Toxicity of oxalysine and oxalysine-containing peptides against *Candida albicans*: regulation of peptide transport by amino acids

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A lysine antimetabolite, L-4-oxalysine [$\text{H}_2\text{NCH}_2\text{CH}_2\text{OCH}_2\text{CH}(\text{NH}_2)\text{COOH}$], and oxalysine-containing di-, tri-, tetra- and pentapeptides inhibited growth of *Candida albicans* H317. Micromolar amounts of amino acids were found to overcome ammonium repression of the di- and tripeptide transport system(s) in strain H317. Several amino acids increased the toxicity of oxalysine-containing di- and tripeptides for *C. albicans* with little or no increase in toxicity of oxalysine or oxalysine-containing tetra- and pentapeptides. L-Lysine completely reversed the toxicity of oxalysine by competing with the transport of oxalysine into the cells. In contrast, L-lysine increased the toxicity of oxalysine-containing di- and tripeptides, but had no effect on the toxicity of oxalysine-containing tetra- and pentapeptides. Incubation of cells with L-lysine for 4 h resulted in a 15-fold increase in the rate of transport of radiolabelled dileucine, indicating that increased sensitivity of *C. albicans* to some toxic peptides in the presence of L-lysine may be attributed to an increased rate of transport of these peptides. Our results indicate that the dipeptide and tripeptide transport system(s) of *C. albicans* are regulated by micromolar amounts of amino acids in a similar fashion to the regulation of peptide transport in *Saccharomyces cerevisiae* and that multiple peptide transport systems differentially regulated by various nitrogen sources and amino acids exist in *C. albicans*.

Introduction

Small peptides with highly variable amino acid composition are taken up by micro-organisms via a carrier-mediated active transport system(s). The regulation of peptide transport systems in enteric bacteria such as *Escherichia coli* and *Salmonella typhimurium* has been studied extensively (Andrews *et al.*, 1986; Guyer *et al.*, 1985; Higgins, 1984; Hiles *et al.*, 1987; Payne, 1980). *S. typhimurium* and *E. coli* possess three distinct systems for peptide transport, with overlapping substrate specificities, termed Opp, Dpp and Tpp for oligopeptide, dipeptide and tripeptide transport systems, respectively. The *opp*-encoded peptide transport system of *S. typhimurium* is constitutive, whereas the *tppB*-encoded system is induced by leucine or anaerobiosis (Jamieson & Higgins, 1984). In *E. coli*, however, the *opp*-encoded system rather than the *tpp*-encoded system is induced by anaerobiosis, alanine, leucine, or peptides containing

alanine or leucine (Andrews *et al.*, 1986; Andrews & Short, 1986). Recent studies by Abouhamad *et al.* (1991) showed that the absolute levels of the constitutively expressed Dpp varied widely between *E. coli* and *S. typhimurium*.

Peptide transport has been studied in less detail in the fungi, but some progress has been made on the regulation of these systems. The oligopeptide transport system of *Neurospora crassa* has been shown to be constitutive (Wolfenbarger & Marzluf, 1975; Wolfenbarger, 1980). We have shown that the peptide transport system of *Saccharomyces cerevisiae* is regulated by micromolar amounts of amino acids (Island *et al.*, 1987, 1991) in addition to its repression by ammonium as a sole nitrogen source (Becker & Naider, 1977, 1980; Moneton *et al.*, 1986 *a, b*; Nisbet & Payne, 1979). Genetic analysis of peptide transport mutants of *S. cerevisiae* allowed us to define PTR1, PTR2 and PTR3 as three components involved in the amino-acid-inducible peptide transport system in this yeast (Island *et al.*, 1991).

Peptide conjugates have been used to deliver toxic moieties into *Candida albicans* via the peptide transport system (Borowski *et al.*, 1979; Kenig & Abraham, 1976;

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Kingsbury *et al.*, 1983; Lichliter *et al.*, 1976; Meyer-Glauner *et al.*, 1982; Milewski *et al.*, 1991a). An understanding of the number of peptide permeases in *C. albicans* and their regulation under different physiological conditions is clearly important in order to design and deliver an efficacious, pathogen-specific drug via 'illicit transport' (Fickel & Gilvarg, 1973; Ames *et al.*, 1973; Hammond *et al.*, 1987; Higgins, 1987) to this opportunistic pathogen. Previous investigations have concluded that the peptide transport system of *C. albicans* is regulated by nitrogen metabolism (Becker & Naider, 1977, 1980; Davies, 1980; McCarthy *et al.*, 1985a, b; Naider & Becker, 1987; Payne *et al.*, 1991; Yadan *et al.*, 1984). For example, peptide uptake is repressed by ammonium ions and stimulated when cells are grown on amino acids as the sole nitrogen source (Logan *et al.*, 1979). Recently, Payne *et al.* (1991) reported stimulation of dialanine transport in *C. albicans* B2630 grown in media containing bacto-peptone or trypticase peptone but not casein hydrolysate. Physiological studies with mutants of *C. albicans* suggest the presence of multiple peptide permeases (McCarthy *et al.*, 1985a, b; Mehta *et al.*, 1984; Milewski *et al.*, 1988; Payne & Shallow, 1985; Yadan *et al.*, 1984) and no evidence has been presented on the differential regulation of these uptake systems.

Oxalysine [$\text{H}_2\text{NCH}_2\text{CH}_2\text{OCH}_2\text{CH}(\text{NH}_2)\text{COOH}$], a lysine analogue which can be produced both chemically and biologically (from fermentation broths of several actinomycetes), is not toxic to mammalian cells and is known to cause growth inhibition of a number of bacteria (McCord *et al.*, 1957; Stapley *et al.*, 1968) and *C. albicans* (Zhang *et al.*, 1979). Here we describe the effect of oxalysine and a series of oxalysine-containing peptides [oxalysyl-(leucyl) $_n$ -glycine] on *C. albicans* in the presence and absence of a variety of nitrogen-containing metabolites.

Methods

Organism and growth conditions. *Candida albicans* H317, a well-characterized (Riggsby *et al.*, 1982) clinical isolate obtained from the Centers for Disease Control (Atlanta, Georgia, USA), was used in all experiments. The strain was maintained on YEPD agar containing (w/v): yeast extract 1%; Bacto-peptone (Difco) 2%; glucose 2%; agar (Difco) 2%. The organism was transferred monthly to a fresh agar slant, incubated for 24 h at 37 °C and stored at 4 °C. Unless mentioned otherwise, cells were grown overnight to exponential phase ($1-2 \times 10^7$ cells ml^{-1}) in minimal medium (MM) which was composed of yeast nitrogen base (Difco) without amino acids containing ammonium sulphate (5 mg ml^{-1}) and glucose (2%, w/v). Individual amino acids were supplemented as indicated at 0.15 mM unless stated otherwise. In some cases, the ammonium sulphate nitrogen source was replaced by one of the following: allantoin (1 mg ml^{-1}), isoleucine (6.6 mg ml^{-1}), proline (4 mg ml^{-1}), peptone (20 mg ml^{-1}), trypticase peptone (20 mg ml^{-1} , BBL), or casein hydrolysate (20 mg ml^{-1} , Sigma). The amounts of allantoin, isoleucine and proline used in the growth medium

as the sole nitrogen source were approximately equivalent to the minimal amount required for optimal growth.

Disk assays. Sensitivity to toxic peptides was measured by using disk assays as described by Island *et al.* (1987). Briefly, cells were grown overnight to the exponential phase of growth in MM or other medium as described in the Tables, washed, and resuspended to 5×10^6 cells ml^{-1} in sterile water. One millilitre of this cell suspension was added to 3 ml molten Noble agar (1.1%, w/v) and overlaid on 20 ml of the same medium as used to prepare the inoculum or MM medium containing a single amino acid supplement as indicated. A disk (6 mm diameter, Whatman 3MM filter paper) was placed on the plate and oxalysine or an oxalysine-containing peptide (0.25 μmol unless indicated otherwise) was spotted onto the disk. Zones of inhibition were measured after 24–48 h incubation at 37 °C. Each test comprised three independent assays and the results represented in all Tables are means of the values obtained. Maximum variation between the three zones of inhibition measured for each test was ≤ 2 mm. A value of 7 mm for the diameter of the zone of inhibition represents a minimal growth inhibition value as the disk diameter was 6 mm.

Transport assays. Uptake of L-leucyl-L-[^3H]leucine was determined as described by Island *et al.* (1987). Briefly, *C. albicans* cultures were grown overnight to exponential phase ($1-2 \times 10^7$ cells ml^{-1}) in MM. Cells were washed and resuspended to 2.5×10^6 cells ml^{-1} in fresh medium with or without 0.15 mM-L-lysine. After incubation for 4 h, cells were harvested, washed and resuspended in glucose (1%, w/v) at 2×10^8 cells ml^{-1} . Five hundred microlitres of cell suspension was added to an equal volume of reaction mixture and incubated at 37 °C. The final concentration of the components in the uptake assay was: glucose (1%, w/v), 4 mM-sodium citrate/potassium phosphate buffer (pH 5.5), and L-leucyl-L-[^3H]leucine (28 μM ; specific activity 20 mCi mmol^{-1} , 740 MBq mmol^{-1}). At various time points, 180 μl portions were removed and filtered through membrane filters (Gelman Membrane Filter, GN-6, 0.45 μm , Gelman Sciences). The yeast cells retained on the filter were washed twice with ice-cold distilled water and the residual radioactivity was measured by liquid scintillation counting using the scintillant Budget Solve (Research Products International). There was no peptide adsorption to the cell surface or sticking to the filters since at 0 °C the counts were at background levels. The uptake results, calculated on the basis of 57% counting efficiency (determined using L-[^3H]lysine as a standard) and the specific activity of the peptide, are expressed as nmol peptide uptake per mg cell protein. The standard deviation was $\leq 10.8\%$ of the mean for data from one experiment and was $\leq 13.1\%$ of the mean for data from five independent experiments.

Competition with L-[^3H]lysine transport by oxalysine in *C. albicans* was determined using an identical procedure to that described for dileucine uptake. The final concentration of the components in the uptake assay was: glucose (1%, w/v), 4 mM-sodium citrate/potassium phosphate buffer (pH 5.5) containing L-[^3H]lysine (43 μM ; specific activity 0.6 mCi mmol^{-1} , 22.2 MBq mmol^{-1}) and various amounts of oxalysine. The standard deviation was $\leq 3\%$ of the mean for uptake values from a single experiment. Standard deviation for data from four independent experiments was $\leq 10\%$ of the mean.

Chemicals and reagents. Oxalysine, Oxa-Leu and Oxa-Gly-Leu were a gift from Shanghai Institute of Materia Medica, Academia Sinica, Shanghai 20031, China. L-Leucyl-L-[^3H]leucine and oxalysine-containing peptides were synthesized by solution-phase techniques (Becker & Naider 1977; Naider *et al.*, 1983). Briefly, α,ϵ -dibenzoyloxycarbonyl-L-oxalysine was coupled with (Leu) $_n$ -Gly-OBzl ($n = 0-3$) using isobutylchloroformate in the presence of *N*-methylmorpholine. The protected peptides [Z-Oxa(Z)-Leu $_n$ -Gly-OBzl ($n = 0-3$), where Z = benzoyloxycarbonyl and OBzl = benzyl ester] were purified to homogeneity,

characterized by NMR spectroscopy and the protecting groups were removed by transfer hydrogenation using Pd black and formic acid. The free peptides were >95% homogeneous as judged by reversed-phase HPLC and thin-layer chromatography. All amino acids used in the synthesis of peptides were the L-form. Nikkomycin X+Z is a mixture of 75% nikkomycin X and 25% nikkomycin Z which was obtained by purifying crude nikkomycin (gift from Drs H. Zahner and W. A. König, Bayer Chemicals, Leverkusen, FRG) by using reversed-phase, high-performance liquid chromatography (Krainer *et al.*, 1987).

Results

Toxicity of oxalysine and oxalysine-containing peptides against C. albicans

Oxalysine and oxalysine-containing peptides inhibited the growth of *C. albicans* H317 in minimal medium (MM) containing ammonium sulphate as the nitrogen source (Table 1). Oxalysine-containing di- and tripeptides were two to four times more toxic per mol of compound than oxalysine. Tetrapeptide and pentapeptide containing oxalysine were 16-fold more toxic than oxalysine. Oxa-Leu was also found to be two-fold more toxic than oxalysine when the MIC was determined in MM liquid medium (data not shown).

Effect of nitrogen source on the toxicity of oxalysine and oxalysine-containing peptides against C. albicans

The nitrogen source in the growth medium has been shown to affect peptide transport in both *S. cerevisiae* and *C. albicans* (Becker & Naider, 1977, 1980; Logan *et al.*, 1979; Nisbet & Payne, 1979; McCarthy *et al.*, 1985a; Moneton *et al.*, 1986a, b; Payne & Shallow, 1985; Payne *et al.*, 1991; Yadan *et al.*, 1984). *C. albicans* can utilize various organic compounds, such as isoleucine, proline, allantoin and peptone as a sole source of nitrogen. Disk sensitivity assays were performed to determine the effect of nitrogen source on the toxicity of oxalysine and oxalysine-containing peptides (Table 2). The largest zone of inhibition with oxalysine was observed with isoleucine as the sole nitrogen source. The zone of inhibition produced by oxalysine was greater in medium with proline as compared to either ammonium sulphate or allantoin as the nitrogen source. Oxalysine did not cause any growth inhibition of *C. albicans* in peptone-containing medium.

Oxalysine-containing peptides also caused maximum growth inhibition in medium with isoleucine as the sole nitrogen source. No significant difference in toxicity of oxalysine-containing peptides was apparent in medium with either allantoin or proline as nitrogen source. Allantoin, an intermediate in the degradation of adenine and guanine (Cooper, 1982), is a non-repressive nitrogen

Table 1. *Toxicity of oxalysine and oxalysine-containing peptides against C. albicans*

The disk sensitivity was done in minimal medium (MM). Oxalysine or oxalysine-containing peptide from a serial twofold dilution series was spotted on a disk placed on a lawn of cells prepared as described in Methods. The least amount of the compound that showed anticandidal activity is given in the Table.

Compound	Minimum inhibitory amount (μ mol per disk)
Oxalysine	0.125
Oxa-Leu	0.062
Oxa-Gly	0.031
Oxa-Gly-Leu	0.062
Oxa-Leu-Gly	0.031
Oxa-Leu-Leu-Gly	0.008
Oxa-Leu-Leu-Leu-Gly	0.008

source for peptide transport in *S. cerevisiae* as compared to ammonium sulphate (Island *et al.*, 1991). Oxalysine-containing di- and tripeptides were only weakly toxic in medium containing ammonium sulphate in contrast to the other nitrogen sources. Ammonium sulphate is known to be a repressive nitrogen source for peptide transport in *C. albicans* (Logan *et al.*, 1979). The influence of the nitrogen source on the toxicity of tetra- and pentapeptides was minor, with both repressive and non-repressive nitrogen metabolites resulting in similar zones of inhibition. Only oxalysine-containing di- and tripeptides showed toxicity in assay medium with peptone; oxalysine-containing tetra- and pentapeptides were non-inhibitory in this medium.

Effect of L-lysine on the toxicity of oxalysine and oxalysine-containing peptides in medium with different nitrogen sources

Previous studies with bacteria have shown that L-lysine reverses the growth-inhibitory effect of oxalysine (McCord *et al.*, 1957; Stapley *et al.*, 1968). We found that L-lysine (0.15 mM) completely reversed the toxicity of oxalysine in all media tested (Table 2). With proline as a nitrogen source a hazy zone of inhibition was observed. In contrast to the result with oxalysine the effect of L-lysine on the toxicity of oxalysine-containing peptides was dependent on the nitrogen source. The presence of L-lysine (0.15 mM) increased the toxicity of oxalysine-containing di- and tripeptides in medium with ammonium sulphate as the nitrogen source; toxicity of oxalysine-containing tetra- and pentapeptides remained unaffected or was slightly depressed. L-Lysine caused a slight decrease in the toxicity of oxalysine-containing peptides in medium containing allantoin, had a slightly greater effect in medium containing proline, and had no

Table 2. Toxicity of oxalysine and oxalysine-containing peptides against *C. albicans*: effect of nitrogen source and induction by L-lysine

Assay medium*	Diameter (mm) of zone of inhibition of plates containing†				
	Oxalysine	Oxa-Gly	Oxa-Leu-Gly	Oxa-(Leu) ₂ -Gly	Oxa-(Leu) ₃ -Gly
YNB w/ (NH ₄) ₂ SO ₄					
Without lysine	12	12‡	19	31	32
With lysine	none	26	23	28	30
YNB w/ allantoin					
Without lysine	12	29	26	27	27
With lysine	none	27	24	23	26
YNB w/ proline					
Without lysine	40	30	27	27	28
With lysine	9‡	26	19	21	22
YNB w/ isoleucine					
Without lysine	50	45	38	36	37
With lysine	none	25	23	26	26
YNB w/ peptone					
Without lysine	none	25	22	none	none
With lysine	none	25	22	none	none

* YNB w/ (NH₄)₂SO₄ is MM as described in Methods; in the other media ammonium sulphate in MM replaced with other nitrogen sources – YNB w/ allantoin is MM with allantoin (1 mg ml⁻¹), YNB w/ isoleucine is MM with isoleucine (6.6 mg ml⁻¹), YNB w/ proline is MM with proline (4 mg ml⁻¹), YNB w/ peptone is MM with peptone (20 mg ml⁻¹). L-Lysine was added to a final concentration of 0.15 mM where indicated.

† Oxalysine or oxalysine-containing peptide (0.25 µmol) was spotted on a disk (diameter 6 mm). Each test comprised three independent assays and the results represented in the Table are means of the values obtained. Maximum variation between the three zones of inhibition for each test was ≤ 2 mm. 'none' indicates no growth inhibition.

‡ Zone of inhibition was not clear.

Table 3. Effect of amino acid addition on toxicity of oxalysine and oxalysine-containing peptides against *C. albicans*

Amino acid added*	Diameter (mm) of zone of inhibition of plates containing†				
	Oxalysine	Oxa-Gly	Oxa-Leu-Gly	Oxa-(Leu) ₂ -Gly	Oxa-(Leu) ₃ -Gly
None	14	12‡	20	31	32
Arginine	9	34	31	30	32
Lysine	none	26	25	28	30
Histidine	13	24	25	29	30
Glutamine	12	35	29	29	30
Aspartic acid	16	31	27	29	28
Serine	11	26	25	29	28

* The effect of addition of each of 20 amino acids (0.15 mM) was determined in MM. The amino acids not listed in the Table (Ala, Asn, Cys, Glu, Gly, Ile, Leu, Phe, Thr, Tyr, Val, Met, Pro and Trp), did not increase or decrease the effect on growth inhibition by oxalysine or oxalysine-containing peptides.

† Oxalysine or oxalysine-containing peptide (0.25 µmol) was spotted on a disk (diameter 6 mm). Each test comprised three independent assays and the results represented in the Table are means of the values obtained. Maximum variation between the three zones of inhibition for each test was ≤ 2 mm. 'none' indicates no growth inhibition.

‡ Zone of inhibition was not clear.

detectable effect on the toxicity of Oxa-Gly or Oxa-Leu-Gly with peptone as the nitrogen source. In medium containing isoleucine as sole nitrogen source, however, L-lysine caused a marked reduction in the zones of inhibition caused by all oxalysine-containing peptides tested.

Toxicity of oxalysine and oxalysine-containing peptides in the presence of amino acids

Studies from our laboratory (Island *et al.*, 1987, 1991) showed that the peptide transport system of *S. cerevisiae* was induced by addition of several amino acids to the

Fig. 1. Effect of L-lysine on the toxicity of Oxa-Gly and Oxa-(Leu)₂-Gly against *C. albicans*. Disk sensitivity assays were done in MM as described in Methods. (a) Oxa-Gly (0.25 µmol) was spotted on a disk and adjacent to each disk was placed another disk containing different amounts of L-lysine: (1) 50 µmol, (2) 25 µmol, (3) 2.5 µmol, (4) 0.25 µmol, (5) 0.025 µmol, (6) 0.0025 µmol or (7) none. (b) Oxa-(Leu)₂-Gly (0.25 µmol) was spotted on a disk and adjacent to each disk was placed another disk containing different amounts of L-lysine: (1) 2.5 µmol, (2) 0.062 µmol or (3) none. Maximum variation for the zones of inhibition was ≤ 2 mm for three independent assays.

medium. Since ammonium sulphate was the only nitrogen source in which L-lysine increased the toxicity of oxalysine-containing di- and tripeptides (Table 2), we determined the effect of addition of each of 20 amino acids (0.15 mM) on the toxicity of oxalysine and oxalysine-containing peptides against *C. albicans* in MM. Arginine, lysine, histidine, glutamine, aspartic acid and serine increased the toxicity of oxalysine-containing di- and tripeptides against *C. albicans*, but

Table 4. Effect of lysine on the toxicity of oxalysine against *C. albicans*

The disk sensitivity assay was done in MM as described in Methods. Different amounts of either L- or D-lysine were spotted on disks placed adjacent to disks containing 0.25 µmol oxalysine.

Amount of lysine on the disk (µmol)	Effect on oxalysine toxicity*	
	L-Lysine	D-Lysine
25.0	+	+
2.5	+	+
0.25	+	—
0.025	+	—
0.0025	+	—
0.00125	+	—
0.00025	—	—
None	—	—

* +, Reversal of oxalysine toxicity; —, no effect.

had little or no effect on oxalysine-containing tetra- or pentapeptide toxicity (Table 3). The amino acids not listed in Table 3 (alanine, asparagine, cysteine, glutamic acid, glycine, isoleucine, leucine, phenylalanine, threonine, tyrosine, valine, methionine, proline and tryptophan) did not increase or decrease the growth inhibition by oxalysine or oxalysine-containing peptides. L-Lysine completely reversed the toxicity of oxalysine for *C. albicans*, whereas the other amino acids had little or no effect on oxalysine toxicity. Using disk sensitivity assays with different amounts of L-lysine on disks placed adjacent to oxalysine-containing disks it was found that the amino acid caused a dose-dependent increase in toxicity of Oxa-Gly with no increase in the toxicity of Oxa-(Leu)₂-Gly (Fig. 1). As little as 25 nmol L-lysine per disk stimulated toxicity of Oxa-Gly. L-Lysine by itself has no toxic effect on *C. albicans* (data not shown).

Effect of L-lysine and D-lysine on the toxicity of oxalysine against *C. albicans*

Disk sensitivity assays were performed in MM using different amounts of L-lysine or D-lysine on disks placed adjacent to a disk containing oxalysine (250 nmol) to determine the least amount of L-lysine or D-lysine needed to reverse oxalysine toxicity. As little as 1.25 nmol of L-lysine was sufficient to reverse the toxicity of oxalysine (Table 4). A 2000-fold greater amount of D-lysine (2.5×10^3 nmol) than L-lysine (1.25 nmol) was required to cause the same extent of reversal of oxalysine toxicity. A small contamination (0.05%) of the D-lysine solution by L-lysine could have resulted in this apparent reversal of oxalysine toxicity.

To further investigate the effect of L-lysine on the toxicity of oxalysine we did transport competition studies

Fig. 2. Transport competition of L-[³H]lysine in *C. albicans* by oxalysine. *C. albicans* cells grown overnight in MM were used to determine transport competition by oxalysine of L-[³H]lysine (43 μ M, specific activity 0.6 mCi mmol⁻¹) as described in Methods. ■, Oxalysine at a 10-fold molar excess; ▲, 100-fold molar excess; ◆, 1000-fold molar excess; ○, control with no oxalysine. Five hundred microlitres of cell suspension (2×10^8 cells ml⁻¹) was added to an equal volume of reaction mixture and incubated at 37 °C. At various time points 180 μ l portions were removed, placed on filters, washed and radioactivity was measured by liquid scintillation counting. The standard deviation was $\leq 3\%$ of the mean for uptake values from a single experiment. Standard deviation for data from four independent experiments was $\leq 10\%$ of the mean.

of L-[³H]lysine in the presence and absence of oxalysine (Fig. 2). Oxalysine at a 100- and 1000-fold molar excess competed with L-lysine for transport into the cells, suggesting that the reversal of oxalysine toxicity by L-lysine may be due to competition for the amino acid permease.

L-Lysine increases the transport of L-leucyl-L-[³H]leucine in C. albicans

We tested whether the increased sensitivity to oxalysine-containing di- and tripeptides in MM containing L-lysine (Table 2) could have been due to an increased transport of these peptides. The results in Fig. 3 show that Leu-Leu reversed the zone of inhibition produced by Oxa-Gly, whereas Leu-Leu had no effect on the zone of inhibition produced by Oxa-(Leu)₂-Gly. These data indicated that Leu-Leu and Oxa-Gly, but not Oxa-(Leu)₂-Gly, competed with each other for transport. Direct assay of uptake of L-leucyl-L-[³H]leucine into *C. albicans* showed that incubation of cells with L-lysine (0.15 mM) for 4 h resulted in a 15-fold increase in the rate of transport of the dipeptide (Fig. 4). Since Leu-Leu and Oxa-Gly share the same transport system it is reasonable to conclude that the increased toxicity of Oxa-Gly in the presence of L-lysine is due to increased transport of this dipeptide.

Fig. 3. Effect of Leu-Leu on the toxicity of Oxa-Gly and Oxa-(Leu)₂-Gly against *C. albicans*. Disk sensitivity assays were done in MM with 0.15 mM-L-lysine as described in Methods. Two hundred and fifty nanomoles of Oxa-Gly (1a, 2a, 3a) or Oxa-(Leu)₂-Gly (4a, 5a) was spotted on a disk and adjacent to each disk was placed another disk containing either 250 nmol Leu-Leu (2b, 4b), or 500 nmol Leu-Leu (1b, 5b), or water (3b). Maximum variation for the zones of inhibition was ≤ 2 mm for three independent assays.

Fig. 4. Transport of L-leucyl-L-[³H]leucine in *C. albicans* in the presence of L-lysine. *C. albicans* cells grown overnight in MM were harvested in the exponential phase ($1-2 \times 10^7$ cells ml⁻¹), washed and resuspended to 2.5×10^6 cells ml⁻¹ in fresh medium with (●) or without (○) L-lysine (0.15 mM). After incubation for 4 h, cells were harvested, washed and resuspended in fresh medium to 2×10^8 cells ml⁻¹. Five hundred microlitres of the cell suspension was added to an equal volume of reaction mixture containing L-leucyl-L-[³H]leucine (28 μ M, specific activity 20 mCi mmol⁻¹) as described in Methods. At various time points 180 μ l portions were removed, placed on filters, washed and radioactivity was determined by liquid scintillation counting. The standard deviation was $\leq 10.8\%$ of the mean for data from one experiment and was $\leq 13.1\%$ of the mean for data from five independent experiments.

Table 5. Effect of ammonium sulphate, casein hydrolysate, trypticase peptone and peptone on toxicity of oxalysine and oxalysine-containing peptides against *C. albicans*

Assay medium*	Diameter (mm) of zone of inhibition of plates containing†				
	Oxalysine	Oxa-Gly	Oxa-Leu-Gly	Oxa-(Leu) ₂ -Gly	Oxa-(Leu) ₃ -Gly
YNB w/ (NH ₄) ₂ SO ₄	12	12‡	19	31	32
YNB w/ casein hydrolysate	none	none	none	none	none
YNB w/ trypticase peptone	none	11	8	none	none
YNB w/ peptone	none	25	22	none	none
YNB w/ peptone + (NH ₄) ₂ SO ₄	none	24	17	none	none

* YNB is yeast nitrogen base without any nitrogen source. Different nitrogen supplementation was added as (NH₄)₂SO₄ (0.5%, w/v), casein hydrolysate (2%, w/v), trypticase peptone (2%, w/v) or peptone (2%, w/v).

† Oxalysine or oxalysine-containing peptide (0.25 µmol) was spotted on a disk (diameter 6 mm). Each test comprised three independent assays and the results represented in the Table are means of the values obtained. Maximum variation between the three zones of inhibition for each test was ≤2 mm. 'none' indicates no growth inhibition.

‡ Zone of inhibition was not clear.

Table 6. Effect of amino acid addition on toxicity of nikkomycin X + Z against *C. albicans*

Amino acid*	Diameter (mm) of zone of inhibition†
None	none
Arginine	32
Lysine	17
Glutamine	27
Aspartic acid	28

* The effect of addition of various amino acids (0.15 mM) was determined in MM. The amino acids Ala, Asn, Cys, Glu, Gly, Ile, Leu, Phe, Thr, Tyr, Val, Met, Pro and Trp did not increase or decrease the effect of growth inhibition by nikkomycin X+Z. Effect of histidine and serine on growth inhibition by nikkomycin X+Z was not determined.

† Nikkomycin X+Z (20 µg) was spotted on a disk (diameter 6 mm). Each test comprised three independent assays and the results represented in the Table are means of the values obtained. Maximum variation between the three zones of inhibition for each test was ≤2 mm. 'none' indicates no growth inhibition.

Effect of ammonium sulphate, casein hydrolysate, trypticase peptone and peptone on the toxicity of oxalysine and oxalysine-containing peptides against *C. albicans*

We performed disk sensitivity assays to determine the toxicity of oxalysine and oxalysine-containing peptides in medium containing ammonium sulphate, casein hydrolysate, trypticase peptone (enzymically hydrolysed peptone) and peptone (Table 5). Payne *et al.* (1991) reported that bacto-peptone and trypticase peptone, but not casein hydrolysate, stimulated dialanine transport in *C. albicans* B2630. We observed that oxalysine-containing peptides did not cause growth inhibition of *C. albicans* H317 in medium containing casein hydrolysate. Only oxalysine-containing di- and tripeptides showed toxicity in medium containing trypticase peptone or peptone; oxalysine-containing tetra- and pentapeptides

showed no growth inhibition. Oxalysine did not cause growth inhibition in media containing casein hydrolysate, peptone or trypticase peptone. We observed that peptone could overcome the repression of dipeptide toxicity by ammonium.

Effect of amino acids on growth inhibition by nikkomycin in *C. albicans*

We tested whether the amino acids that increased the toxicity of oxalysine-containing di- and tripeptides in MM had any effect on the toxicity of the peptidyl nucleoside nikkomycin X+Z (Table 6). The peptidyl nucleosides enter *C. albicans* via the peptide transport system (Decker *et al.*, 1991; Krainer *et al.*, 1991; McCarthy *et al.*, 1985 a; Yadan *et al.*, 1984). The amino acids arginine, lysine, glutamine and aspartic acid overcame repression of peptide transport by ammonium sulphate and resulted in marked growth inhibition by nikkomycin X+Z (20 µg per disk). The amino acids which had no effect on oxalysine-containing peptide toxicity (Table 3) did not reverse ammonium repression of nikkomycin toxicity.

Discussion

The data presented show that oxalysine and oxalysine-containing peptides inhibit the growth of *C. albicans* H317. The nitrogen source in the medium markedly influenced the toxicity of these compounds (Table 2). Zones of inhibition were larger for oxalysine-containing peptides with isoleucine, proline or allantoin as the nitrogen sources as compared to the zones observed with ammonium sulphate as the nitrogen source. Increased zones of inhibition with proline as the nitrogen source

could be due to increased transport of peptides: *S. cerevisiae* grown with proline as the nitrogen source transported peptides at about 10 times the rate of ammonia-grown organisms (Becker & Naider, 1977; Moneton *et al.*, 1986*b*; Nisbet & Payne, 1979), and *C. albicans* grown with isoleucine as the nitrogen source had six to eight times the peptide transport activity (trimethionine uptake) of cells grown with ammonium chloride as the nitrogen source (Logan *et al.*, 1979). Toxicity of polyoxins which are transported by the peptide transport system has been shown to be dependent on the nitrogen source (Becker *et al.*, 1983; Mehta *et al.*, 1984). It is clear that general nitrogen metabolism can have an important influence on the efficacy of peptide drugs.

We found that in addition to the regulation of toxicity by the nitrogen source for growth, micromolar amounts of certain amino acids can control peptide transport in *C. albicans*. Thus, addition of L-lysine or several other amino acids overcame repression by ammonium and increased toxicity to oxalysine-containing di- and tripeptides (Table 3). A similar observation of induction of dipeptide transport in minimal medium by several amino acids has been made in *S. cerevisiae* (Island *et al.*, 1987, 1991). At the present time we are unable to explain why L-lysine (0.15 mM) caused a marked reduction in the zones of inhibition with oxalysine-containing peptides in medium containing proline or isoleucine as the nitrogen source. Lack of effect of L-lysine or other amino acids on the toxicity of oxalysine-containing tetra- or pentapeptides suggests differential regulation of the permeases that transport these peptides, or that these peptides may enter *C. albicans* via a process other than a peptide transport system. Current evidence, though not conclusive, indicates that *C. albicans* has at least two peptide permeases, one specific for di- and tripeptides and the other for tri- and oligopeptides (McCarthy *et al.*, 1985*a, b*; Mehta *et al.*, 1984; Milewski *et al.*, 1988; Payne & Shallow, 1985; Shallow *et al.*, 1991; Yadan *et al.*, 1984). Our results are consistent with the notion of at least two peptide permeases in *C. albicans* that are operative even under noninducing conditions. The oxalysine-containing tetra- and pentapeptides may also be able to enter cells by fluid-phase endocytosis. Fluid-phase endocytosis is operative in *C. albicans* (Basrai *et al.*, 1990) and we have observed that Lucifer Yellow (LY), the marker for fluid-phase endocytosis, does not accumulate in cells in the presence of peptone, apparently due to rapid exocytosis of the internalized LY. Exocytosis of oxalysine-containing tetra- and pentapeptides in peptone-containing medium may prevent the peptides from reaching an intracellular level required to cause growth inhibition.

Oxalysine-containing di- and tripeptides caused growth inhibition of *C. albicans* H317 in medium with trypticase peptone or peptone but not in medium

containing casein hydrolysate (Table 5). We also observed that bacto-peptone appeared to overcome repression of dipeptide transport by ammonium ions. These results are consistent with the stimulation of dialanine transport in *C. albicans* B2630 grown in medium containing bacto-peptone or trypticase peptone but not in casein acid hydrolysate (Payne *et al.*, 1991). However, casein acid hydrolysate and bacto-peptone contain a heterogeneous mixture of amino acids and peptides, respectively, and it is difficult to ascribe any effects to individual components using these complex nitrogen sources. Additionally, Payne *et al.* (1991) have shown that bacto-peptone and not proline can override ammonium repression of dialanine transport. Hence, the authors concluded that peptides and not amino acids override ammonium repression of peptide transport. We systematically analysed the effect of 20 amino acids on growth inhibition of *C. albicans* using oxalysine-containing peptides and our results (Table 3) suggest that specific amino acids overcome repression by ammonium ions and cause induction of di- and tripeptide transport system(s) of *C. albicans*.

There are several possible explanations of the increased sensitivity of *C. albicans* toward oxalysine-containing di- and tripeptides in the presence of L-lysine and other amino acids: (a) the rate of hydrolysis of the peptide intracellularly could be increased; (b) the intracellular target of the drug could have altered sensitivity in the presence of amino acids; or (c) the uptake characteristics of the peptide could be affected in the presence of amino acids. The first possibility, of increased hydrolysis of the peptide in the presence of certain amino acids, may be ruled out since amino acids increase sensitivity of *C. albicans* to both oxalysine-containing peptides and nikkomycin X+Z (Table 6). The peptidyl nucleoside nikkomycin X+Z inhibits chitin synthase activity in an intact form and is resistant to peptidase cleavage (Becker *et al.*, 1983; Decker *et al.*, 1991; Krainer *et al.*, 1991; Milewski *et al.*, 1991*b*). The second possibility, of alteration of intracellular target by certain amino acids, is unlikely based on the different targets of action of the two anticandidal agents; nikkomycin inhibits chitin synthase whereas oxalysine inhibits RNA synthesis (Zheng *et al.*, 1991). The increased transport of radiolabelled dileucine in the presence of L-lysine (0.15 mM) (Fig. 4) supports an L-lysine-mediated increase in transport. Differences in the sensitivity of *S. cerevisiae* to toxic dipeptides in the presence of different nitrogen sources were also reported to be due to changes in peptide transport (Moneton *et al.*, 1986*a*).

L-Lysine completely reversed the toxicity of oxalysine and increased the toxicity of oxalysine-containing di- and tripeptides to *C. albicans* in MM. These data confirm

previous conclusions for distinct amino acid and peptide transport systems in *C. albicans*. They also support the argument that the peptides are not hydrolysed extracellularly. If this were the case, the released oxalysine should be competed for by L-lysine. As seen from the data in Table 2 this is clearly not the case.

Lysine transport in *C. albicans* is mediated by two systems: one system is highly specific and the other has a broad specificity (Rao *et al.*, 1986). It is possible that more oxalysine enters cells grown in the presence of proline by a less specific transport system, and this may explain why a slight zone of inhibition is seen even in the presence of lysine in medium containing proline as the nitrogen source (Table 2). Reversal of oxalysine toxicity by lysine was specific to L-lysine (Table 4) and the reversal observed with D-lysine could have been due to a very minor contamination of L-lysine in the D-lysine preparation. A partial reversal of oxalysine toxicity by high concentrations of D-lysine was also seen by Stapley *et al.* (1968) in studies with *E. coli* and they concluded this to be due to contaminating L-lysine in the D-lysine preparation.

Growth inhibition of *C. albicans* with oxalysine-containing peptides coupled with the fact that oxalysine is not toxic to mammalian cells may allow design of a pathogen-specific drug to be delivered by 'illicit transport' (Fickel & Gilvarg, 1973; Ames *et al.*, 1973; Hammond *et al.*, 1987; Higgins, 1987) via the peptide transport system. Our results on regulation of peptide transport in *C. albicans* indicate that in such drug testing and screening particular attention must be given to medium composition.

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