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Review Article

Histatins: antimicrobial peptides with therapeutic potential

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Abstract

Histatins are a group of antimicrobial peptides, found in the saliva of man and some higher primates, which possess antifungal properties. Histatins bind to a receptor on the fungal cell membrane and enter the cytoplasm where they target the mitochondrion. They induce the non-lytic loss of ATP from actively respiring cells, which can induce cell death. In addition, they have been shown to disrupt the cell cycle and lead to the generation of reactive oxygen species. Their mode of action is distinct from those exhibited by the conventional azole and polyene drugs, hence histatins may have applications in controlling drug-resistant fungal infections. The possibility of utilising histatins for the control of fungal infections of the oral cavity is being actively pursued with the antifungal properties of topical histatin preparations and histatin-impregnated denture acrylic being evaluated. Initial clinical studies are encouraging, having demonstrated the safety and efficacy of histatin preparations in blocking the adherence of the yeast *Candida albicans* to denture acrylic, retarding plaque formation and reducing the severity of gingivitis. Histatins may represent a new generation of antimicrobial compounds for the treatment of oral fungal infections and have the advantage, compared with conventional antifungal agents, of being a normal component of human saliva with no apparent adverse effects on host tissues and having a mode of action distinct to azole and polyene antifungals.

Histatins — salivary antimicrobial peptides

Histatins are small histidine-rich cationic peptides ranging in size from 7 to 38 amino-acid residues in length. They are secreted by the parotid and sub-mandibular salivary glands in man and some higher primates and are present in saliva at concentrations in the range 50–425 $\mu\mu$ (Helmerhorst et al 1997). Histatins manifest an in vivo IC50 (the concentration which inhibits growth by 50%) value of approximately 1.4 μM (Helmerhorst et al 1997). Histatins 1, 3 and 5 contain 38, 32 and 24 amino-acid residues, respectively, and the sequence of the first 22 amino acids of each histatin is identical. Histatins 1 and 3 are products of different genes while histatin 5 is a proteolytic cleavage product of histatin 3 (Oppenheim et al 1988).

Histatins represent a group of antimicrobial peptides with some antibacterial properties and significant antifungal properties (Oppenheim et al 1988). The oral cavity is susceptible to a range of bacterial and fungal infections and histatins may have evolved to control infection in this region. Higher levels of histatins are present in the saliva of patients with recurrent oral candidosis than in un-infected controls, indicating their potential role in curtailing disease in cases of persistent infection (Bercier et al 1999). The elevated levels present in cases of recurrent infections may assist in curtailing more serious infection or the dissemination of the infection to other parts of the body. In contrast, however, the production of histatins decreases with age, as the incidence of oral fungal infection rises (Johnson et al 2000).

Histatins are active against a range of pathogenic yeast and filamentous fungi resistant to conventional azole and polyene antifungal drugs (Helmerhorst et al 1999a). Histatin 5 is the most active histatin against the pathogenic yeast *Candida albicans*, which is a normal component of the body flora but is capable of causing lesions in the mouths of immuno-compromised patients. Histatin 5 retards the transition of *C. albicans* from the blastospore to the hyphal stage of growth — a process that may assist in arresting tissue penetration by the fungus (Helmerhorst et al 1997). Histatins are also

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capable of killing the yeast *Cryptococcus neoformans* (Tsai 1998) and *Candida dubliniensis* (a close relative of *C. albicans*) is susceptible to the effects of histatin 3 (Fitzgerald et al 2003). Although not normally associated with disease of the oral cavity, conidia of the pulmonary pathogen *Aspergillus fumigatus* demonstrate greater susceptibility to histatin 5 than amphotericin B (Helmerhorst et al 1999a). The efficacy of histatins in controlling fungal infection may be seen in patients who experience xerostomia (dry mouth), where there is an increased incidence of oral fungal infections in the absence of saliva containing these antimicrobial peptides. Their presence in saliva in normal patients helps to curtail (rather than eliminate entirely) the numbers of *C. albicans* in the oral cavity and thus prevent disease.

Structure of histatin 5

Histatin 5 may be distinguished from the other histatins by the ability to form α-helices in aqueous trifluoroethanol. This ability was initially thought to be important for the mode of action of histatin 5 but Situ et al (2000) demonstrated that a histatin variant (called 3P), with reduced ability to form a helix, that was formed by replacing three residues with proline, had an antifungal ability comparable with that of histatin 5.

The fungicidal activity of histatin 5 resides in a region of 11–24 residues at the C terminal referred to as the functional domain (Driscoll et al 1995) or dh-5 (Helmerhorst et al 1997). Mutations in histatin 5 were created by replacing single residues and producing the altered protein in the bacterium *Escherichia coli*. Variant M21 was created by replacing Lys-13 with Thr, while M71 had Lys-13 replaced by Glu. Both variants demonstrated reduced fungicidal activity, indicating that the Lys-13 region was required for antifungal activity (Tsai et al 1996). A similar strategy was adopted in generating variants with multi-site substitutions in the dh-5 region. Two of the synthetic peptides (dhvar1 and dhvar 2) demonstrated enhanced anti-*Candida* properties having an IC50 (the concentration which inhibited growth by 50%) values of 0.6 $\mu\mu$ and 0.8 $\mu\mu$, respectively, compared with an IC50 value of 1.4 $\mu\mu$ for histatin 5 (Table 1). Peptides hvar1 and dhvar 2 demonstrated the ability to inhibit the growth of *C. albicans* in an agar assay, in contrast to histatin 5, and manifested antibacterial activity against *Prevotela intermedia, Streptococcus mutans* and methicillin-resistant *Staphylococcus aureus* (Helmerhorst et al 1997). The potential therapeutic application of dhvar 1 and dhvar 2 was abandoned when it was discovered that both displayed haemolytic activity in-vivo (Helmerhorst et al 1997).

Mode of action of histatins

Histatins demonstrate significant antifungal activity and represent an important element of the local immune response in the oral cavity. Understanding their mode of action may allow an enhancement (or fine-tuning) of their activity and open the possibility of employing them as antifungals, either as free agents or incorporated into materials that would normally be located in the oral cavity (e.g. denture acrylic, prosthetic implants). While histatins can manifest significant anti-*Candida* properties, their mode of action remains to be fully characterised.

Histatin 5 was demonstrated to be capable of killing *C. albicans* pre-loaded with calcein without inducing significant release of the fluorescent dye, indicating that lysis of the cell membrane was not a significant effect of the peptide (Baev et al 2002). Histatins are also capable of killing spheroplasts (osmotically fragile cells retaining fragments of cell wall) of *C. albicans*, demonstrating that the fungal cell wall was not a target for their action (Driscoll et al 1996). Histatins appear to target a specific binding site in the fungal cell. Using cells fractionated with glass beads and separated by SDS-PAGE it was possible to demonstrate binding of ^{125}I-histatin 5 to a protein of approximately 67 kDa, which may reside in the plasma membrane of the intact cell (Edgerton et al 1998). Recent work has demonstrated that histatin 5 binds heat shock protein 70 (Ssa1/2) — a protein located in the fungal cell envelope. Fungal mutants deleted in the genes for Ssa1/2 synthesis show a low level of susceptibility to histatin 5 compared with the unaltered parental isolate (Li et al 2003). It is unclear how histatin gains access to this binding site but it may cross the cell wall of *C. albicans* using an existing transporter and then target the binding site in the plasma membrane.

One of the first indications that histatin 5 targets the mitochondrion came from the observation that non-respiring cells were resistant to its activity (Helmerhorst et al 1999b). Fungal cells incapable of respiration (respiratory-deficient or petite mutants) are resistant to the action of histatins and *C. albicans* cells treated with the respiration inhibitor sodium azide are resistant to histatin 5 (Gyurko et al 2000). FITC-labelled histatin 5 accumulated in respiring *C. albicans* cells but not in those cells treated with azide, indicating the requirement for mitochondrial

Table 1 Amino-acid sequences of histatin 5 and derivatives.

Peptide	Sequence	IC50	Antifungal on agar
Histatin 5	DSHAKRHHGYKRKFHEKHHSHRGY	1.4 μM	No
Dhvar 1	KRLFKELKFSLRKY	0.6 μM	Yes
Dhvar 2	KRLFKELLFSLRKY	0.8 μM	Yes

Adapted from Helmerhorst et al (1997).

function for histatin activity. The histatin 5 appeared to accumulate in discrete regions of the cell, which were later identified as mitochondria using a specific mitochondrial stain (mitotracker orange).

Histatin 5 shares a number of structural similarities with mitochondrial pre-sequences, which are basic peptides that target the mitochondrion. Consequently, once histatin 5 crosses the plasma membrane, it may be attracted to the mitochondrion in a similar manner as mitochondrial pre-sequences (Helmerhorst et al 1999b). It has been postulated that cardiolipin (diphosphatidylglycerol), a negatively charged phospholipid in the mitochondrial membrane, which attracts mitochondrial pre-sequences, may serve to attract histatin 5 towards the mitochondrion (Helmerhorst et al 1999b). Once histatin binds the mitochondrion, it appears to induce the release of ATP into the cytoplasm. *C. albicans* exposed to histatin 5 demonstrate a reduction in intracellular ATP levels and there is a corresponding elevation in extracellular ATP. Cells treated with the respiration uncouplers CCCP, azide or cyanide and subsequently exposed to histatin 5 show unchanged ATP levels, indicating that respiration is essential for histatin activity to occur (Helmerhorst et al 1999b; Koshlukova et al 1999). These finding suggest the existence of a relationship between ATP efflux and cell death. ATP may exit *C. albicans* cells via ATP-binding cassette (ABC) proteins. One suggestion for the fungicidal activity of histatin 5 is that, following non-lytic release of ATP from the cell, extracellular ATP activates a purigenic-like receptor that triggers cell death (Koshlukova et al 1999).

The loss of ATP in itself could also contribute to the demise of the cell. In addition to their effect on ATP levels within fungal cells, recent work has indicated that the fungicidal activity of histatin 5 may be mediated through the generation of reactive oxygen species (ROS) (Helmerhorst et al 2001). Treatment of *C. albicans* with histatin-5 induced the formation of ROS as measured using an oxygen radical sensitive probe (dihydroethidium). ROS could disrupt the cell organelle structure or function, or induce lethal DNA damage. Histatin 5 has also been shown to disrupt the cell cycle in *C. albicans*, halting cells in the G1 phase with a concomitant effect on the regulation of cell volume homoeostasis, which is correlated with ATP loss (Baev et al 2002).

The current state of knowledge regarding the mode of action of histatins can be summarised as follows (Figure 1): histatins bind a specific receptor in *C. albicans* , cross the plasma membrane and are released into the cytoplasm (A in Figure 1); they specifically target the mitochondrion (B) and induce the non-lytic release of ATP, perhaps through activation of ABC proteins (C); the released ATP activates purigenic receptors (D) or the energy loss (E) inactivates the cell's ability to respire; histatins cause G1 arrest (F); the generation of ROS may damage membranes, DNA or organelles (G) and represents the final insult leading to the demise of the fungal cell (Helmerhorst et al 2001).

Therapeutic potential of histatins

Histatin 5 possesses potent antifungal properties and has the advantage over conventional synthetic azole or polyene antifungals of being a naturally occurring compound in man, with no known cross-reactivity with human cells or tissues. These qualities make it an ideal compound for development as an antifungal agent in the treatment of fungal infections of the oral cavity. In addition, the distinct mode of action displayed by histatin 5, compared with that of azoles or polyenes (Tsai & Bobek 1997), may facilitate its use in situations where resistance to conventional antifungals has emerged (White et al 1998). It has

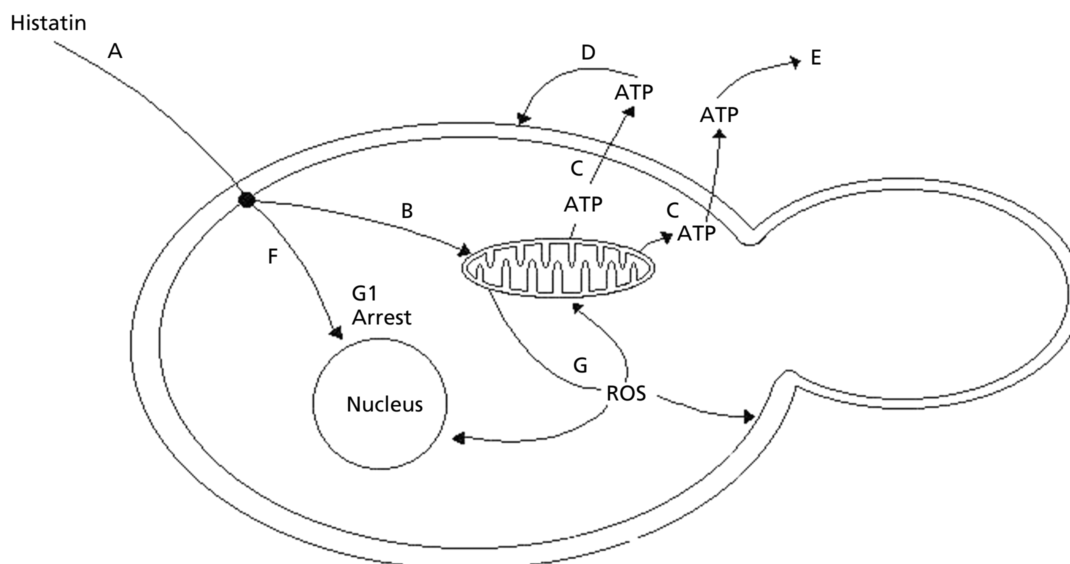

Figure 1 The effect of histatin 5 on *C. albicans*. Histatin 5 targets the mitochondrion (B), induces the release of ATP (C) and affects the ability of the cell to respire (E). ATP loss may activate a cell death pathway (D). Histatins also cause the generation of reactive oxygen species (G) and induce G1 phase arrest of the nucleus (F).

been suggested that the antifungal potential of histatins could be exploited by inclusion in topical gels or denture acrylic, or as a component in the formulation of artificial saliva to combat xerostomia (Tsai & Bobek 1998).

Topical histatin gels have been tested for their efficacy in controlling gingivitis in man (Paquette et al 2002). Histatin concentrations of 0.0625, 0.125 and 0.375% were self-applied by patients twice daily for the treatment of gingivitis and plaque development. Administration of gels improved the condition but, more importantly, this work demonstrated the safety and tolerance of using repeated doses of histatin gels in controlling oral disease.

The ability to localise histatins in dentures, or other synthetic oral implants, would be beneficial in that the antifungal properties of the histatin would prevent colonisation of the material itself and leaching from the structure might also contribute to the control of yeast in the oral cavity at sites distant from the structure. Surface-modified denture resin manufactured from poly(methyl methacrylate) allowed enhanced adsorption and desorption of histatin 5 (Edgerton et al 1995). The modified material demonstrated anti-*Candida* activity and inhibited the adherence of the yeast, thus preventing the development of a biofilm on the surface. The adherence of *C. albicans* to oral tissues and dental appliances is critical to its success in colonising the oral cavity (Cotter & Kavanagh 2000) and the ability of histatin 5 to inhibit this phenomenon may retard fungal colonisation and dissemination.

A number of histatin variants have been assessed for their antifungal activity. Variant M21 (described earlier) was found to be less potent than histatin-5 (Tsai et al 1996). Dhvar1 and dhvar 2 (multi-site substitutions in the dh-5 region) displayed lower antifungal activity than histatin 5 (Helmerhorst et al 1997). The variants displayed activity against a number of bacteria but their haemolytic activity rendered them unsuitable for future development. The efficacy of dhvar 1 and histatin 5 was assessed using xanthan as a delivery vehicle (Ruissen et al 1999). The results demonstrated an increase in cidal activity of the peptides in the presence of xanthan and indicated that this medium would be suitable for use in situations where retention of the peptide was critical. The nature of the matrix can influence the activity and retention of histatins to a significant degree. In a study of three polymeric materials, it was established that optimum activity was achieved in uncharged hydroxyethylcellulose rather than xanthan or negatively charged carboymethylcellulose but that in saliva substitutes xanthan is more suitable because of its rheological properties (Ruissen et al 2002). It was suggested that xanthan should be further evaluated as a vehicle for delivering histatins but that the reductive effect of this polymer should be counteracted by increasing the dose of histatin present in the medium.

Conclusion

Histatins are a group of antimicrobial peptides that show pronounced antifungal activity and are particularly active against the yeast *C. albicans*. They target the mitochondrion and have a mode of action distinct from those of the conventional azole and polyene antifungals. Histatins may have applications in situations where conventional drugs are of limited use due to the appearance of drug resistance. The fact that histatins are a normal component of human saliva indicates that they should be well tolerated if applied clinically. The ability to synthesise quantities of recombinant histatins may allow their use as topical gels or incorporation into denture acrylic for the control of a range of acute and chronic oral fungal infections.

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