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Effect of an Arginine-Glycine-Aspartic Acid-Containing Peptide on Hematogenous Candidal Infections in Rabbits

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The adherence of *Candida albicans* yeast cells to the subendothelial extracellular matrix, fibronectin, laminin, and type I and IV collagen was tested. Fibronectin (10^{-7} M) and a peptide, PepTite-2000 (Telios Pharmaceuticals Inc., San Diego, Calif.), containing the sequence arginine-glycine-aspartic acid (RGD) inhibited *Candida* adherence to these targets by greater than 90%. When *C. albicans* was perfused over ex vivo rabbit aortic endothelium, there was no significant difference in the amount of adherence in the presence or absence of the RGD-containing peptide. However, the RGD-containing peptide reduced the number of *Candida* organisms present in liver, brain, heart, and kidneys ($P < 0.05$) of rabbits 4 h after intravenous inoculation of 5×10^7 *C. albicans* yeast cells. The peptide also reduced the number of macroscopic *Candida* abscesses in the kidneys of rabbits 72 h after intravenous inoculation of 10^7 *C. albicans* yeast cells ($P < 0.05$). Inhibition of *Candida* adherence in vitro and in vivo may occur because the peptide blocks a fungal receptor that is necessary for adherence.

Disseminated candidiasis is the gravest manifestation of disease with the opportunistic *Candida* species (7) and is most commonly caused by *Candida albicans* (17). The hallmark of disseminated candidiasis is the presence of metastatic sites of infection throughout the body. The establishment of these metastatic sites presumably occurs following the adherence of the fungus to the endothelium (11, 18) or the subendothelial basement membrane (12), also known as the subendothelial extracellular matrix (ECM). If this premise is true, i.e., that adherence of the fungus to the vasculature is an integral step in the pathogenesis of disseminated disease, then interruption or inhibition of the adherence process may ameliorate the disease process (10).

We have recently described a fibronectin receptor on *C. albicans* (13) which appears to mediate the adherence of yeast cells to various ECM proteins such as laminin, type I and IV collagen, and fibronectin. The adherence of the fungus to these proteins and to subendothelial ECM can be abolished with exogenous plasma fibronectin (13) and a synthetic peptide modeled after fibronectin (12a). Here we report the effect of PepTite-2000, a synthetic peptide containing the arginine-glycine-aspartic acid (RGD) sequence of fibronectin, on the adherence of *C. albicans* yeast cells to proteins, subendothelial ECM in vitro, to ex vivo aorta, and to organs in vivo.

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MATERIALS AND METHODS

Cultures. Seven clinical isolates of *C. albicans* were maintained on Sabouraud dextrose agar slants (Difco, Detroit, Mich.) and were transferred monthly. After determining the effects of the synthetic peptide on the adherence of these isolates to type I collagen, only one *C. albicans* isolate was used thereafter. To perform the adherence assay and for animal injections, a loopful of yeast cells was removed from the slants, inoculated into 50 ml of Sabouraud dextrose broth (Difco), and incubated at room temperature with shaking for 20 h. This yielded stationary-phase yeast cells. The yeast cells were washed three times with Earle's balanced salt solution with calcium and magnesium (EBSS; GIBCO Grand Island, N.Y.) by centrifugation. The yeast cells were counted in a hemacytometer and resuspended to the desired concentrations in EBSS.

Proteins and peptides. The human plasma fibronectin used for inhibition studies was isolated by previously described methods (24). Immobilized target proteins were prepared by adding 200 μg of laminin, type IV collagen, and fibronectin (Collaborative Research Inc., Bedford, Mass.) per well to 24-well tissue culture trays (GIBCO) and were allowed to adsorb overnight. Type I collagen (Collagen Corporation, Palo Alto, Calif.) was adsorbed in a similar manner at 140 μg per well. Subendothelial ECM prepared by the method of Gospodarowicz et al. (5) was purchased from Accurate Chemical and Scientific Corp., Westbury, N.Y. PepTite-2000 is a biocompatible peptide containing the RGD sequence and is modeled after fibronectin. It is approximately a 23-mer; the sequence is a proprietary secret. GRADSP is a control peptide that lacks the RGD sequence. PepTite-2000 and the peptide GRADSP were purchased from Telios Pharmaceuticals Inc., San Diego, Calif.

In vitro adherence assay. Trays containing ECM or ECM proteins were washed three times with EBSS. Each well was then covered with 200 μl of EBSS containing 1.25×10^3 CFU of *C. albicans* with or without 100 μg of fibronectin or 1,000 μg of PepTite-2000. Trays were then incubated for 30 min without agitation at 37°C, and then each well was washed three times with EBSS. Molten Mycosel agar (BBL,

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Cockeysville, Md.) at 56°C was added to each well, and the number of adherent CFU was determined after overnight incubation of the tray at 37°C. Aliquots of the original yeast cell suspensions were also spread onto Mycosel agar in order to determine the total number of CFU added per well and to determine whether there was any inhibitory effect of PepTite-2000 on fungal growth. Samples of yeast suspensions containing PepTite-2000 were removed at different time periods during the adherence assay and were observed microscopically for aggregation of yeast cells. Furthermore, wells were observed by inverted-phase microscopy for aggregation of yeast cells.

Animals. Seventy-six female New Zealand White rabbits weighing approximately 3.5 kg each were housed separately and given water and food ad libitum. Animals were anesthetized with ketamine and xylazine, and then *C. albicans* with or without PepTite-2000 in 1 ml of EBSS was administered intravenously (i.v.) through a central ear vein. Ten animals were initially used to standardize the *C. albicans* inoculum required to produce renal cortical abscesses 72 h after i.v. injection but not to kill the animal acutely. Animals were sacrificed by CO_2 narcosis, after being anesthetized with ketamine and xylazine. Use of the animals was in accordance with a locally approved protocol.

Ex vivo adherence assay. Ten animals were anesthetized, given 5,000 U of heparin i.v., and sacrificed by CO_2 narcosis. A midline incision of the thorax and abdomen was made, and the thoracic and abdominal aorta were dissected free of the adnexa. A 14-gauge catheter was placed at the arch and directed caudad. A small cut was made at the ilial bifurcation, and Ringer's solution (pH 7.4) at 26°C maintained at 130 cm above the aorta was perfused through the aorta until the effluent from the ilial bifurcation was clear of blood. A plastic rod was then passed through the bifurcation toward the arch, and the aorta was sectioned at the arch and the bifurcation and was placed in 0.2 M Tris (pH 7.4). A piece of the thoracic aorta (about 4 to 5 cm) was removed and carefully stripped of adnexa, turned endothelial side out, and drawn over the plexiglass support rod of a Baumgartner perfusion chamber (a gift of H. Baumgartner). The perfusion chamber is made of plexiglass and consists of two parts. One part is a rod of 3.5 mm in diameter with holes to allow the inflow of perfusate from connecting silastic tubing. The other part is a tube (4.4-mm inside diameter) which fits over the rod and has a port connected to silastic tubing, allowing for outflow of the perfusate (25). The chamber was used in a closed system with a reservoir of perfusion fluid, consisting of 5 ml of EBSS with or without 1 mg of PepTite-2000 per ml and 10^7 *C. albicans* yeast cells per ml, which was pumped through the chamber continuously for 30 min at 26°C by a peristalic pump (LKB-Pharmacia, Uppsala, Sweden). The perfusate was returned to the reservoir through silastic tubing to be pumped over the aorta again. The flow rate yielded a wall shear rate on the endothelium of 515 dynes/s. Following perfusion of the aortas with *C. albicans* for 30 min, 15 ml of EBSS was then perfused through the chamber as a washing step; the rod containing the thoracic aortic segment was removed; and ~0.5-cm-long strips of aorta were removed, being careful not to include any segment with an intercostal artery, weighed, and then homogenized and diluted in Sabouraud dextrose broth, as explained below. Results are presented as CFU per gram of tissue.

In vivo experiments. Animals were divided into pairs, one animal serving as a control that received only *C. albicans* in EBSS i.v. and an experimental animal, which was matched with the control for weight, that received *C. albicans* plus 1,000 μg of PepTite-2000 in EBSS i.v. Five pairs of animals constituted an experiment. Two types of animal experiments were performed, one which involved sacrificing the animals 4 h after i.v. injection and another that involved sacrificing the animals 72 h after i.v. injection.

(i) The 4-h experiments. Animals were sacrificed 4 h after i.v. administration of 5×10^7 CFU of *C. albicans*. The abdomen and thorax of each animal were opened in an aseptic manner. Sections of the brain, lung, heart, liver, spleen, muscle, and both kidneys were removed, weighed, and placed in 1 ml of Sabouraud dextrose broth (Difco); the tissue was homogenized with a motor-driven Teflon grinder (Tri-R Instruments, Inc., Rockville Center, N.Y.). Serial 10-fold dilutions of the homogenate were prepared, and 100 μl of each dilution was spread onto three Mycosel agar plates (BBL). The plates were incubated overnight at 37°C, and the CFU was determined the following day. Results are expressed as CFU per gram of tissue of each organ.

(ii) The 72-h experiments. Rabbits were sacrificed 72 h after i.v. administration of 10^7 CFU of *C. albicans* with or without 1,000 μg of PepTite-2000. Both kidneys were removed and placed on a sheet of paper, their outlines were traced with a pencil, and then the kidneys were placed in 10% formalin. The paper silhouette was weighed, as was a 1-cm^2 piece of paper, and the weight was converted to surface area (this number was multiplied by 2 in order to estimate the surface area on two sides of the kidney). After fixation in formalin, the total number of visible renal cortical abscesses per square centimeter for the same (or comparable) regions on each kidney was determined, a mean value was obtained, and results are expressed as the number of abscesses per kidney.

Statistics. Significant differences in percent adherence in the in vitro and ex vivo assays were determined by comparing means with a two-tailed Students *t* test (6), and data are given as means ± standard deviations. No assumptions about the distribution of data in the in vivo experiments were made, and the significance of differences in these experiments was determined by use of a one-tailed Wilcoxon rank sum test (6). Mean values are shown to demonstrate the central tendencies. P values of <0.05 were considered significant.

RESULTS

In vitro experiments. Preliminary experiments were performed with type I collagen as the target substrate; it was immobilized in the wells of plastic tissue trays. Seven different *C. albicans* isolates (from blood, urine, and sputum) were tested for their ability to adhere to type I collagen in the presence of PepTite-2000. PepTite-2000 inhibited adherence of all isolates by >95% (Table 1). Isolate 1 was also tested in the germinated form, and PepTite-2000 was effective against this form as well. GRADSP did not inhibit yeast adherence to type I collagen. Inhibition by PepTite-2000 was not due to a pH effect, since at 1,000 μg/ml, PepTite-2000 in EBSS has a pH of 7.4. Furthermore, the protein was not toxic to the fungus; on the contrary, colonies appeared to grow more rapidly on agar in the presence of the peptide. Yeast cells and germinated forms showed no evidence of clumping or aggregating in the presence of the peptide, as observed by direct and inverted microscopy.

If PepTite-2000 was placed in the plastic wells at 200 μg per well and allowed to adsorb to the plastic surface, it reduced the adherence of yeast cells to the wells. For example, yeast cells in EBSS had 89 ± 3% adherence to

TABLE 1. Inhibition of adherence of *C. albicans* strains to type I collagen in the presence of 1,000 μg of PepTite-2000 per ml

Isolate	% Inhibition of adherence[a]
1	97
2457	92
2453	97
2466	96
2472	96
2396	96
2448	96

[a] Percent inhibition = 100 − (percent adherence of PepTite-2000-treated yeast cells/percent adherence of EBSS-treated yeast cells).

plastic well bottoms, whereas there was only 32 ± 3% adherence to PepTite-2000-treated wells, representing an inhibition of adherence of 64%.

PepTite-2000 inhibited *C. albicans* yeast cell adherence to type I collagen in a dose-dependent manner. For instance, in EBSS alone there was 74 ± 2% adherence, at 1 and 10 μg/ml there were 84 ± 1 and 83 ± 2% adherence, respectively, and at 100, 500, and 1,000 μg/ml there were 55 ± 4, 6 ± 3, and 5 ± 2% adherence, respectively. Therefore, there was maximal inhibition of adherence somewhere between a concentration of 100 and 500 μg of PepTite-2000 per ml, or approximately 10^{-5} M.

Because *C. albicans* has been shown to possess a fibronectin receptor (13) and PepTite-2000 is modeled after fibronectin, we compared the amount of inhibition achieved with PepTite-2000 with that achieved with fibronectin for adherence to different ECM proteins and subendothelial ECM.

First, *C. albicans* yeast cells were preincubated with EBSS alone or in EBSS with 100 μg of human plasma fibronectin per ml or 1,000 μg PepTite-2000 per ml for 2 h at 37°C. The yeast cells were then washed by centrifugation in EBSS, and their capacities to adhere to type I collagen were compared. Yeast cells in EBSS alone had 65 ± 2% adherence, whereas fibronectin- and PepTite-2000-treated yeast cells had 22 ± 1 and 3 ± 1% adherence, respectively. This indicated that both these proteins adhere to the surface of yeast cells even after vigorous washing. We then examined the ability of human plasma fibronectin and PepTite-2000 to inhibit the adherence of *C. albicans* to other ECM proteins, type IV collagen, laminin, and fibronectin. Fibronectin and PepTite-2000 both markedly reduced yeast cell adherence to all the immobilized proteins (Table 2). Both also diminished yeast cell adherence to the complex target of subendothelial ECM. For example, when yeast cells were suspended in EBSS alone, there was 90 ± 4% adherence to ECM, whereas

TABLE 2. Inhibition of *C. albicans* adherence to ECM proteins with human plasma fibronectin and PepTite-2000

Immobilized target protein	% Adherence ± SD		
	EBSS alone	Fibronectin (100 μg/ml)	PepTite-2000 (1,000 μg/ml)
Type I collagen	80 ± 10	5 ± 4[a]	6 ± 3[a]
Type IV collagen	79 ± 8	5 ± 1[a]	3 ± 3[a]
Laminin	79 ± 19	25 ± 8[a]	1 ± 2[a]
Fibronectin	82 ± 12	5 ± 2[a]	3 ± 7[a]

[a] $P < 0.05$ for all treatment groups.

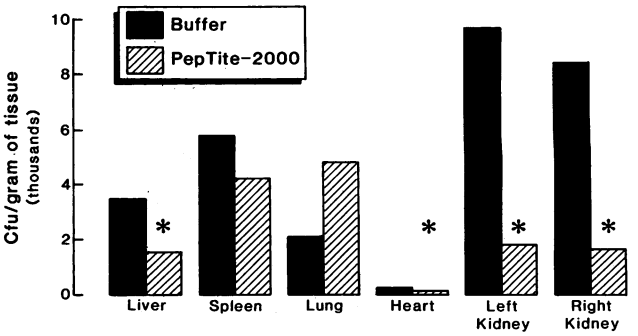

FIG. 1. Comparison of CFU of *C. albicans* per gram of tissue in rabbits receiving 5 × 10^7 *C. albicans* i.v. along with 1 mg of PepTite-2000 or buffer alone. *, $P < 0.05$.

with human plasma fibronectin and PepTite-2000 there were 23 ± 4 and 2 ± 2% adherence, respectively.

Ex vivo experiments. We then attempted ex vivo experiments by perfusing *C. albicans* over endothelium in the presence or absence of PepTite-2000. There was no significant difference between the number of adherent CFU per gram of tissue. PepTite-2000-treated yeast cells had 610,700 CFU/g of tissue ($n = 9$), versus 502,200 CFU/g of tissue in the control group ($n = 9$).

In vivo experiments. Because PepTite-2000 was such an effective inhibitor of *C. albicans* adherence to ECM proteins and to subendothelial ECM, we performed in vivo experiments to determine whether the presence of PepTite-2000 would affect the outcome of metastatic candidal disease since it is possible that subendothelial ECM may be a target for fungal adherence in vivo (10). The first experiments involved administering *C. albicans* i.v. with or without 1,000 μg of PepTite-2000 per rabbit and then homogenizing the target organs 4 h after i.v. injection. In these experiments there were significantly fewer CFU in PepTite-2000-treated animals than in EBSS-treated animals in the liver, heart, and both kidneys. There was no significant difference in the CFU in lungs and spleen (Fig. 1). Three other matched pairs of rabbits were checked for the distribution of the fungus to the brain and skeletal muscle. PepTite-2000-treated animals had 5% of the CFU per gram of tissue in the brain compared with that in the controls, and *C. albicans* could not be detected in skeletal muscle in either PepTite-2000-treated rabbits or controls.

These results demonstrate that PepTite-2000 affects the early steps of metastatic *Candida* lesions. Because hematogenous dissemination of yeast cells results in macroscopic renal cortical abscesses, we determined whether abscesses could be prevented by treatment with PepTite-2000. Rabbits were injected i.v. with 10^7 CFU of *C. albicans* per animal with or without 1,000 μg of PepTite-2000. PepTite-2000-treated animals had significantly fewer abscesses per kidney (Fig. 2). Typical results of these experiments are shown in Fig. 3. Kidneys were sectioned, and there was no evidence of shunting of abscesses to the medulla in PepTite-2000-treated animals. To determine whether the fewer abscesses present in the kidneys occurred because of increased trapping of yeast cells in the lungs, a 14-gauge catheter was inserted into the iliac artery of two animals and passed to the level of the aortic arch. The animals received 10^7 CFU of *C. albicans* with or without 1,000 μg of PepTite-2000 in 1 ml of EBSS. The catheters were removed, the artery was ligated, and the animals were sacrificed 72 h later. The rabbit that

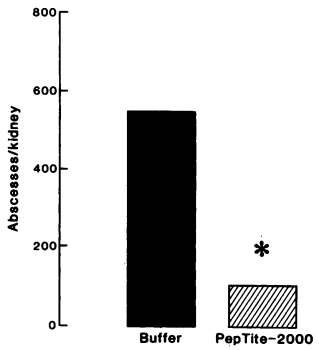

FIG. 2. Comparison of abscesses per kidney of rabbits receiving 10^7 *C. albicans* i.v. along with 1 mg of PepTite-2000 or buffer alone. *, $P < 0.05$.

received only EBSS had 2,555 abscesses per kidney, whereas the PepTite-2000-treated rabbit had 788 abscesses per kidney. This demonstrates that simple trapping in the lungs did not account for the reduction of metastases in the kidneys.

Experiments were subsequently performed in which 1,000 μg of PepTite-2000 was injected prior to and after the administration of yeasts. In three of five animals pretreated with PepTite-2000, renal abscesses were fewer in number than they were in five EBSS-treated rabbits ($P > 0.05$). In other experiments, 1,000 μg of PepTite-2000 given 15 min after the administration of yeast cells demonstrated no significant reduction in renal abscesses in any animal.

DISCUSSION

The ability of *C. albicans* to bind numerous human proteins such as fibrinogen (2), laminin (3), fibronectin (13, 23), and iC3b (8) may be related to the pathogenicity of this microorganism. These proteins all contain the RGD sequence, a ligand recognized by many mammalian integrins (20). Integrins are highly conserved glycoprotein receptors that are present throughout phylogeny (1). These receptors are heterodimers, consisting of α and β subunits that are important in anchoring cells to basement membranes and ECM.

Proteins antigenically similar to α subunits (16) and of a size similar to those of α subunits (9) of integrins have been described on *C. albicans*. A protein that is antigenically identical to the β_1 subunit of integrins has also been detected in *C. albicans* (15). Previous work by us (13) and others (4) suggests that the amino acid sequence of RGD may be recognized by receptors on *C. albicans*. Preliminary work by others (3a, 4a) confirms that *C. albicans* possesses receptors that are capable of recognizing the sequence of RGD.

It is interesting that tumor metastases arise by blood-borne tumor cells that adhere to the microvasculature, and this interaction may be mediated by integrin receptors (19). RGD peptides have been shown to inhibit the adherence of tumor cells in vitro and to inhibit metastases in vivo (20).

PepTite-2000 is an amino acid sequence modeled after the RGD cell-binding domain of fibronectin. In the in vitro experiments reported here, it was as effective as plasma fibronectin in inhibiting the adherence of *C. albicans* to ECM proteins and subendothelial ECM, albeit at a greater concentration, i.e., 10^{-4} to 10^{-5} M versus 10^{-7} M for fibronectin. The peptide was not toxic to the fungus and did not promote aggregation of yeast cells. Inhibition appeared to occur in part by the binding of the peptide to *C. albicans*, which is perhaps similar to the binding of fibronectin to *C. albicans* (13). Interestingly, unlike RGD (data not shown) or fibronectin (13), when it was adsorbed to wells, PepTite-2000 inhibited yeast adherence to plastic. This result implies that the molecule in solution has a different conformation than the molecule adsorbed to a surface. This could be explained by an interaction between the yeast cell and the adsorbed peptide, in which the preferred ligand would be hidden when the peptide is adsorbed to a surface.

The experiments involving the perfusion of *C. albicans* over the aortic endothelium showed no difference between PepTite-2000-treated yeast cells and those without the peptide present. This was not unexpected, since endothelial cells contain surface receptors that are capable of binding RGD peptides, which could actually cause an increase in *Candida* adherence because the peptide would serve as a bridge between the endothelium and *C. albicans*. Furthermore, in preliminary studies Frey et al. (4a) found that PepTite-2000 in amounts <1 mg/ml actually enhances *Candida* adherence to human umbilical vein endothelial cells in vitro, whereas there was inhibition of adherence by PepTite-2000 at a concentration of 1 mg/ml. In addition, RGD peptides may cause aortic relaxation (14), which could lead to greater exposure of subendothelial ECM and, hence, greater adherence of *C. albicans* (12).

The animal experiments document the ability of PepTite-2000 to affect the distribution and fate of i.v. administered *C. albicans* yeast cells. The hearts, kidneys, and livers of animals treated with PepTite-2000 had significantly fewer CFU of fungus ($P < 0.05$), whereas there was no difference in the CFU of the lungs and spleens of treated and untreated animals ($P > 0.05$). Similarly, PepTite-2000 significantly reduced the number of renal abscesses that were formed ($P < 0.05$). When the peptide was given before or after the yeast cells rather than simultaneously with the yeast cells, there was no significant effect on the number of metastatic abscesses that formed.

The mechanism by which PepTite-2000 reduces CFU and abscesses in vivo is unknown, but it is not solely due to the shunting of organisms from the liver and kidneys to the lungs, as shown by a reduction in the number of renal

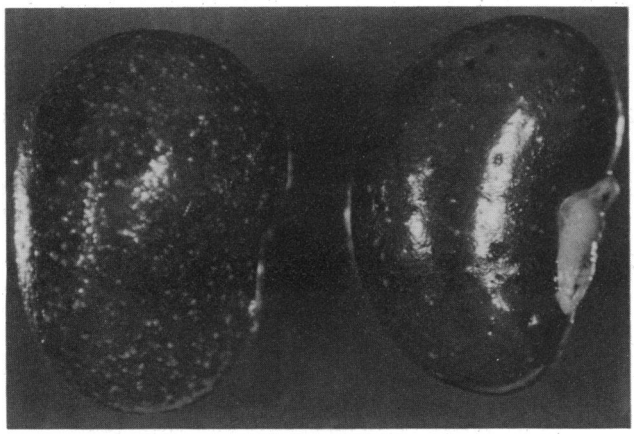

FIG. 3. Photograph of rabbit kidneys. The kidney on the left is from an animal that received 10^7 *C. albicans* i.v. in buffer. The kidney on the right is from an animal that received *C. albicans* plus 1 mg of PepTite-2000.

abscesses when yeast cells were introduced at the level of the aortic arch. In this circumstance, the fungi did not pass through the lungs before reaching the kidneys. Therefore, the inhibitory effect was expressed at the level of the kidneys.

The in vitro data suggest that yeast cells may be incapable of sticking to the vascular surface after contact with PepTite-2000, perhaps because PepTite-2000 blocks a specific receptor. However, the ex vivo results and recent work by Sawyer et al. (22) suggest that other mechanisms may contribute to the overall reduction in culturable *C. albicans*. Sawyer et al. (22) found that increased killing of *C. albicans* occurs when *C. albicans* is perfused into isolated mouse livers along with certain agents. Some agents decreased the adherence of the perfused *C. albicans* to the liver and caused enhanced killing of perfused *C. albicans* by resident hepatic macrophages. The most recent work of Sawyer (21) with mouse fibronectin and PepTite-2000 in this model demonstrates increased macrophage killing of *C. albicans* coated with fibronectin or PepTite-2000.

In conclusion, results of the experiments described here demonstrate the alteration of the interaction of *C. albicans* with target substrates by a novel peptide, PepTite-2000. PepTite-2000 and similar peptides are worthy of further work to determine what effect they may have on the interaction (including adherence) of *C. albicans* with inert surfaces, such as plastic, as well as relevant biological surfaces.

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