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Original Research

Effects of 12 beticolins, *Cercospora beticola* toxins, on proliferation of *ras*-transformed adrenocortical cell

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KEY WORDS beticolin; cell division; transformed cell line; adrenal cortex; calcium; oncogene protein p21 (*ras*); confocal microscopy

ABSTRACT

AIM: To explore different effects of 12 beticolins, *Cercospora beticola* toxins, on *ras*-transformed adrenocortical cell growth inhibition and their functional mechanism. **METHODS**: Beticolin-induced inhibition was measured with survival cell number determined by an automated photocolorimetric method. The penetration of beticolin was examined by confocal microscopy. Ras protein determined by Lowry method were separated by 14 % SDS-PAGE and electroblotted to Immobilon-P transfer membrane and detected with pan-Ras (Ab-3) monoclonal antibody. The Ca^{2+} chelation by beticolin was investigated using a calcium ionophore. **RESULTS**: Cell growth inhibition was found dose- and time-dependently at submicromolar level for beticolin-1, -2, and -13 ($IC_{50} \leqslant 250$ nmol/L) and for beticolin-0, 6, and -11 (400 nmol/L < $IC_{50} \leqslant 500$ nmol/L). The inhibition by beticolin-1 was immediate, independent of cell culture step and not reversible for 3-day treatment. Beticolin-3 and -4 were slightly active (1 μmol/L < $IC_{50} \leqslant 2$ μmol/L) and beticolin-7, -9, -12, and -5 were inactive at micromolar level. The beticolin-induced cell growth inhibition was correlated with the hydrophobicity of these compounds. Beticolin-1 fluorescence in RTAC cells was detected by confocal microscopy whereas beticolin-3 and -12 were not even after a 24 h incubation period. Beticolin-1-induced cell growth inhibition was partially reverted by calcium ionophore suggesting a role of intracellular Ca^{2+} chelation by beticolin-1 on cell growth inhibition. Furthermore, beticolin-1 blocked up Ras p21 translocation to membrane and induced accumulation of Ras in the cytosol as an inactive form by different ways. **CONCLUSION**: Beticolins with high hydrophobicity inhibit tumorigenic cell proliferation by different ways.

INTRODUCTION

Beticolins are secondary metabolites extracted from a phytopathogenic fungus *Cercospora beticola* that causes leaf spot disease of sugar beets[1,2]. Their isolation and purification have been performed[3] and some structures have been elucidated by X-ray diffraction, nuclear magnetic resonance, and mass spectrometry[4-6]. These toxins exhibited antibiotic activity and phytotoxicity[1] and modified plant cell phenomena including K^+ uptake and H^+ extrusion, depolarization of trans-membrane electrical potential, and a specific inhibition of a plasmalemma K^+-Mg^{2+}-dependent ATPase *in vitro*[7]. Interaction between beticolins and hydrophobic membranous components has been analysed by fluorescence[8]. These studies have been extended to pharmacological aspects in a controlled model system of mammalian cells, the *ras*-transformed adrenocortical (RTAC) cells. RTAC cells were obtained by stable transfection of freshly trypsin-dissociated rat adrenocortical cells with a plasmid containing the mutated oncogene c-Ha-ras^{EJ}[9]. They are proliferative and tumorigenic cells characterized by the expression of the mutated c-Ha-ras^{EJ} and overexpression of a cellular *ras* gene. The overproduced Ras protein was immunologically identified as Kirsten (Ki) type Ras[10]. As Ki-Ras is the frequent Ras oncoprotein in

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human cancer[11,12], the RTAC cells are the valuable model systems for studying interactions between tumorigenic animal cells and exogenous compounds and for evaluating the potential pharmacological properties of foreign molecules. It was previously shown that beticolin-1 and -2 inhibited the RTAC cell proliferation at submicromolar concentrations and modulated the 11β-hydroxysteroid production in these cells as a function of time and concentration[13]. Laser scanning confocal microscopy experiments led to the presumption that beticolins accumulate in cell organelles and that the cytochrome $P450_{11\beta}$ catalyzing the 11β-hydroxylation of steroids in adrenocortical mitochondria might be one of the targets for beticolins[13].

In this article, we report that twelve beticolins display differential inhibitory effects on proliferation of RTAC cell line. Characterization and classification of these toxins have been made by comparing their inhibiting potentialities at 500 nmol/L with their concentrations allowing 50 % of the maximum inhibition (IC_{50}). Differential penetrations of three typical beticolins into RTAC cells were investigated by laser scanning confocal microscopy. The interactions of beticolins with RTAC cells were discussed with regards to their hydrophobicity measured by their retention time in reversed phase liquid chromatography. To understand the mechanism of beticolin action, we investigated the reversion of beticolin-induced cell growth inhibition by a calcium ionophore and the effect of beticolins on the translocation of Ras p21.

MATERIALS AND METHODS

Chemicals Beticolin-0 to -7, -9, and -11 to -13 were isolated from a *Cercospora beticola* strain[3]. Their purities were assessed by high performance liquid chromatography (HPLC) on an octadecyldimethylsilyl (C18) grafted column[3]. Molecular structures of beticolin-0 to -4, and -13 were determined or confirmed by X-ray diffraction analyses[5,6], that of beticolin-6 with nuclear magnetic resonance and mass spectrometry. These structures are shown in Fig 1. Structures of beticolin-5, -7, -9, and -11 are under study, nevertheless their molecular weights were determined (M_r 622 for beticolin-0, -11, -12, and -13; M_r 638 for beticolin-1, -2, and -6; M_r 654 for beticolin-3 and -4; M_r 656 for beticolin-5, -7, and -9). A calcium ionophore was obtained from Sigma (St Louis, MO, USA). All the solvents used were of analytical grade.

Adrenocortical cell cultures Primary newborn rat adrenocortical cells were transfected with a plasmid p^{EJ} containing the mutated oncogene c-Ha-*ras*^{EJ} encapsulated in a liposome[9]. Cell lines were selected by passage through immunodepressive mice[9]. Several RTAC cell lines or sublines were established and characterized[9,13]. The GM16-RTAC cell line was mainly used in this work at around the 50th passage. Each passage in subculture was obtained by trypsin dissociation of the cell monolayer and by seeding 0.5×10^6 cells in a flask of a 25-cm^2 culture area, filled with 5 mL of serum supplemented medium (SSM). These culture medium contained 2.5 % fetal calf serum, 2.5 % newborn calf serum (Gibco, Gaithersburg, MD), Ham's F10 (Gibco) and William's E (Flow Laboratory) media (1:1, v/v). An average cell number of 4.5×10^6 cells per flask were obtained at confluence. To measure cell proliferation, 5×10^3 cells were seeded with 0.2 mL of SSM in each well of a 96-well dish. Cell number was determined by an automated photocolorimetric method[13,14]. Cells attached to the plastic substratum were stained with methylene blue for 10 min then washed several times with water. Release of methylene blue from cells was obtained by treating them with 0.2 mL of HCl (0.1 mol/L) and the absorbance was measured at 630 nm. A linear regression was found between the absorbance and cell number (absorbance = $1.09 \times 10^{-5} \times$ cell number + 1.64×10^{-3}; regression coefficient 0.995; $n = 8$). To access beticolin effect on cell growth, cell incubations with beticolins were carried out each day by introducing the compound in dimethylsulfoxide-water (1:9, v/v) solution into fresh medium. A calcium ionophore was added as an ethanol solution. Each control or test was performed in octoplicate. The treatments started from the cell attachment, 3 h after seeding. The controls were incubated with the same volume of the drug vehicle alone.

Subcellular fractionation and immunoblotting Cells were washed with PBS and then cellular lysate was fractionated at 30 000 × *g* into cytosolic or soluble (S) and membranous or precipitated (P) fractions as described previously[18]. According to Lowry's method[19], aliquots of 15 μg protein of S and 5 μg protein of P were separated by 14 % SDS-PAGE and electroblotted to Immobilon-P transfer membranes (Millipore, Bedford, MA). Ras proteins were detected with pan-Ras (Ab-3) monoclonal antibody (Oncogene Science, Uniondale, NY). Blots were developed using a horseradish peroxidase-conjugated anti-mouse antibody, enhanced-chemiluminescence reagents, and hyperfilm-ECL

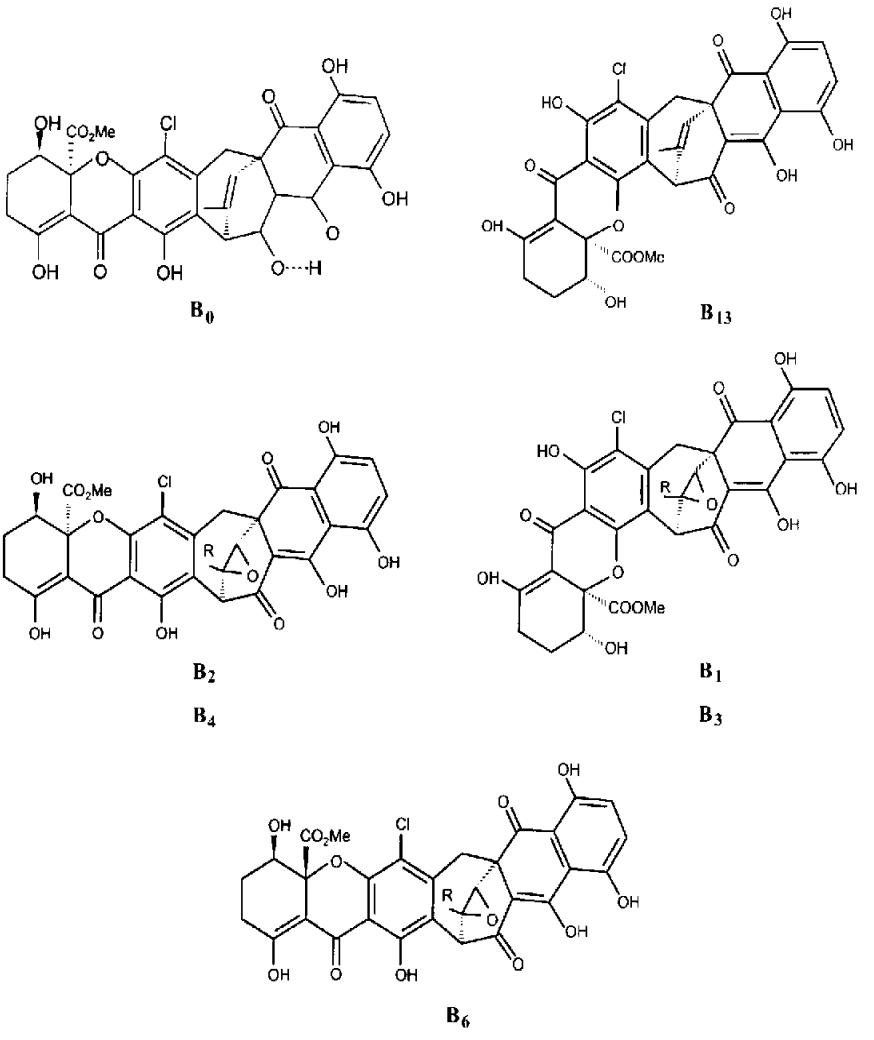

Fig 1. Structures of some beticolins. Beticolin-0, -1, -2, -3, -4, and -13 structures have been determined by X-ray diffraction; beticolin-6 by comparing NMR and MS data. R = CH_3 for B2, B1, and B6; R = CH_2OH for B4 and B3.

(Amersham Inc, UK).

Confocal microscopy Living cells were viewed under a Leica TCS 4D confocal microscope using an oil immersion objective lens 1.4 NA × 60. The fluorescence excitation wavelength was set at 488 nm and emission at 515 nm. The focal depth was 1 micron.

RESULTS

Differential cellular growth inhibition induced by beticolins RTAC cells were incubated with beticolins (500 nmol/L) for 6 d and inhibition of cell growth was analyzed every day. Beticolin-1, -2, and -13 showed a significant inhibitory effect on cell growth, which increased up to 93 %, 90 %, and 88 %, respectively. For beticolin-0, -11, and -6 with inhibitory capacity 74 %, 53 %, and 51 %, the effect was noticeable for longer periods. Beticolin-4 and -3 with 32 % and 14 % showed a slight effect. Beticolin-7, -9, -12, and -5 displayed no effect on cell growth compared to control. A dose-response effect was observed between 0.1 nmol/L and 10 μmol/L for beticolin-0 to -4, -6, -11, and -13. Typical dose response curves were determined on the 6th day of treatment for beticolin-1 and -3 whereas almost no response was observed with beticolin-12 at concentration up to 10 μmol/L (Fig 2). According to the percentage of inhibition at 500 nmol/L or the IC_{50} on the 6th day of treatment, beticolins could be divided into four groups. A strong inhibitor group involved beticolin-1, -2, and -13 ($IC_{50} \leqslant 250$ nmol/L).

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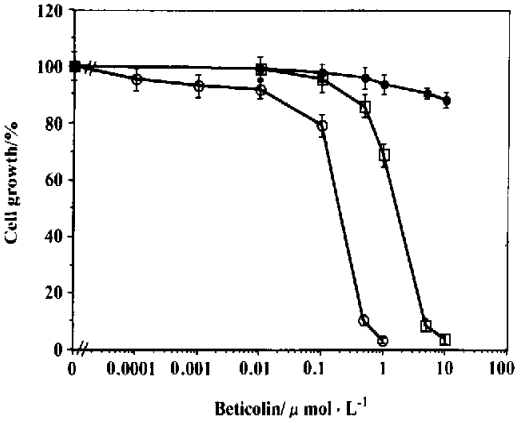

Fig 2. Dose-dependent inhibition of RTAC cells incubated with beticolins representative of three different groups. Inhibition of cell growth was measured on the 6th day of incubation. The results were average of a typical experiment repeated three times. $n=3$. $\bar{x} \pm s$. Beticolins used were beticolin-1 (○), beticolin-3 (□), and beticolin-12 (●).

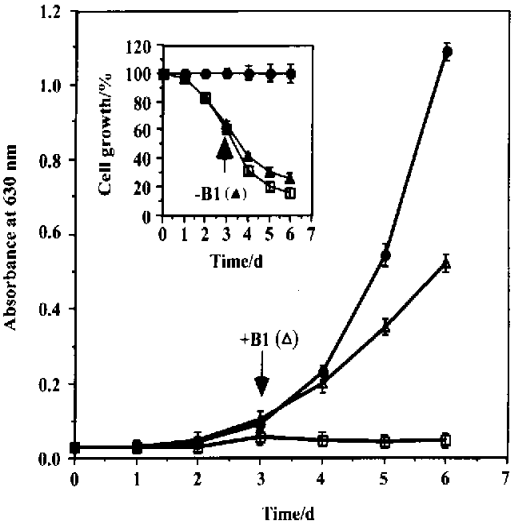

Fig 3. Effect of the beginning or withdrawal of beticolin-1-treatment on RTAC cell growth. Cell treatment by beticolin-1 (500 nmol/L) started from the beginning of the culture (□), or after 3 d (△), or control (●). Inset: cell treatment by beticolin-1 (500 nmol/L) for 6 d (□); cell treatment was stopped after 3 d (▲), control (●). $n=3$. $\bar{x} \pm s$.

Beticolin-0, -11, and -6 were included in the moderate group (400 nmol/L < IC_{50} ≤ 500 nmol/L). Beticolin-4 and -3 was in the group of weak inhibitory effect (1 μmol/L < IC_{50} ≤ 2 μmol/L). The forth group was beticolin-7, -9, -12, and -5 with no significant effect. Inhibition of cell growth induced by beticolin-1 was started at the 1st day of culture and at the beginning of exponential cell growth (the 3rd day) (Fig 3). This showed that beticolin-1 acted not only immediately on cell growth but also at any step of cell growth development. When beticolin-1 was added in the cell culture medium for 3 d and then omitted until the 6th day of culture, the cell growth inhibition was not reversed compared to a continuous treatment (Fig 3, inset). This result indicates that the inhibitory effect induced by beticolin-1 on cell proliferation is not reversible when cells have been treated during 3 d.

Differential penetration of beticolins into cultured RTAC cells Differential effect of beticolins on cell growth might be dependent upon their penetration into the cells. This hypothesis was explored by the kinetics of beticolin-1, -3, and -12 uptake by RTAC cells with confocal laser scanning microscopy by taking advantage of the autofluorescence of beticolins[8].

RTAC cells attached on a glass slide were incubated in a SSM culture medium containing beticolins 1 μmol/L for 1, 8, and 24 h. The fluorescence of beticolin-1 was detected in the cytoplasm and cytoplasmic organelles after 1 h at a level above the cell fluorescence background (Fig 4A, B, and C). In this case, the fluorescence increased with time. Semi-quantitative measurements of fluorescence intensities showed that cells incubated with beticolin-3 and -12 did not differ from the control at 1, 8, or 24 h of incubation (Fig 4D, E, and F). Conversely, curves obtained with beticolin-1 showed an increasing number of spots at high intensities when the incubation time increased (Fig 4D, E, and F). These results suggest that beticolin-1 not -3 nor -12 can penetrate into cells during these incubating periods.

Effect of calcium ionophore on beticolin-inhibited cell growth Beticolins were described as agents chelating divalent ions such as magnesium[17] and calcium[1]. A tightly regulated calcium concentration was an important factor for regulating cell proliferation[18]. In order to test whether beticolin-induced inhibition of cell proliferation was due to the decrease of intracellular calcium concentration, it was investigated whether the addition of a calcium ionophore could reverse the beticolin-induced growth inhibition. When RTAC cells were incubated with calcium ionophore from 1 nmol/L up to 1000 nmol/L in the presence of beticolin-1 250 nmol/L, the inhibition of cell growth induced by beticolin-1 was partially reversed (Fig 5). In presence

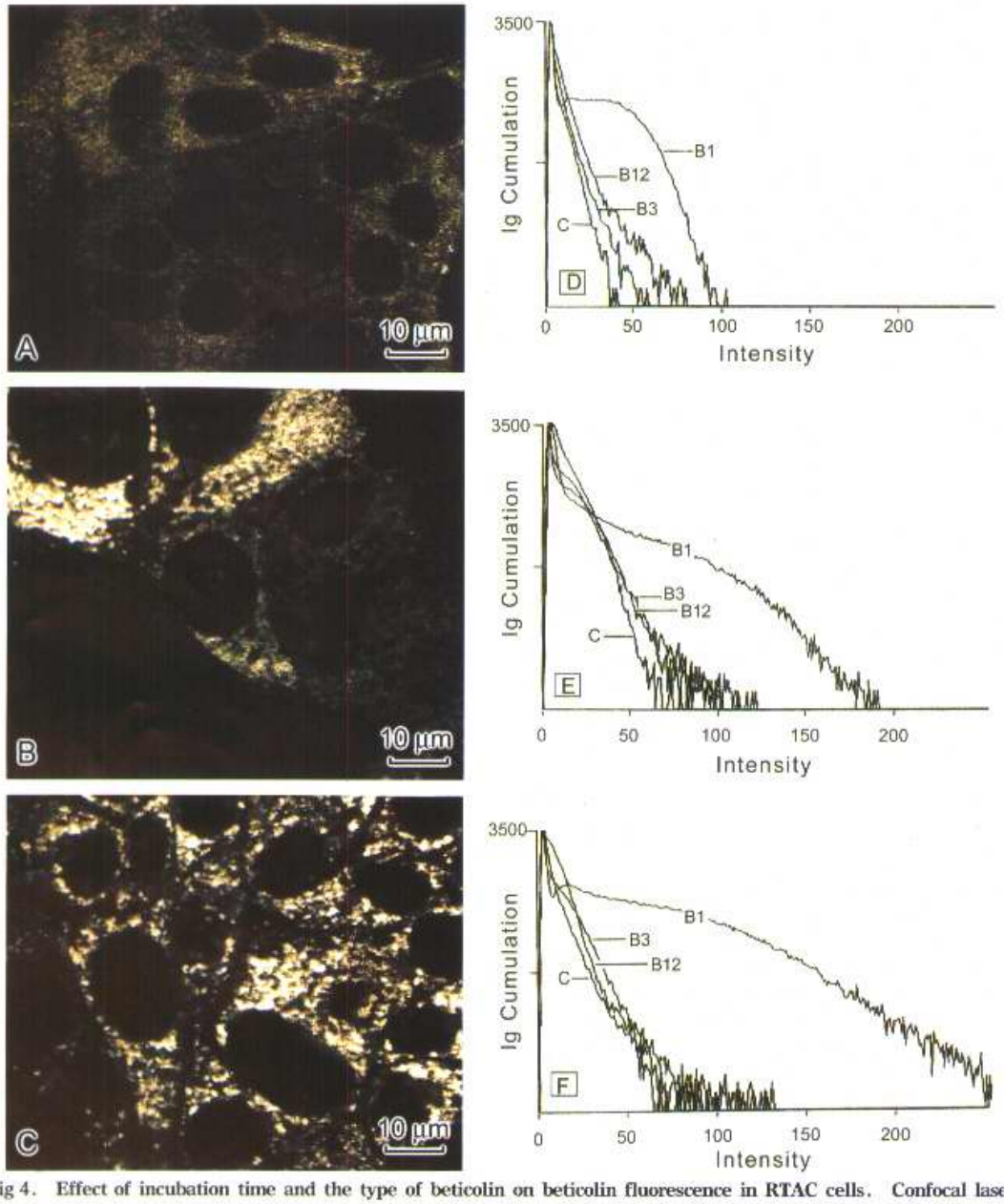

Fig 4. Effect of incubation time and the type of beticolin on beticolin fluorescence in RTAC cells. Confocal laser scanning pictures showing beticolin-1 fluorescence in RTAC cells after 1 h (A), 8 h (B), or 24 h (C) incubations. Semi-quantitative measurement by determining the number of items in function of fluorescent intensities of spot in image for different times of incubation: 1 h (D), 8 h (E), or 24 h (F) with beticolin-1, -3, and -12 and control.

of calcium ionophore above 250 nmol/L, beticolin-1-induced inhibition of cell growth increased and at 400 nmol/L rised above to the level as with beticolin-1 alone. Without beticolin-1, the calcium ionophore inhibited cell growth in a dose-dependent manner, showing that an excess of intracellular calcium was also cell growth inhibitor (Fig 5, inset).

Effect of beticolin-1 and -3 on Ras p21 translocation Since beticolin-induced inhibition of cell growth by a calcium ionophore was only partially reversed, we wanted to search for another site of action. As RTAC cells were Ras dependent for their growth, we

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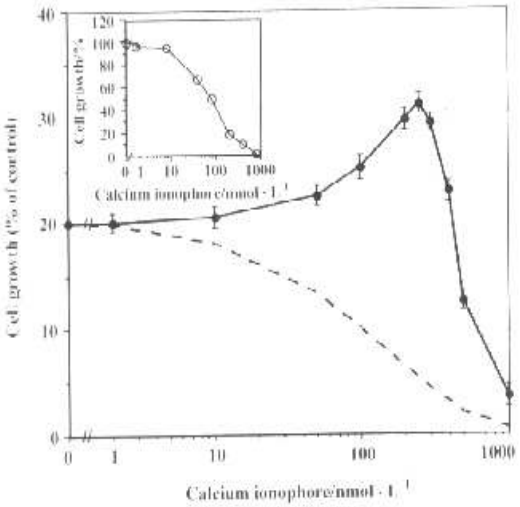

Fig 5. Partial reversion by calcium ionophore on beticolin-1-induced inhibition of RTAC cell growth. RTAC cell received beticolin-1 250 nmol/L on the first day of culture and various concentrations of calcium ionophore. Inhibition of cell growth was measured on the 6th day of incubation. In dotted line: theoretical inhibition of calcium ionophore plus beticolin-1 on cell growth. Inset: effect of calcium ionophore concentrations on cell growth.

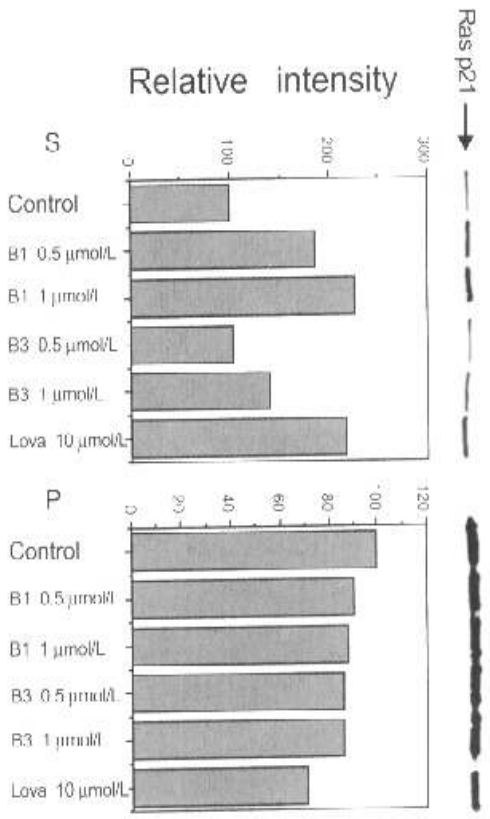

Fig 6. Inhibition of p21 translocation induced by beticolin-1, beticolin-3, or lovastatin. GM16 cells were incubated with beticolin-1 (B1), beticolin-3 (B3) and lovastatin (Lova) at the indicated concentrations for 24 h. Semiquantitative estimation of electrophoresis spots was obtained by microdensitometry and given as relative intensity compared to control (100 %). The figure represents a typical experiment repeated at least three times.

investigated if beticolins altered the process of Ras modifications leading to Ras translocation from cytoplasm to plasma membrane. Inhibition of Ras translocation could result in a cytosolic form of Ras devoid of transforming activity and could explain cell growth inhibition. The effects of beticolin-1 and -3 on Ras membrane association were investigated. Amounts of Ras proteins in the cytosolic or membranous fractions of RTAC cells exposed to beticolin-1 and -3 0.5 and 1 μmol/L for 24 h were assessed. SDS PAGE and Western blotting analysis showed a 2-fold and 2.5-fold increase in Ras p21 of the cytosolic fraction when beticolin-1 was incubated at 0.5 and 1 μmol/L respectively with RTAC cells. Conversely, beticolin-3 had no effect on the accumulation of Ras p21 in the cytosolic fraction at 0.5 μmol/L and only a weak effect (1.4-fold increase) at 1 μmol/L. For comparison, lovastatin at 10 μmol/L was shown to accumulate (2.2-fold) Ras protein in cytosolic fraction (Fig 6). Beticolin-2 presented an effect on Ras accumulation similar to those of beticolin-1 (results not shown). Ras protein accumulation in the cytosolic fraction of beticolin-1- or -2-treated cells indicated that these toxins inhibited the translocation of Ras proteins. Beticolin-3 at the used concentration had a very weak effect on Ras translocation.

Correlation between cell growth inhibition by beticolin and its retention time The percentage of cell growth inhibition induced by beticolins at 500 nmol/L was correlated to its retention time measured by reversed phase HPLC with a C18 grafted column (Fig 7). All the molecules giving less than 50 % cell growth inhibition had a retention time lower than 17 min which could be considered as a threshold of beticolin activity.

DISCUSSION

Beticolines could be separated with a large range of retention times by reversed phase HPLC with a C18 grafted column[3,10], showing that beticolins have very

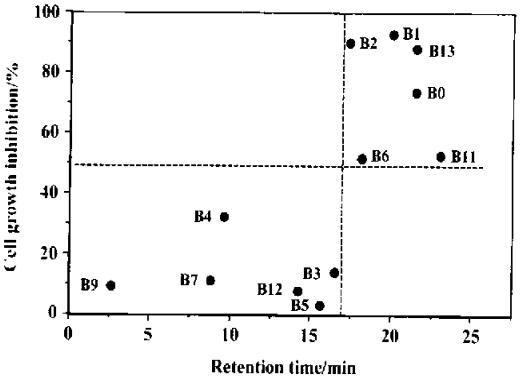

Fig 7. Correlation between RTAC cell growth inhibition and hydrophobicity measured by C18-HPLC retention times for twelve beticolins. Retention times in min were obtained in reverse-phase HPLC on a C18 grafted column.

different hydrophobicities. The hydrophobicity of a molecule is well known to control its distribution between a membrane lipid or protein phase and an aqueous phase. Therefore, these molecules have the profound significance on their biological function and activity[19]. Retention times of beticolins can be used to measure their hydrophobicity. Beticolins with higher hydrophobicity, ie, with longer retention time, are stronger inhibition on cell growth. The dose-response study brought more details about characterization of beticolins: they can be divided into very active, moderately active, weak active, and completely inactive molecules. This explanation is consistent with images obtained by laser scanning confocal microscopy showing a time-dependent internalization of fluorescent beticolin-1 into the cell. Beticolin-3 and -12 did not induce any fluorescence inside the cell after 24-h incubation. These results suggest that the higher the hydrophobicity of beticolin is, the more efficient their internalization is and the higher their inhibitory capacity is. Only after their internalization could beticolins interact with a second messenger or a protein of the transduction cascade responsible for cell mitosis.

The mode of inhibition of RTAC cell growth by beticolins was examined according to two hypotheses. Firstly, beticolins induced a chelation of intracellular divalent ions such as calcium and magnesium[1,17] that decreased their intracellular concentration. It was recently emphasized that intracellular calcium was essential for cell growth and survival[18]; but its increase could also activate the apoptosis processes[18]. This hypothesis is consistent with the fact that beticolin-induced inhibition of cell growth was partially reverted by calcium ionophore 250 nmol/L. Nevertheless, only 13 % of cell growth restoration showed that another mechanism was also involved in the inhibition process. Secondly, we postulated that beticolins inhibited Ras protein translocation to the inner plasma membrane where growth and differentiation signals were transferred[20]. Such a mechanism was evidenced in numerous types of cell for another fungus toxin, lovastatin, a known inhibitor of HMG-CoA reductase[20]. It was believed that Ras translocation blockage by lovastatin was the major cause of inhibition of proliferation in Ras-dependent mammalian cells[21,22]. A marked accumulation of Ras p21 in the soluble fraction of beticolin-1-treated cell was detected by immunoblotting; this accumulation of Ras was similar to that observed with lovastatin-treated cells. These results led us to postulate that beticolin-1 could exert its action through post-translation modification of Ras proteins.

Our data show that beticolin-induced inhibition of cell growth results from multiple target interactions with these toxins. Cell internalization of beticolins is largely dependent on their hydrophobicity. Inside the cell, the internalized beticolins interact with Ca^{2+} ions resulting in a minor effect on cell growth. Beticolins inhibit RTAC cell proliferation mainly through Ras translocation blockage. Therefore, beticolins could be interesting pharmacological tools for research against Ras-dependent cancer.

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真菌 *Cercospora beticola* 的 12 种毒素贝第高林对癌基因 *ras* 转导的肾上腺细胞生长的作用

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关键词 贝第高林; 细胞分裂; 转化细胞系; 肾上腺皮质; 钙; 原癌基因蛋白质 p21 (*ras*); 共聚焦显微镜检查

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