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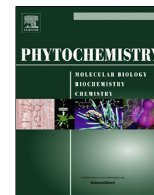

The haploinsufficiency profile of α-hederin suggests a caspofungin-like antifungal mode of action

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ABSTRACT

The leaves of common ivy (*Hedera helix*) contain the cytotoxic saponin α-hederin, which is inhibitory to *Candida albicans* at low concentrations. To investigate the mode of action of α-hederin, a haploinsufficiency screen was carried out using a library of 1152 *Saccharomyces cerevisiae* deletion strains. An ethanol ivy extract containing α-hederin was used in the initial screen to reduce the amount of compound required. Strains exhibiting disproportionately low growth were then examined in more detail by comparing growth curves in the presence and absence of α-hederin. This approach identified three hypersensitive strains carrying gene deletions for components of the transcription related proteins SWI/SNF, RNA polymerase II and the RSC complex. Saponin cytotoxicity is often attributed to membrane damage, however α-hederin did not induce hypersensitivity with an aminophospholipid translocase deletion strain that is frequently hypersensitive to membrane damaging agents. The haploinsufficiency profile of α-hederin is most similar to that reported for drugs such as caspofungin that inhibit synthesis of the fungal cell wall. Screening with plant extracts rather than isolated compounds, provides a valuable shortcut in haploinsufficiency screening provided hypersensitive strains are then confirmed as such using purified active principles.

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Introduction

Common ivy (*Hedera helix* L., Araliaceae) is a species of climbing plant with a European natural distribution (Valcarcel and Vargas, 2010). Ivy leaves contain the saponin α-hederin which exhibits *in vitro* activity against pathogenic yeasts. Minimal inhibitory concentrations (MICs) for *Candida glabrata* and *Candida albicans* have been reported as 25 and 5 μg/ml, respectively (Favel et al., 1994; Moulin-Traffort et al., 1998). A study using electron microscopy to examine the effect of α-hederin on *C. albicans* morphology revealed degradation of cellular organelles and the plasma membrane as well as alterations in the cell wall (Moulin-Traffort et al., 1998). More recently Lorent et al. (2013) studied the effect of α-hederin on unilamellar vesicles and proposed a mechanism based on a curvature-driven permeabilization caused by saponin sterol interactions. Whilst these studies point towards a membrane permeabilization mechanism it is not clear if this is the single overriding mode of action or if other processes are involved in α-hederin cytotoxicity. In the present study we use yeast haploinsufficiency profiling to assess α-hederin activity in a library of yeast strains knocked-down for virtually all essential gene products (Smith et al., 2010) and in doing so identify three genes with related functions that modulate α-hederin activity.

Yeast haploinsufficiency profiling is a technique that employs a library of yeast deletion strains to identify genes associated with the mode of action of cytotoxic compounds (Giaever, 2003; Hughes et al., 2004). The reason yeast is chosen as the test organism is because it is genetically tractable and a proven model for both human cells and eukaryotic microorganisms (Smith et al., 2010). Diploid *Saccharomyces cerevisiae* deleted for one copy of an essential gene will typically express 50% of the gene product in question and thus the yeast library represents a collection of yeast strains each expressing half the normal level of a different protein (Giaever et al., 1999). The basic premise behind the technique is that when such a yeast deletion library is grown in the presence of a sub-lethal concentration of a cytotoxic compound, the yeast strain that is deleted for the compound's drug target will be disproportionately sensitive to the compound and therefore exhibit a slower growth rate (Giaever et al., 1999). This phenomenon is termed drug-induced haploinsufficiency (Giaever et al., 1999). In practice, more than one yeast deletion strain may exhibit hypersensitivity, as proteins that act in pathways that influence the target protein will have a disproportionate effect when their levels are reduced.

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Yeast haploinsufficiency profiling has been used to confirm the mode of action of cytotoxic compounds where the mode of action is already known (Smith et al., 2010). It has also been used to reveal previously unknown modes of actions of compounds that were previously thought to be well characterized (Giaever, 2003). Whilst cytotoxic compounds are widely reported in the phytochemical literature there is a general paucity of information regarding their modes of action. This reflects the fact that until recently there were few effective methods with which to determine the cellular target of a cytotoxic compound, and in practice those that were identified owed much to serendipitous discovery (Lum et al., 2004). In the present study a plant extract led approach to haploinsufficiency screening is used to circumvent the need for large quantities of active compound. Once hypersensitive strains are identified with the plant extract, their hypersensitivity is confirmed using purified active principle.

Results and discussion

Activity-guided fractionation confirms α-hederin as the yeast inhibitory component of ivy

Although previous studies have identified α-hederin as a cytotoxic principle in *H. helix* (Moulin-Traffort et al., 1998) we wanted to confirm through activity-guided fractionation that it is responsible for the inhibitory effect of the leaf extract on *S. cerevisiae*. Fresh leaf material was extracted with solvents of differing hydrophobicity (water, methanol, ethanol, acetone, ether, ethyl acetate and hexane) and assayed with *S. cerevisiae* NCPF3178 (which carries no gene deletions) to establish the optimum extraction solvent. The ethanol extract had the greatest activity with an MIC of 0.25% (v/v) compared with 0.5% (v/v) for methanol and acetone; all other extracts displayed no activity. Assay of HPLC fractions confirmed that the activity of the leaf extract was attributable to a single HPLC peak. Subsequent activity-guided purification yielded a compound with NMR spectra consistent with α-hederin (Fig. 1).

Hypersensitivity to α-hederin is not observed with a neo1Δ/NEO1 deletion strain

The phytochemical literature on saponins suggests that their cytotoxic mode of action is attributable to their ability to interact with and damage lipid bi-layers such as the plasma membrane (Bomford, 1980; Coleman et al., 2010). To investigate if this is the case in yeast a growth curve was measured with the yeast strain *neo1Δ/NEO1* which expresses reduced levels of a putative aminophospholipid translocase that plays a role in maintaining lipid asymmetry in membranes (Hua and Graham, 2003). Hypersensitivity with *neo1Δ/NEO1* is frequently seen with membrane damaging agents (Hoepfner et al., 2014). As a control experiment we tested the known membrane damaging compound chlorpromazine, an anti-psychotic drug whose side effects have been established to be caused by a membrane damage mechanism (Suwalsky et al., 2008). As expected, the *neo1Δ/NEO1* strain exhibits hypersensitivity to chlorpromazine, revealed by a greater difference between *neo1Δ/NEO1* treated and untreated growth curves compared to those of the BY4743 control strain (Fig. 3f and g). However hypersensitivity was not observed between *neo1Δ/NEO1* and α-hederin (Fig. 3a and e). Thus if α-hederin cytotoxicity in yeast is associated with membrane damage it is either a different form of membrane damage to that induced by chlorpromazine or is not a sufficiently overriding component of the mode of action in yeast to produce hypersensitivity with the *neo1Δ/NEO1* deletion strain.

A haploinsufficiency screen identifies three genes with related functions

To carry out the haploinsuffiecicny screen of 1152 yeast deletion strains, a liquid medium method (Prescott et al., 2014) was used as this allows for plate reader measurements of cell density where the numerical readout from replicates can be averaged. Additionally the initial screen of yeast deletion strains was carried out using a sub-lethal dose of the ethanol ivy leaf extract which LC–MS analysis revealed to be highly enriched in α-Hederin (Fig. 2). This extract-led approach provided a shortlist of potentially hypersensitive yeast strains which were then examined in more detail by comparing growth curves with and without extract and α-hederin.

Three yeast strains *arp7Δ/ARP7*, *rpb7Δ/RPB7*, and *rsc58Δ/RSC58* were found to exhibit hypersensitivity. Growth curves for each strain with and without ivy leaf extract and α-hederin are shown in Fig. 3. In each case, hypersensitivity is revealed by a greater difference between the treated and untreated growth curves compared to that observed with the BY4743 strain (Fig. 3a). The yeast strain *arp7Δ/ARP7* which expresses reduced levels of a subunit of the chromatin remodeling protein SWI/SNF is the most sensitive (Fig. 3b). The second most sensitive strain *rpb7Δ/RPB7* (Fig. 3c) expresses reduced levels of subunit B16 of RNA polymerase II which in eukaryotes is the principal polymerase for nuclear transcription of DNA to mRNA (Cramer et al., 2000). The third most sensitive strain *rsc58Δ/RSC58* (Fig. 3d) expresses reduced levels of a component of another chromatin remodeler, the RSC complex. Both SWI/SNF and the RSC complex belong to the same family of ATP-dependent chromatin remodeling complexes (classified by homology of protein subunits) and the Arp7 protein is in fact

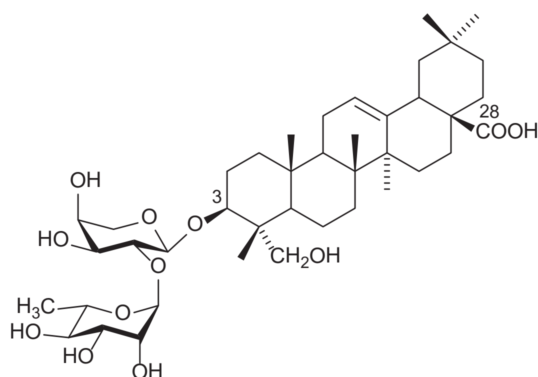

Fig. 1. α-Hederin, the cytotoxic principle of ivy leaves.

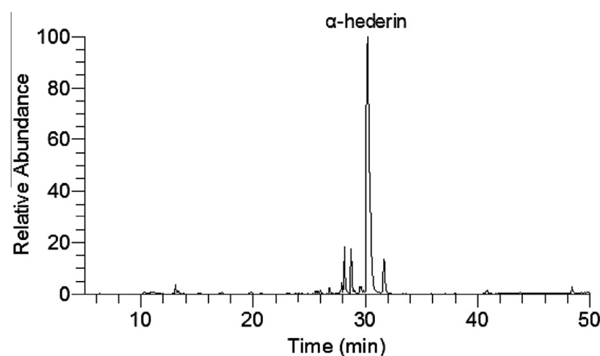

Fig. 2. Base ion chromatogram from an LC–MS analysis (positive mode) of the extract from *Hedera helix* used in the haploinsufficiency screen.

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