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Identification of Fungal H^+-ATPase Inhibitors by Microfractionation and HPLC-HRMS-SPE-NMR

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Background - Fungal Fight

A large number of fungal proteins have been proposed as potential targets for novel antifungal agents.1 However, current available antifungal agents are primarily targeting the intracellular membrane biosynthesis2 and thus need to enter the fungus to act. In our search for novel and more efficient antifungal compounds, we are focusing on the plasma membrane (PM) H^+-ATPase enzyme as target.

Plants are exposed to a wide array of phytopathogenic fungi in their natural habitat, and have been forced to develop antifungal metabolites in order to survive.3,4 Hence, as previously suggested by Mark and coworkers, it is reasonable to assume that some plants have the PM H^+-ATPase enzyme as target for the antifungal metabolites. However, plant extracts are very complex mixtures, and the traditional bioassay-guided fractionation used for identification of individual bioactive compounds is very time-consuming and suffers from inherently low resolution during the fractionation process. To circumvent this, we have developed a bioanalytical platform that combines high-resolution microplate cryogenic probe detection to allow characterization of metabolites with high resolution. We expected that this would allow for the discovery of novel antifungal agents, primarily targeting the intracellular membrane biosynthesis, for the treatment of fungal diseases.

Results - Crude extract screening

• From 48 plants to 20 plants

Extracts were tested in three different concentrations and those showing inhibition higher than 95% for all concentrations or a concentration-dependent activity profile were selected for semi-HR screening.

Results - HR-screening

• From 2 plants to 2 compounds

The two plants (Haemorhabdus foliolosus and Sauvagia erecta) showing distinct chromatographic peaks correlated with PM H^+-ATPase inhibition in semi-HR screening were subjected to high-resolution screening (assay resolution: 5.33 data points per min).

From H. foliolosus two peaks (peak 1 and 2) were correlated with > 80% inhibition of the PM H^+-ATPase. However, despite the noticeable inhibition in both crude extract screening and semi-HR resolution assay, S. erecta did not show any peak correlated to a defined inhibition profile in high-resolution assay. This can be attributed to the possible loss of aggregate activities of multiple constituents due to lower resolution complexity of the tested compounds in the HR screening compared to both semi-HR and crude extract screening.

Concluding remarks

• Thorough investigation of 48 plant extracts for fungal PM H^+-ATPase inhibitors led to identification of two active metabolites, i.e., Chebulagic acid (1) and Tellimagrandin II (2).
• Systematic combination of crude extract screening, high-resolution screening and HPLC-HRMS-SPE-NMR analysis allowed optimized workflow.
• High-resolution PM H^+-ATPase inhibition assay allows subsequent HPLC-SPE-NMR analysis to be targeted bioactive constituents only.
• Cryogenic probe detection (1.7 mm) allowed characterization of metabolites (with high PM H^+-ATPase inhibition) direct from analytical-scale HPLC of crude extract.

The setup - HR screening/HPLC-SPE-NMR

Empowered by the separation capabilities of analytical HPLC, high-resolution bioassay-guided HPLC-HRMS-NMR has been shown to provide a platform that can substitute the traditional bio-guided fractionation with considerable reduction of workload and improved efficiency.5 In this approach, chromatographically separated fractions will be subjected to a given bio-activity test to afford the so-called biochromatogram. The resulting biochromatogram will then be used as a tool for targeted analysis of active constituents. Such biochromatogram can be acquired through micro-fractionation followed by offline assay.

Results - Semi-HR-screening

• From 20 plants to 2 plants

The 20 samples selected for semi-HR-resolution screening (assay resolution: 2.68 data points per min) were assayed for their ability to inhibit the PM H^+-ATPase.

Results - HPLC-HRMS-SPE-NMR

Detailed analysis of HRMS and NMR data acquired via HPLC-HRMS-SPE-NMR led to identification of the two active metabolites as Chebulagic acid (1) and Tellimagrandin II (2). Moreover, minimum inhibitory concentrations of the two compounds were assessed against growth of two fungal strains (Candida albicans and Saccharomyces cerevisiae).

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References