effects and prevention of metastases through immunomodulatory activity.[3] Low-molecular-weight secondary metabolites from fungi that display potential anticancer activity target a large set of processes including apoptosis, angiogenesis, metastasis, cell cycle regulation, and signal transduction cascades.[4]

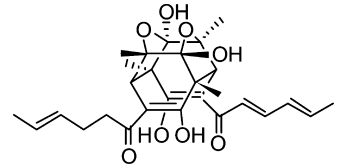
The present study deals with the characterization of the antiproliferative properties of 14 compounds isolated from terrestrial (including phytopathogenic and toxigenic) fungi. These 14 compounds included bislongiquinolide (**1**),[5] its 16,17-dihydro derivative (**4**),[5] dihydrotrichodimerol (**5**),[5] cavoxin (**2**),[6] cyclopaldic acid (**3**),[7] flufuran (**6**),[8] fusapyrone (**7**),[9] phyllostin (**8**),[10] scytolide (**9**),[10] seiricardines B (**10**)[11] and C (**11**),[11] seiricuprolide (**12**),[12] seiridin (**13**),[13] and verrucarin E (**14**).[14]

The growth inhibitory activity of the 14 compounds was determined using four human cancer cell lines that display certain levels of resistance to apoptosis and two apoptosis-sensitive cancer cell lines. The former group included U373 glioblastoma,[15] A549 non-small-cell lung cancer,[16] SKMEL-28 melanoma cell line,[17] and OE21 esophageal cancer.[18] The latter group comprised human Hs683 oligodendroglioma[19] and mouse B16F10 melanoma.[17]

Only two of the 14 compounds under study, i.e., **1** and **5**, displayed significant growth inhibitory activity ($IC_{50} < 100\ \mu M$) in the whole set of cancer cell lines under study. The mean $IC_{50} \pm$ SEM values obtained for **1** and **5** were as follows: U373 (**1** = 4 ± 1 μM; **5** = 25 ± 3 μM), A549 (**1** = 11 ± 2 μM; **5** = 33 ± 3 μM); SKMEL-28 (**1** = 8 ± 1 μM; **5** = 33 ± 3 μM); OE21 (**1** = 9 ± 1 μM; **5** = 28 ± 3 μM); Hs683 (**1** = 22 ± 3 μM; **5** = 34 ± 3 μM); B16F10 (**1** = 3 ± 1 μM; **5** = 3 ± 1 μM). These data indicate that compounds **1** and **5** displayed similar in vitro growth inhibitory activity in apoptosis-sensitive cancer cell lines as well as those that display significant levels of resistance to pro-apoptotic stimuli. Compounds **1** and **5** belong to the bisorbicillinoid group of compounds that includes all dimeric sorbicillin-derived natural products, e.g., bisvertinoquinol, the bisvertinols, bisvertinolone, trichodimerol, trichodermolide, sorbiquinol, bislongiquinolide, the trichotetronines, bisorbicillinol, demethyltrichodimerol, bisorbicillinolide, and bisorbibetanone, which were all isolated from a new fungal source.[5]



Bislongiquinolide (**1**)



Dihydrotrichodimerol (**5**)

Figure 1. Chemical structures of bislongiquinolide (**1**) and dihydrotricodimerol (**5**).

Bislongiquinolide (**1**) is also named trichotetronine.[20,21] To the best of our knowledge, no potential anticancer activity of **1** has been reported to date. In contrast, preliminary data describing anticancer activity of **5** have already been reported by Liu et al.[22] Dihydrotrichodimerol (**5**) has recently been reported to activate peroxisome proliferator-activated receptor-Y (PPAR-Y),[23] which exerts a major role in cancer cell biology.[24] Dihydrotrichodimerol (**5**) has also been reported to suppress the production of tumor necrosis factor-α (TNF-α) and nitric oxide in LPS-stimulated RAW264.7 cells.[23]

As compounds **1** and **5** displayed similar activity in vitro for the cancer cell lines that display certain levels of resistance to apoptosis or those that are sensitive to apoptosis, sorbicillin-derived compounds could represent a novel class of compounds that may

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overcome the natural resistance to apoptosis of those cancers associated with dismal prognoses, such as gliomas,[25] melanomas,[26] pancreatic cancers,[27] non-small-cell lung cancers,[28] esophageal cancers,[29] and, above all, metastatic cancers.[30,31] It must be kept in mind that more than 80% of the chemotherapeutics used today to combat cancers are pro-apoptotic agents.

Among the remaining 12 compounds under study, cavoxin (**2**), which is a fungal metabolite belonging the calchone group of natural products,[7] displayed growth inhibitory activity for three of the six cancer cell lines in the study (IC_{50} range: 25−88 μM). Flufuran (**6**) and verrucarin E (**14**) displayed in vitro activity for two cancer cell lines, with IC_{50} values of 73−88 μM for **6** and 44−80 μM for **14**. Compounds **6** and **14** are both hetero-pentacyclic aromatic compounds that differ only at the heteroatom and the substituent group at C-3.[8,14] Scytolide (**9**) also displayed activity in only two cells lines (IC_{50}: 31−78 μM). This weak activity shown by compound **9** was lost by phyllostin (**8**), its close shikimate metabolite, which as well as **9** belongs to the hexahydrobenzo[1,4]-dioxine group.[10] This result shows that the presence in scytolide[11] of the exocyclic methylene group at C-3 conjugated with the lactone carbonyl group, which is a driving force for nucleophilic additions at the β-position, is an important structural feature for in vitro growth inhibitory activity, as demonstrated in the current study. Compound **12** displayed inhibitory activity ($IC_{50} < 100$ μM) in two out of six cancer cell lines, while the remaining four compounds (**3**, **4**, **7**, and **13**) were inhibitory for only one cancer cell line. Compounds **10** and **11** displayed no activity ($IC_{50} > 100$ μM) for the six cancer cells lines under study. Two reference natural products, i.e., narciclasine[15] and lycorine,[32] extracted from Amaryllidaceae plants,[15,32] were used as reference compounds. Narciclasine displayed IC_{50} values in the range 0.05−0.1 μM in the six cancer cell lines under study,[15] while lycorine displayed IC_{50} values of 2−10 μM.[32]

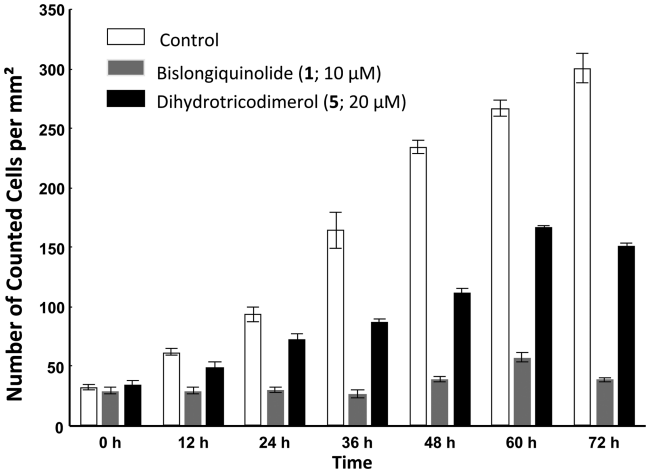

Figure 2. Determination of the mean number of mouse B16F10 melanoma cells (±SEM) present in G×20 microscopic fields of 1 mm^2. Each experimental condition was carried out in triplicate, and five microscopic fields were analyzed for each experimental condition; thus a total of 15 fields for a given experimental condition was performed.

Determination of the in vitro IC_{50} growth inhibitory concentration of a given compound using the MTT colorimetric assay provides no information as to whether the compound is cytotoxic or cytostatic.[32,33] A cytotoxic compound is a compound that kills cancer cells as a direct consequence of its anticancer activity, for example by inducing apoptosis. A cytostatic compound impairs proliferation of cancer cells, and cancer cells in which proliferation is impaired too much will die. Computer-assisted phase-contrast microscopy, i.e., quantitative videomicroscopy, makes it possible to determine whether a compound is cytotoxic or cytostatic.[32,34] The data obtained by means of quantitative videomicroscopy (Figure 2) validated those provided by the MTT colorimetric assay. Indeed, 10 μM **1** and 20 μM **5** markedly impaired the in vitro mouse B16F10 melanoma cell population development over time (Figures 2 and 3). Compounds **1** (Figure 3) and **5** (data not shown) induced their activity against the cancer cells used through cytostatic and not cytotoxic effects. Indeed, it is clearly the impairment of cell proliferation rather than the direct cell killing that translates to the activity displayed by these two compounds in vitro (Figure 3).

Experimental Section

Fungal Metabolites. Bislongiquinolide, its 16,17-dihydro derivative, and dihydrotrichodimerol (**1**, **4**, and **5**) were all isolated from a solid culture of *Trichoderma citrinoviride* as homogeneous solids.[5] Cavoxin (**2**) was isolated as yellow needles from the culture filtrates of *Phoma cava*.[34] Cyclopaldic acid (**3**)[7] and seiricuprolide (**12**)[12] were both isolated as white needles from the culture filtrates of *Seiridium cupressi*. Fusapyrone (**7**) was isolated as a homogeneous oil from the solid culture of *Fusarium semitectum*.[9] Flufuran (**6**) was isolated as a white crystalline compound from the culture filtrates of *Aspergillus flavus*.[8]

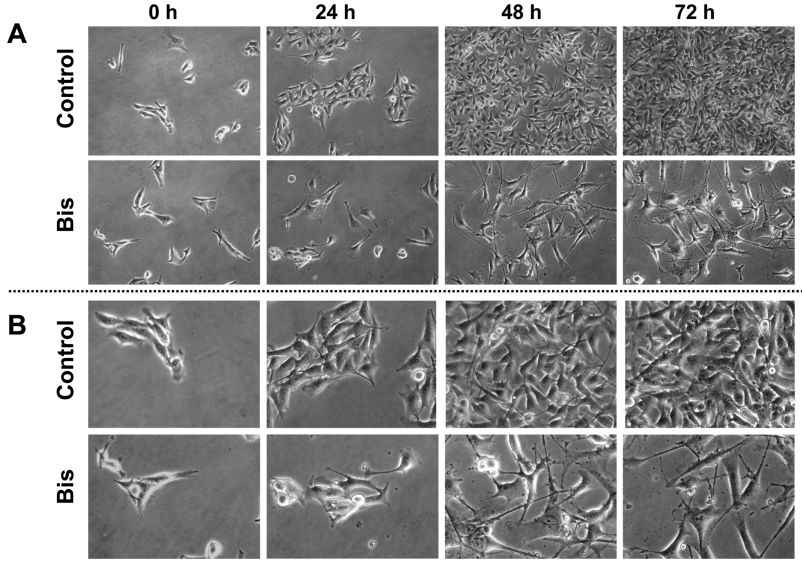

Figure 3. Morphological illustrations of the effects of 10 μM **1** ("Bis") on the development of the mouse B16F10 melanoma cell line over time. (A) Microscopic fields analyzed at low magnification, i.e., G×20. (B) Microscopic fields analyzed at a higher magnification, i.e., G×200.

Phyllostin (**8**) and scytolide (**9**) were isolated, as white needles and a homogeneous solid, respectively, from the culture filtrates of *Phyllosticta cirsii*.[10] Seiricardines B (**10**)[11] and C (**11**)[11] and seiridin (**13**)[13] were isolated as homogeneous oils from the three *Seiridium* species cited above. Verrucarin E (**14**) was obtained as a white crystalline solid from the culture filtrates of *Myrothecium verrucaria*.[14]

Determination of IC_{50} Growth Inhibitory Values. The overall growth level of human cancer cell lines was determined using the colorimetric MTT (3-[4,5-dimethylthiazol-2-yl])-2,5-diphenyltetrazolium bromide, Sigma, Belgium) assay.[32,33] Briefly, the cell lines were incubated for 24 h in 96-microwell plates (at a concentration of 10 000 to 40 000 cells/mL culture medium depending on the cell type) to ensure adequate plating prior to cell growth determination. The assessment of cell population growth by means of the MTT colorimetric assay is based on the capability of living cells to reduce the yellow MTT to the blue product formazan, by a reduction reaction occurring in the mitochondria. The number of living cells after 72 h of culture in the presence (or absence: control) of the various compounds is directly proportional to the intensity of the blue color, which was measured quantitatively by spectrophotometry with a Biorad model 680XR (Biorad, Nazareth, Belgium) at a 570 nm wavelength (with a reference of 630 nm). Each experiment was carried out in sextuplicate.

Five human and one mouse cancer cell line were used. The five human cancer cell lines included U373 glioblastoma[15] (ECACC code 89081403), Hs683 oligodendroglioma[19] (ATCC code HTB-138), A549 (DSMZ code ACC107) NSCLC,[16] OE21 esophageal cancer[18] (ECACC code 96062201), and SKMEL-28 melanoma[17] (ATCC code HTB-72) cells. We also used B16F10 mouse melanoma[17] (ATCC code CRL-6475) cells. The cells were cultured in RPMI (Invitrogen, Merelbeke, Belgium) media supplemented with 10% heat-inactivated fetal calf serum (Invitrogen). All culture media were supplemented with 4 mM glutamine, 100 μg/mL gentamicin, and penicillin−streptomycin (200 U/mL and 200 μg/mL) (Invitrogen).

Computer-Assisted Phase-Contrast Microscopy (Quantitative Videomicroscopy). The direct visualization of the effects of **1** and **5** on B16F10 mouse melanoma cell proliferation and cell migration was carried out by means of computer-assisted phase-contrast microscopy (quantitative videomicroscopy), as detailed elsewhere.[32,34] B16F10 cells were monitored for 72 h in the absence (control) or the presence of 10 μM **1** and 20 μM **5**. Movies were built from the time-lapse image sequences obtained and enabled rapid screening of cell viability.[32,34] In each control or compound-treated condition, the B16F10 cell population growth level was evaluated by the ratio between the number of cells counted in the first and last frames of the image sequences. All the cell counts were performed in triplicate.

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