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Carvone and Perillaldehyde Interfere with the Serum-Induced Formation of Filamentous Structures in *Candida albicans* at Substantially Lower Concentrations than Those Causing Significant Inhibition of Growth

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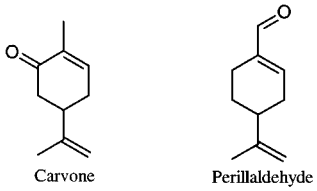
Abstract: Carvone and perillaldehyde were shown to inhibit the transformation of *Candida albicans* to a filamentous form at concentrations far lower and more biologically relevant than the concentrations necessary to inhibit growth. This morphological transformation is associated with *C. albicans* pathogenicity; hence these naturally occurring monoterpenes are potential lead compounds in the development of therapeutic agents against *C. albicans* infection.

Candida albicans is a fungus that is part of the normal human flora and is not necessarily associated with human disease. However, *C. albicans* can cause mild to fatal opportunistic infections and can be particularly dangerous in people with compromised immune systems such as cancer patients undergoing immunosuppressive therapy and AIDS patients. It is the most common fungal pathogen associated with cancer[1] and is a particular problem in patients with neutropenia, a condition of lowered neutrophil count that can result from chemotherapy or radiation therapy. Three major forms of the disease are known: genital candidiasis, oral candidiasis, and invasive or deep candidiasis.[2] Occasionally other forms are found such as ocular candidiasis or infection of the skin and nails.[2] Of the three major forms, the first two, genital and oral are generally treatable, although they may be quite persistent. The most dangerous form of infection, invasive or deep candidiasis, where the fungus invades internal organs, has a mortality rate of 40−60%.[3] Fungal infection, primarily *Candida* and to a lesser extent *Aspergillus*, is the major cause of infectious death among patients with chemotherapy-induced myelosuppression (neutropenia).[4] In fact, it is a major cause of death among patients with acute leukemia. At some institutions 40% or more of the mortality associated with such patients is due to fungal infection, primarily by *Candida*.[5]

Pathogenicity is associated with conversion of the fungus from a cellular yeast to a filamentous form.[1,6,7] Patients with serious disease generally have filaments of *C. albicans* penetrating the infected tissue. Filamentous penetration through the gastrointestinal tract is also probably the origin of a majority of deep or invasive *Candida* infections. Various organs can be affected such as the liver, spleen, lungs, heart, and brain, each manifesting different symptoms.[2]

C. albicans can be converted from the cellular yeast to the filamentous form in vitro by exposure to serum. Serum can induce a variety of filamentous structures (i.e., germ tubes, hyphae, and pseudohyphae).[8] This in vitro conversion provides a convenient model system for the testing of potential therapeutics aimed at preventing conversion of *C. albicans* from the relatively benign, cellular yeast form to the pathogenic, filamentous forms.



Carvone and perillaldehyde, as well as other related monoterpenes, have been shown to have a variety of antiproliferative effects in microbial[9] as well as mammalian cell lines.[10,11] However, their usefulness has been limited by the high concentrations necessary to cause a significant

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biological effect. We show that carvone and perillaldehyde can cause inhibition of growth in the cellular yeast form of the organism at high concentrations. However, both compounds can cause inhibition of the tranformation of the organism from the relatively benign, cellular yeast form to the pathogenic, filamentous form at much lower levels. Hence, these natural products are potential lead compounds in the development of chemotherapeutic agents against *C. albicans* infection.

Organism and Growth Conditions. A clinical isolate of *C. albicans*, strain H-317, was obtained from the Centers for Disease Control and Prevention. The yeast was maintained in the budding or cellular yeast form in suspension culture using either defined media[12,13] or YEPD media [1% w/v yeast extract (DIFCO), 2% w/v bacto peptone (DIFCO), 2% w/v dextrose (Fisher Scientific)] at room temperature. Overnight cultures (10 mL, initial cell density (2.5−10) × 10^6 cells/mL) were subcultured and grown in defined media or YEPD media in the presence of monoterpene [*S*-(+)-carvone or *S*-(−)-perillaldehyde] or vehicle (DMSO) control (final concentration of DMSO, 0.1%) (total volume, 5 mL) for 2 h or overnight at room temperature. Growth in the cellular yeast form was monitored by measuring the optical density at 600 nm.

Serum Induction of Filamentous Structures. After a 2 h preincubation in the presence of monoterpene or vehicle control (see above) a portion of the cells (to form a 1/10 dilution) was added to PBS (pH 7.4) (140 mM NaCl, 3 mM KCl, 8.6 mM $NaHPO_4$, 2.3 mM KH_2PO_4, adjusted to pH 7.4 with HCl) containing 10% fetal bovine serum (BioWhittaker) and grown in the presence of monoterpene [*S*-(+)-carvone or *S*-(−)-perrillaldehyde] or vehicle (DMSO) control (final concentration of DMSO, 0.1%) (total volume, 5 mL), at 37 °C. After an overnight incubation with monoterpene or vehicle control in the presence of serum, images were recorded using phase contrast microscopy, and the amount of the filamentous form was determined.

Quantification of Filamentous Structures. To obtain quantitative data concerning the relative amount of the culture in the filamentous form, the following procedure was used. Cells were first passed through a Pasteur pipet to aid in the breakup of clumps of cells. PBS (5 mL, pH 7.4) was then added, and the suspension was mixed using a Vortex mixer at maximum speed and centrifuged at 800*g* for 10 min. This washing step was done to remove serum proteins and was repeated three times using 10 mL of PBS (pH 7.4) for each subsequent wash. The supernatant from the last wash, when tested by Biorad protein assay, showed no detectable level of protein, indicating that serum proteins had been removed. Finally, the cells were taken up in 1 mL of PBS (pH 7.4) and passed over a polypropylene 105 μm mesh screen (Fisher Scientific), and the filtrate was collected. The screen was then washed with several 1 mL aliquots of PBS (pH 7.4) and the flow-through collected in the same tube. The cells passing through were in the cellular yeast form. The material remaining on the screen, primarily filamentous material, was washed off the screen with PBS (pH 7.4) to a separate tube. The filamentous fractions from this step were adjusted to 5 mL with PBS and disrupted with glass beads. The total protein was then determined for each fraction by Biorad protein assay.

Results and Discussion

Effects of Carvone and Perillaldehyde on the Conversion of *C. albicans* from a Cellular Yeast to a Filamentous Form. Figure 1 shows photomicrographs of *C. albicans* cells treated with *S*-(+)-carvone (250 μM) or

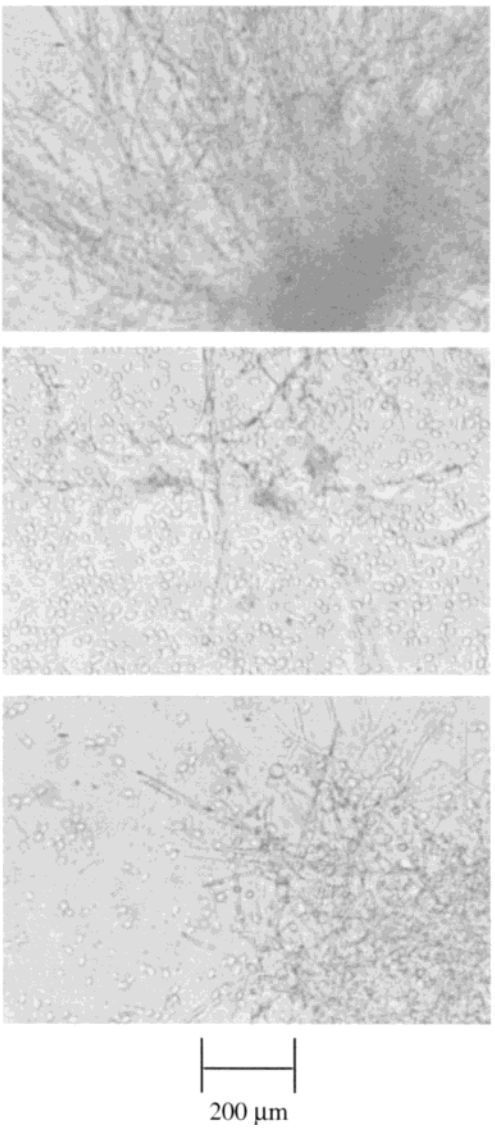

Figure 1. Cells treated with vehicle (DMSO), top, *S*-(+)-carvone (250 μM), middle, or *S*-(−)-perillaldehye (125 μM), bottom, in YEPD media for 2 h, then in PBS containing 10% serum for an additional 22 h. The top figure appears somewhat distorted due to the large size of the tangled clump of filaments present in the sample.

S-(−)-perillaldehyde (125 μM) and vehicle control 22 h after exposure to serum. The control shows the typical tangled filamentous structures associated with serum exposure with very few cells in the cellular yeast form. Far fewer filaments were observed by microscopy in the monoterpene-treated samples, and the pictures shown are typical of the filaments that were found. These pictures demonstrate that the few clumps of filaments that were observed were smaller and with less well developed filaments. Additionally, a large number of cells in the cellular yeast form can be seen.

Quantification of the Filamentous Form of *C. albicans* after Exposure to Serum. We have developed a technique to partially separate and quantify the filamentous form of *C. albicans*. The examination of potential therapies in vitro that are based on blocking the transformation of *C. albicans* from a cellular yeast to a filamentous form are hampered by the difficulty in quantifying the relative amounts of the two forms of the organism. This is due to the propensity of the organism to form clumps of cells which leads to large tangled filamentous structures, where the individual filaments are not discernible. The

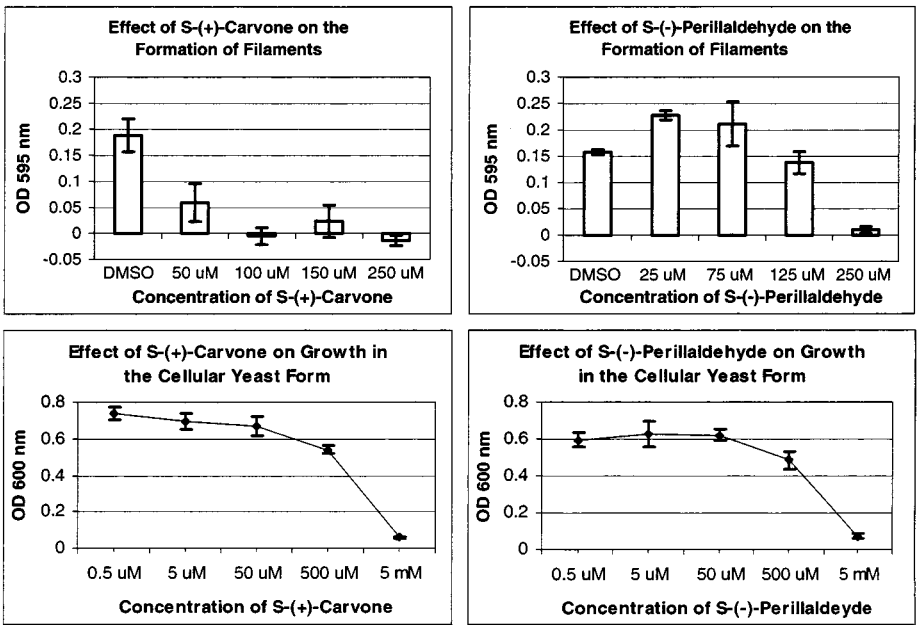

Figure 2. Cells were grown with the indicated concentration of monoterpene. The top panels show the total protein from the filamentous fraction, by Biorad protein assay, of cells that were treated with the indicated concentration of monoterpene in media for 2 h and with the indicated concentration of monoterpene in PBS containing 10% serum overnight. The bottom panels show the optical density at 600 nm of a 1/10 dilution of samples that were grown with the indicated amount of monoterpene in media in the absence of serum. All samples were grown in defined media (see text). Duplicate readings of duplicate samples (upper panels) or duplicate readings of triplicate samples (lower panels) were taken. Values represent the mean value, and the error bars are ±SD. Very similar results were also found using YEPD media (data not shown).

method that we describe provides a way to separate the majority of the cells in the cellular yeast form from the cells in the filamentous form. Microscopic examination showed that the cellular yeast fraction is almost completely free of filaments; however, the filamentous fraction still contains a small number of cellular yeast cells that are associated with large filamentous structures (data not shown). Hence the separation can only provide relative and not absolute numbers for the separation. The upper panels in Figure 2 show the amount of total protein in filamentous fractions of samples treated with several concentrations of carvone and perillaldehyde or vehicle control.

Effects of Carvone and Perillaldehyde on the Growth of the Cellular Yeast Form of *C. albicans*. The lower panels in Figure 2 show the results of various concentrations of carvone and perillaldehyde on the growth of *C. albicans* in the cellular yeast form by OD 600 measurement. At very high concentration (5 mM) significant inhibition of growth was seen. Carvone and perillaldehye at lower concentrations had little or no effect on cell growth in the cellular yeast form.

These results indicate that the monoterpenes carvone and perillaldehyde are potentially useful lead compounds in the development of chemotherapuetics directed against *C. albicans* infection. We have shown that these compounds interfere with the morphological change of *C. albicans* from the relatively benign, cellular yeast form to the pathogenic, filamentous form in vitro. This represents a different strategy from the current strategies used to treat *Candida* infection that are aimed at killing the organism. There is a clear need for the development of new strategies to treat *Candida* infection especially in light of the widespread development of resistance to the azole compounds currently in use.[14] The results described above open a promising area of research with regard to *C. albicans* and possibly other serious fungal infections. We are planning to study the effects of monoterpenes, of a variety of different skeletal types, on the morphological transformation associated with pathogenicity in *C. albicans* as well as their effects on other dimorphic fungi.

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