

The Antihistaminic Chlorpheniramine Inhibits *in vitro* Growth of Several Fungi Isolated from Harvested Fruits

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Chlorpheniramine (CPA) is an antihistaminic that changes the conformation of DNA and inhibits polyamine biosynthesis in mammalian cells. In the present work, we tested the effect of CPA on four genera of fungi species (*Alternaria alternata*, *Botrytis cinerea*, *Cladosporium cladosporioides* and three *Penicillium* spp.) grown *in vitro*. Similar growth inhibitions of these genera were produced by 0.5 mM iprodione, CPA and histidinol, but CPA was the most effective. The CPA sensitivities of the two *B. cinerea* strains were different. Putrescine did not restore the fungal growth inhibited by CPA.

KEY WORDS: Chlorpheniramine; histidinol; iprodione; benomyl; harmaline; polyamines.

INTRODUCTION

Chlorpheniramine (CPA) is a synthetic compound which antagonizes the subtype-H₁, histamine receptor of mammalian cells (4). Its structure resembles those of natural 1,4-diamines (histamine, serotonin, and ornithine-derived polyamines) (7,16). Chlorpheniramine might also function well as a fungicide and, in fact, CPA, like several families of active fungicides, is a chiral compound with heterocyclic rings and a chlorophenyl moiety (13).

Because of its 1,4-diamine structure, CPA is also characterized as an inhibitor of the synthesis of polyamines as it inhibits ornithine decarboxylase (ODC, EC 4.1.1.17), the key enzyme in animal cells for the biosynthesis of putrescine, spermidine and spermine (7). CPA and other histamine analogs mimic several functions of the ornithine-derived polyamines (10,15). Molecular methods and infrared and Raman spectroscopy studies demonstrate that the CPA-nucleic acid interaction involves mainly the phosphodiester bonds and guanine residues (9). Like all histamine and ornithine-derived polyamines, CPA is reported to modulate animal-cell growth (8). In addition, cell growth shows a biphasic response to polyamines and other histaminine-related compounds like N,N-diethyl-2-[4-(phenylmethyl) phenoxy] ethanamine (DPPE) and histidinol (2).

Optimum intracellular concentrations of ornithine-derived polyamines are essential for prokaryotic and eukaryotic cells (fungi included) to survive and grow (5). Polyamine biosynthesis and polyamine analogs are described (and have been patented) as antiproliferating agents that inhibit growth of animal and plant pathogens (1,11,14).

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In addition, fungal growth inhibition by exogenous, natural or synthetic, diamines is also reported (3,6).

We tested the hypothesis that CPA, like other antitumoral compounds, inhibits the growth of mammalian cells and fungi (17). We tested the effects of CPA on the *in vitro* growth of several saprophytic fungi isolated from harvested and stored fruits. In view of the characteristic chemical structure of CPA, we planned our work to compare the effects of CPA on fungal growth with those of the fungicide iprodione (which also has a chlorphenyl moiety), histidinol (another histamine-related compound) and the fungicide benomyl.

MATERIALS AND METHODS

Fungal strains used. Six of the nine fungal strains were isolated from harvested and stored fruits of cherimoya (*Annona cherimola*) in the “La Mayora” Experimental Station (C.S.I.C.) and maintained on potato dextrose agar (PDA). In addition, we tested two strains of *Botrytis cinerea* isolated from tomato and one *Penicillium* from orange fruits. Table 1 gives the strains used and their original hosts.

TABLE 1. Fungal strains used and their original hosts

Strain	Fungus	Host
CH1	<i>Cladosporium cladosporioides</i>	cherimoya fruit
CH7	<i>Alternaria alternata</i>	cherimoya fruit
CH8	<i>Alternaria alternata</i>	cherimoya fruit
CH9A	<i>Penicillium brevicompactum</i>	cherimoya fruit
CH9B	<i>Penicillium corylophilum</i>	cherimoya fruit
CH24	<i>Alternaria alternata</i>	cherimoya fruit
N	<i>Penicillium digitatum</i>	orange fruit
T1	<i>Botrytis cinerea</i>	tomato stem
T2	<i>Botrytis cinerea</i>	tomato fruit

The 1,4-diamine compounds and the fungicides used. We tested the inhibitory efficacy of five chemicals – three 1,4-diamine compounds, inhibitors of ornithine decarboxylase: L-histidinol dihydrochloride, (\pm)-chlorpheniramine, and harmaline (all from Sigma Chemical); and two commonly used postharvest fungicides: benomyl (Benlate[®], containing 50% a.i.) and iprodione (Rovral[®], 50% a.i.), which were used to compare their fungicidal effects with those of the 1,4-diamines.

Preparation of the media with 1,4-diamines and with fungicides. The fungal growth in the presence of the inhibitors was measured on Czapek-Dox agar (CDA), a completely synthetic medium, without vegetable extracts that could contain polyamines. The 1,4-diamine or fungicide used was sterilized by filtering through Acrodisc[®] 0.2 μ m filters. The compounds were diluted and placed into sterilized CDA media (pH 7.3) to obtain a series of different concentrations: 0, 10, 50, 100 and 500 μ M. In a second series of experiments 100 μ M of the three 1,4-diamines (histidinol, chlorpheniramine and harmaline) were placed together with 100 μ M of putrescine to determine whether the putrescine could restore the effect of the polyamines.

Fungal growth in vitro. To avoid possible deterioration of the 1,4-diamines, the media were inoculated 24 h later by placing a 5 mm disk of the fungal strain punched from the edge of an actively growing colony, in 9 cm petri dishes. Three replicates were made for

each fungal strain, each chemical and each concentration of chemical. The cultures were maintained at 26°C in darkness. A Delta-T Devices image analyzer was used to measure the growth of the fungal colonies in mm² at intervals of 2, 4, 7, 9, 11, 16 and 18 days.

Statistical treatment of the results. All assays were conducted in triplicate. Data were analyzed using the SSPSS[®] statistical package from IBM. The significant differences of the factor under consideration were determined by the Student-Newman-Keuls (SNK) test to a level of 0.05.

RESULTS AND DISCUSSION

The growth of the fungal strains in the presence of different concentrations of CPA is shown in Figure 1. The inhibitions of fungal growth by CPA were very similar to those of cultured mammalian cells. When tested on four fungal genera grown *in vitro*, CPA was a more potent fungicide than iprodione; however, the inhibitions produced by CPA, iprodione and histidinol were very similar. The polyamine harmaline had no effect and the results are not reported.

The growth rates of *Alternaria alternata* were very similar and sensitive to the presence of CPA (Table 2); the response of strain CH24 is shown in Figure 1. The *Penicillium* and *Cladosporium* species were also sensitive to the presence of the antihistaminic but to a lesser extent. In contrast, the inhibition of the growth of species of *P. corylophilum* type (CH9B) up to 500 µM CPA was non-significant (Table 2). The effect of the antihistaminic was biphasic (Fig. 1). At a concentration of 50 µM CPA increased fungal growth, but higher concentrations inhibited it; this also occurs in some mammalian cell lines (8). This biphasic pattern is a very common fungal response to toxins. Histamine analogs modulate the proliferation of several mammalian cell types and act through membrane and soluble receptors (2,4).

Figure 2A displays the responses to CPA of two different strains of *B. cinerea* in Czapek-Dox agar that show markedly different growth kinetics. The most proliferative strain (T1) was not at all sensitive to the antihistaminic (0–500 µM). On the contrary, 250 µM CPA reduced the growth of the T2 strain by 30% during the exponential phase of growth. In addition, there was no direct relationship between growth rate and CPA sensitivity: cultured human cancer cells exhibit a similar behavior (8). Differences in membrane proteins and/or in amine catabolism might explain the different qualitative effects.

The growth inhibited by compounds that inhibit ODC and deplete polyamines may be restored by adding an alternative source of polyamines to the culture, for example putrescine. In our preliminary experiments we confirmed this hypothesis by introducing 1 mM putrescine into the *B. cinerea* T2 culture medium. This putrescine concentration was used by others for similar experiments with *B. cinerea* and other fungi species with different ODC inhibitors (14). Putrescine did not reverse the inhibitory effect of CPA, and this suggests that the latter's effect on fungal growth is not caused simply by an inhibition of ODC activity. Similar results have been reported for cultures of mammalian tumor cells (8). On the other hand, in this work, the antifungal effect of CPA increased by more than 20% when 1 mM putrescine was present in the medium (Fig. 2b). Both 1,4-diamines in excess could be lethal for eukaryotic cells (15). In fact, *B. cinerea* may possess diamine oxidase, and its action on putrescine is known to generate hydrogen peroxide and free radicals, both of which would damage cells (5). The possibility that the CPA effect could

be cancelled by putrescine was tested on *Alternaria alternata* (CH24) cultures, but using lower ($100\ \mu\text{M}$) concentrations of both putrescine and CPA. However, in this case, the addition of putrescine did not significantly reverse the growth inhibition of *A. alternata* cultures produced by CPA. The level of inhibition was $21\pm 1\%$ in the presence of $100\ \mu\text{M}$ CPA, and $18\pm 1\%$ when both 1,4-diamines were added.

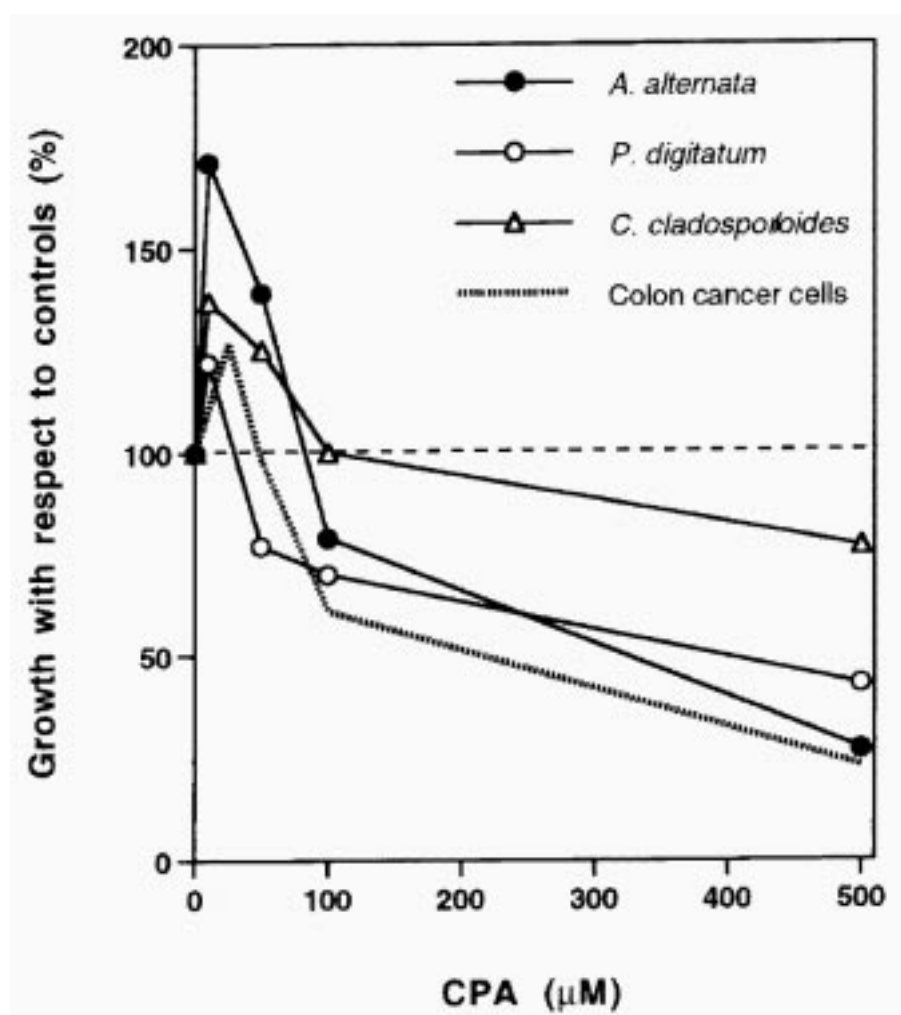


Fig. 1. Relative growth *in vitro* of different fungi species in the presence of different chlorpheniramine concentrations. Data are taken on day 7 after seeding, during the exponential phase of growth. For comparison, data obtained *in vitro* with a colon cancer line culture (ref. 8) during the exponential phase of growth are superimposed.

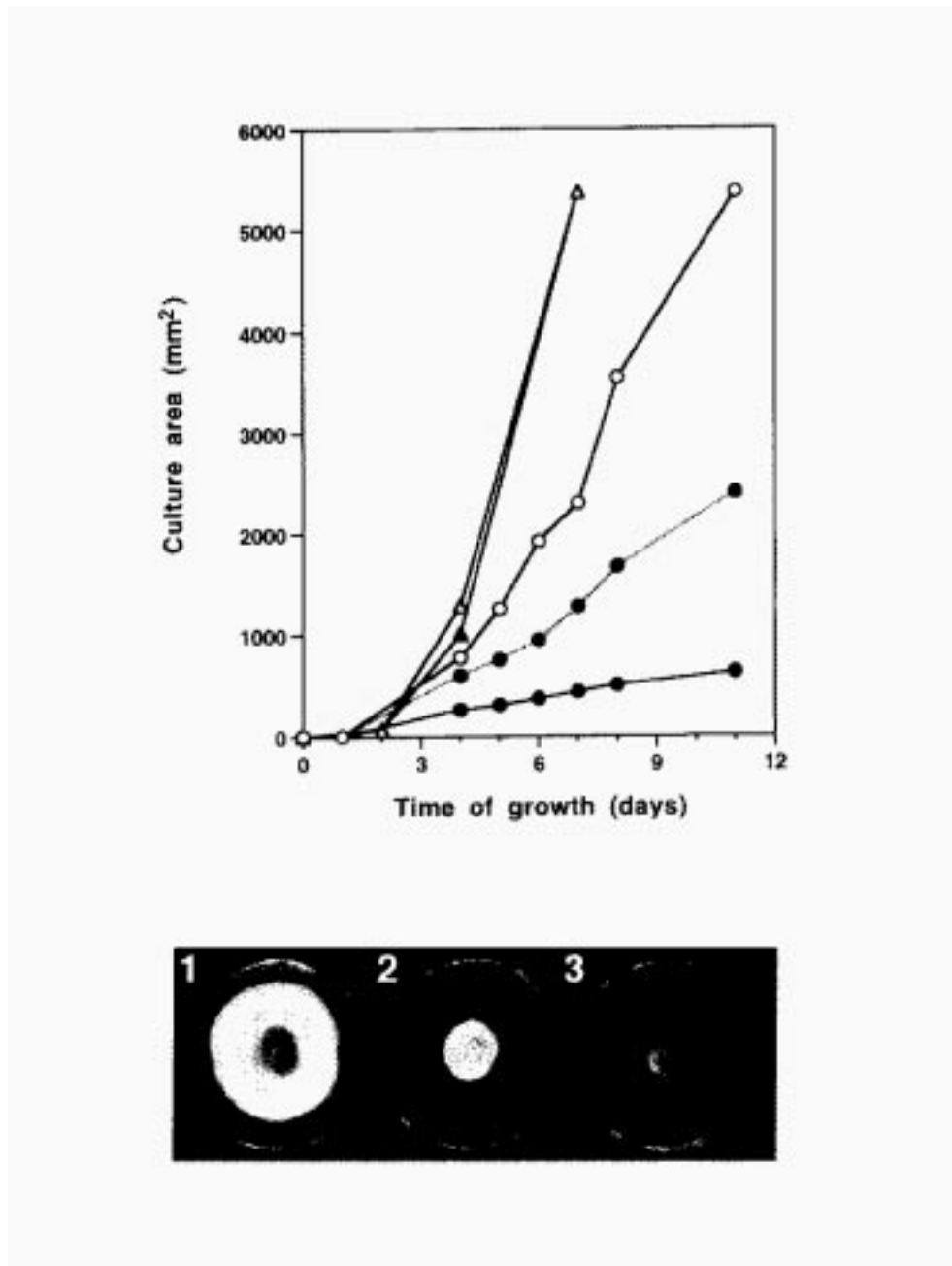


Fig. 2. Response of *Botrytis cinerea* to treatment with chlorpheniramine (CPA). A (top). Growth kinetics of two different strains: T1 is represented by triangles and T2 by circles. White symbols are control cultures in the absence of CPA; dotted line, 250 μ M CPA, T2 strain; continuous line, 500 μ M CPA, both strains. B bottom. Representative photo of *B. cinerea* T2 culture in Czapek-Dox agar alone (1), in the medium supplemented with 1 mM CPA (2), or supplemented with both 1 mM CPA and 1 mM putrescine (3), on day 8 of growth.

To compare the effects of CPA with those of other fungicides, we measured the growth of *A. alternata* cultures in the presence of 0.5 mM CPA, iprodione, benomyl, or the histamine analog L-histidinol (Table 2). The *A. alternata* strains used seem to be partially resistant to iprodione and benomyl; however, CPA used at the same concentration reduced fungal growth by more than 75%. Table 2 also shows the results obtained with *A. alternata* and the other species on day 7 of culture (exponential growth phase). For species other than *A. alternata* and that of *P. corylophilum* type (CH9B), benomyl was the most effective antifungal compound. Even when CPA inhibition of these species was less than that produced in *A. alternata*, it was always higher than the effect observed with equal concentrations of iprodione. Except for the strain CH9B, *P. corylophilum* type, iprodione, histidinol and CPA had similar effectiveness on every species tested. All three compounds have a heterocyclic aromatic ring and an amine group in their aliphatic moiety. The amine of the lateral chain forms a 1,4-diamine structure with other N atoms of the heterocyclic ring. In addition, iprodione and CPA also have a chlorphenyl moiety.

TABLE 2. Percentage of growth inhibition *in vitro* by different compounds (added in 500 μ M amounts at pH 7.3) with respect to controls (untreated cultures) on day 7 of culture (exponential growth phase)

Species	Benomyl	Iprodione	L-histidinol	CPA
<i>A. alternata</i> (CH7)	29*	47*	32*	82*
<i>A. alternata</i> (CH8)	23*	35*	18*	77*
<i>A. alternata</i> (CH24)	51*	57*	55*	85*
<i>P. brevicompactum</i> (CH9A)	98*	0	0	30*
<i>P. corylophilum</i> (CH9B)	0	1	30	20
<i>P. digitatum</i> (N)	99*	20*	0	55*
<i>C. cladosporioides</i> (CH1)	89*	0	10	22*

* $P < 0.05$ with respect to controls (Student-Newman-Keuls test).

In conclusion, the results of this work suggest that there may be several mechanisms by which CPA regulates the growth of different fungal strains. One mechanism could be that CPA mimics the effect of natural polyamines, histamine included, in a similar way to its action in mammalian cells. Some authors suggest that the growth regulator function of CPA and other polyamines could change DNA conformation when the polyamines interact with the DNA molecule (9,12). On the other hand, because CPA and iprodione have common structural characteristics and similar effects on the growth of different genera of fungi, there is the possibility that they might have affinities for an analogous target group. The present findings may lead to greater insight into the cellular physiology of histamine and its analogs in fungal growth and, ultimately, help to develop new fungicide compounds.

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