Carrot Phytoalexin Alters the Membrane Permeability of *Candida albicans* and Multilamellar Liposomes

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The biochemical basis for the antimicrobial effect of the carrot phytoalexin 6-methoxymellein (6-MM) was examined. At fungistatic concentrations 6-MM retarded the ability of *Candida albicans* to incorporate radioactive thymidine, uridine and leucine into biopolymers. When *C. albicans* was incubated with 6-MM, 260-nm-absorbing materials and ^3H -labelled compounds leaked from the cells. The inhibitory effects of 6-MM on cell growth and membrane functions were, however, reduced as the concentration of divalent metal cations added to the medium was increased. 6-MM interacted with multilamellar liposomes constituted from phosphatidylcholine, cholesterol and dicetyl phosphate, or from phosphatidylcholine only, resulting in the release of glucose trapped in these liposomes. These results suggest that 6-MM exerts its toxic effects on susceptible cells as a result of its interaction with their membranes and disturbance of membrane-associated functions.

INTRODUCTION

Phytoalexins are host-synthesized, low-molecular-mass antibiotic compounds whose synthesis from distant precursors is induced in plants in response to microbial infection or treatment of plant tissues with a wide range of naturally occurring or synthetic compounds called elicitors (Dixon & Key, 1983). The mechanism of action of phytoalexins is not fully understood though some evidence suggests that they alter the properties of the plasma membrane (Hargreaves, 1980; Weinstein & Albersheim, 1983). It has also been reported that some phytoalexins inhibit electron transport in mitochondria (Kaplan *et al.*, 1980; Boydston *et al.*, 1983).

The carrot phytoalexin 6-MM is toxic to a wide range of organisms. It inhibits the growth of bacteria, fungi (Kurosaki & Nishi, 1983), and animal and plant cells (Coxon *et al.*, 1973; Kurosaki *et al.*, 1984). The mechanism by which 6-MM stops the growth of cells is not yet understood, though recently we have shown that it inhibits basal and calmodulin-stimulated activity of bovine cyclic nucleotide phosphodiesterase (PDE) (Amin *et al.*, 1986) and calcium/calmodulin-dependent protein phosphorylation in cultured carrot cells (Amin *et al.*, 1987). In this paper, we report on the effect of 6-MM on cell permeability of a pathogenic strain of *Candida albicans*, and on its interaction with multilamellar liposomes.

METHODS

Micro-organism and culture. *Candida albicans* strain ATCC 18804 was grown on yeast nitrogen base (Difco) supplemented with 0.5% (w/v) glucose. Cell growth was followed by measuring the optical density of cultures at 595 nm. Cultures in the early exponential growth phase were used for experiments. Cell density at the beginning of experiments was $1.5\text{--}2.5 \times 10^6$ cells ml^{-1} .

Isolation and purification of 6-MM. 6-MM was isolated and purified from carrot root slices infected by the fungus *Chaetomium globosum*, as described by Kurosaki & Nishi (1983). 6-MM was dissolved in ethanol and was added to cultures to a final concentration of 0.1–0.5 mM. The ethanol concentration was adjusted to 1%. Controls contained the same ethanol concentration.

Abbreviations: 6-MM, 6-methoxymellein; PDE, phosphodiesterase.

Incorporation of ^3H -labelled metabolites. [methyl- ^3H]Thymidine [13.0 Ci mmol^{-1} (481 GBq mmol^{-1})], [$5\text{-}^3\text{H}$]uridine [26.6 Ci mmol^{-1} ($984.2\text{ GBq mmol}^{-1}$)] and L-[$4,5\text{-}^3\text{H}$]leucine [60.0 Ci mmol^{-1} ($2.22\text{ TBq mmol}^{-1}$)] were obtained from New England Nuclear. Incorporation of these radioactive precursors into biopolymers of *C. albicans* was determined as described by Fraser & Creanor (1974). Isotopes were added to the cultures to a concentration of $0.5\text{--}1\text{ }\mu\text{Ci ml}^{-1}$ ($18.5\text{--}37\text{ kBq ml}^{-1}$). At intervals, 0.5 ml samples of the cultures were taken out and mixed with 5 ml ice-cold 10% (w/v) trichloroacetic acid (TCA). The cells were collected by filtration on Whatman GF/A glass-microfibre filter paper and washed three times with 5% (w/v) TCA. Acid-insoluble radioactivities on the dried filters were measured by liquid scintillation spectrometry using a commercial scintillation liquid, ACS II.

Leakage of 260-nm-absorbing materials. To a sample (3 ml) of a suspension of *C. albicans* in a salts solution [$(\text{NH}_4)_2\text{SO}_4$, 0.5% (w/v); K_2HPO_4 , 0.1% (w/v); $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05% (w/v); NaCl , 0.01% (w/v) and $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, 0.01% (w/v); pH 5.5], 6-MM ($10\text{ }\mu\text{l}$) was added. The suspension was kept on a reciprocal shaker at 27°C , then centrifuged at 1500 g for 15 min ; the resulting supernatant was then centrifuged for 10 min . 6-MM in the supernatant was extracted twice with hexane and the absorbance at 260 nm of the aqueous layer was recorded.

Leakage of ^3H -labelled metabolites. *C. albicans* cells were collected by centrifugation and resuspended in fresh medium containing [^3H]thymidine, [^3H]uridine or L-[^3H]leucine [$1\text{ }\mu\text{Ci ml}^{-1}$ (37 kBq ml^{-1})]. The cells were incubated with these labelled metabolites for 2 h on a reciprocal shaker at 27°C . Cells were collected by centrifugation, washed three times with water and resuspended in water to which 6-MM was added. At intervals, 0.5 ml samples of the cell suspension were filtered using Whatman GF/A glass-microfibre filters. The filters were then washed with water (1 ml) and the radioactivities of the filtrates were measured.

Preparation of liposomes. Multilamellar liposomes were prepared by the technique of Bangham *et al.* (1965). The liposomes consisted of egg-yolk phosphatidylcholine or phosphatidylcholine, dicetyl phosphate and cholesterol (molar ratio $10:1:5$). These materials were purchased from Sigma. The dried lipid film, in a 50 ml round bottom flask, was swollen in 0.5 ml 0.3 M -glucose containing $0.5\text{ }\mu\text{Ci}$ (18.5 kBq) D-[$\text{U}\text{-}^{14}\text{C}$]glucose as a marker. Untrapped glucose was removed by dialysing the liposome suspension at room temperature against isotonic (0.85% , w/v) saline solution for 5 h (five changes, 2 l each). The liposome suspension was then diluted with isotonic saline solution to give a final concentration of $0.1\text{ }\mu\text{mol}$ phosphatidylcholine (ml saline) $^{-1}$.

Measurement of liposomal permeability. The permeability of liposomes was estimated from the rate of release of the trapped D-[^{14}C]glucose. The [^{14}C]glucose released in the aqueous solution was separated from the liposomes by the membrane filtration method described by Oku *et al.* (1980) using a 10 ml glass syringe equipped with a membrane filter unit (Millipore filter type GS; pore size $0.2\text{ }\mu\text{m}$). The extent of 6-MM-induced release of glucose is expressed as percentage glucose release and was calculated as follows (HsuChen & Feingold, 1973).

$$\text{Percentage glucose released} = \frac{[^{14}\text{C}]\text{glucose released in the presence of 6-MM} - \text{blank control}}{\text{total amount of } [^{14}\text{C}]\text{glucose trapped} - \text{blank control}}$$

The total amount of [^{14}C]glucose trapped was determined by adding 0.5 ml 10% (v/v) Triton X-100 to 0.5 ml liposomes and heating in a boiling water bath for a few minutes before measurement of the radioactivity. The [^{14}C]glucose released by 6-MM was determined by incubating 0.5 ml liposomes with $10\text{ }\mu\text{l}$ 6-MM in dimethyl sulphoxide (DMSO) or DMSO only (blank control) for 30 min at $22 \pm 2^\circ\text{C}$. Liposomes were then diluted to 3 ml with isotonic saline and transferred to a glass syringe; 1.5 ml of this suspension was then filtered through a membrane filter and the [^{14}C]glucose in the filtrate was determined by measuring the radioactivity.

RESULTS AND DISCUSSION

Effect of 6-MM on the synthesis of macromolecules in C. albicans

6-MM retarded equally the ability of *C. albicans* to incorporate the ^3H -labelled metabolites into biopolymers (Fig. 1). At the minimum inhibitory concentration for growth of 6-MM (0.5 mM) there was a $6\text{--}10\text{ min}$ lag before detectable inhibition of the incorporation of [^3H]thymidine, [^3H]uridine and [^3H]leucine into their respective macromolecules; the time lag was longer when the phytoalexin was applied at lower concentration. The existence of the time lag and the fact that a relatively high concentration of 6-MM was required for significant inhibition of the incorporation of these precursors rules out the possibility that 6-MM is a specific inhibitor of nucleic acid or protein synthesis. A similar conclusion was reported for the pterocarpin phytoalexin glycinol (Weinstein & Albersheim, 1983). The decrease in rate of incorporation of the metabolites might result from their reduced uptake from the medium into the cells.

Fig. 1. Effect of 6-MM on the incorporation of (a) [${}^3\text{H}$]thymidine, (b) [${}^3\text{H}$]uridine and (c) [${}^3\text{H}$]leucine into biopolymers in *C. albicans*. At the indicated intervals, samples of the cultures were taken, mixed with cold 10% (w/v) TCA, filtered through glass-microfibre filters and washed with 5% (w/v) TCA. The radioactivity on the dried filters was counted. ○, Control; ▽, 6-MM (0.1 mM); ▲, 6-MM (0.5 mM). Each point represents the mean of three separate experiments with $\pm$ SE less than 10% of the mean.

Fig. 2. Effect of 6-MM on the leakage of 260-nm-absorbing materials from *C. albicans*. Washed cells of *C. albicans* were suspended in a buffered salt solution. After the addition of 6-MM, the cell suspensions were incubated at 27 °C on a reciprocal shaker. At intervals, samples of suspensions were centrifuged and the supernatants were extracted twice with hexane to remove 6-MM. The absorbance at 260 nm of the aqueous solutions was measured. ○, Control; ▽, 6-MM (0.1 mM); ▲, 6-MM (0.5 mM). Each point represents the mean of three separate experiments with $\pm$ SE less than 10% of the mean.

Effect of 6-MM on cell permeability

Cells incubated with 6-MM leaked 260-nm-absorbing materials (Fig. 2). This suggested that 6-MM might bind to the cell membrane and alter the membrane permeability, a view confirmed as follows. When the cells were labelled with [${}^3\text{H}$]thymidine, [${}^3\text{H}$]uridine or [${}^3\text{H}$]leucine and then incubated with 6-MM, the cells leaked ${}^3\text{H}$ -labelled metabolites (Fig. 3). Loss of labelled metabolites from cells and increase in conductivity of the incubation medium have previously been reported for some phytoalexins (Smith, 1982), membrane acting antibiotics (Storm *et al.*, 1977) and chemotherapeutic agents (Swamy *et al.*, 1974).

Reversal of 6-MM action by metal cations

We have previously shown that 6-MM inhibited basal and calmodulin-stimulated PDE, and that Mg^{2+} and Ca^{2+} were able to reduce the inhibitory effects of the phytoalexin. We suggested that 6-MM might interact with these metal cations which are essential for enzyme activity and for calmodulin activation (Amin *et al.*, 1986). In this study (Table 1), we also found that the inhibitory effect of 6-MM on the uptake of ${}^3\text{H}$ -labelled metabolites was reduced when a large amount of Mg^{2+} was added to the medium. Inhibition of cell growth (Fig. 4a) and the induced leakage of 260-nm-absorbing materials (Fig. 4b) caused by 6-MM depended on the

Fig. 3. Effect of 6-MM on the leakage of (a) $[^3\text{H}]$ thymidine, (b) $[^3\text{H}]$ uridine and (c) $[^3\text{H}]$ leucine from labelled cells of *C. albicans*. Suspensions of *C. albicans* in growth medium containing the labelled metabolites were kept at 27°C for 2 h on a reciprocal shaker. Labelled cells were collected by centrifugation, washed and resuspended in distilled water. 6-MM was added to the cell suspensions, and at intervals samples of the suspensions were taken out, filtered on a glass-microfibre filter and washed with water; the radioactivity in the filtrates was then counted. $\bigcirc$, Control; ∇ , 6-MM (0.1 mM); $\blacktriangle$, 6-MM (0.5 mM). Each point represents the mean of two separate experiments, the results of which agreed to within 10%.

Table 1. Reversal by Mg^{2+} of the effect of 6-MM on the uptake of $[^3\text{H}]$ thymidine, $[^3\text{H}]$ uridine and $[^3\text{H}]$ leucine by *C. albicans*

C. albicans cells suspended in growth medium with the additions indicated were incubated with the ^3H -labelled metabolites for 20 min. Uptake was determined as described in Fig. 1, except that the cells were washed with cold water. Results are the means of three separate experiments $\pm$ SE.

Addition	$10^{-3} \times \text{Uptake (c.p.m. per } 10^6 \text{ cells)}$		
	$[^3\text{H}]\text{Thymidine}$	$[^3\text{H}]\text{Uridine}$	$[^3\text{H}]\text{Leucine}$
None	4.6 ± 0.40	4.9 ± 0.35	10.3 ± 1.60
6-MM (0.5 mM)	1.9 ± 0.15	2.6 ± 0.21	4.8 ± 0.42
MgSO_4 (10 mM)	5.3 ± 0.48	4.3 ± 0.15	8.9 ± 1.2
MgSO_4 (10 mM) + 6-MM (0.5 mM)	3.6 ± 0.51	4.2 ± 0.37	7.6 ± 0.56

concentration of the divalent metal cations and were greatly reduced when these cations were present in high concentrations in the incubation medium. These results are analogous to those reported for the effect of these metal cations on the membrane disruption and growth inhibition of bacteria and fungi caused by polymyxin (Storm *et al.*, 1977) and miconazole (Swamy *et al.*, 1974), respectively. Kotani *et al.* (1977) also reported that ATP leakage from yeast cells treated with killer toxin was inhibited by the addition of Ca^{2+} .

Effect of 6-MM on the permeability of multilamellar liposomes

Imai *et al.* (1975) showed that polymyxin-induced permeability changes of phospholipid liposomes require the presence of negatively charged amphipathic molecules such as dicetyl phosphate. On the other hand, the susceptibility of various membranes to the action of the antifungal agent amphotericin B requires the presence of sterols in the membrane (Bolard, 1986; Kerridge, 1986). In the experiment shown in Fig. 5, 6-MM induced the release of $[^{14}\text{C}]\text{glucose}$ trapped in liposomes constituted from phosphatidylcholine, dicetyl phosphate and cholesterol as well as those constituted from phosphatidylcholine alone. These results indicate that neither cholesterol nor negatively charged molecules are required for 6-MM-induced permeability changes in liposomes. The ability of 6-MM to alter the permeability of biological membranes seems to be independent of the presence of specific molecules such as sterols or negatively

Fig. 4

Fig. 4. Reversal by metal cations of the effect of 6-MM on (a) cell growth and (b) leakage of 260-nm-absorbing materials from cells of *C. albicans*. For the determination of the growth inhibition of *C. albicans* by 6-MM, *C. albicans* was precultured for 24 h in 5 ml of growth medium supplemented with the indicated concentration of the metal cation. After the addition of 6-MM, the cultures were incubated at 27 °C on a reciprocal shaker for further 24 h and the optical density at 595 nm of the cell suspensions was recorded. The optical density at 595 nm of controls without 6-MM was taken as 100%. Experimental procedures for the determination of leakage of 260-nm-absorbing materials were the same as in Fig. 2 except that salt solutions were supplemented with the indicated concentration of the metal cation. In this experiment, 6-MM concentration was 0.5 mM. ○, Ca^{2+} ; ●, Mg^{2+} . Each point represents the mean of three separate experiments with $\pm$ SE less than 10% of the mean.

Fig. 5

Fig. 5. Effect of 6-MM on the permeability of multilamellar liposomes. Multilamellar liposomes containing [^{14}C]glucose were incubated at $22 \pm 2^\circ\text{C}$ with 6-MM for 30 min. Liposomes were separated by filtration through a membrane filter (Millipore GS; $0.2\ \mu\text{m}$) and the [^{14}C]glucose in the filtrate was measured. The total amount of [^{14}C]glucose trapped in liposomes was taken as 100%. ○, Liposomes constituted from phosphatidylcholine; ●, liposomes constituted from phosphatidylcholine, dicetyl phosphate and cholesterol (molar ratio 10:1:5). Each point represents the mean of two experiments, the results of which agreed to within 10%.

charged molecules. This may explain the reason for the indiscriminate effects of this phytoalexin on eukaryotic and prokaryotic cells (Coxon *et al.*, 1973; Kurosaki & Nishi, 1983; Kurosaki *et al.*, 1984), and suggests also that the membrane is the operative target for 6-MM toxicity in susceptible cells.

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