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Publication date: 2014

Citation for published version (APA):
**Identification of Fungal H^+\text{-ATP}ase 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. However, current available antifungal agents are primarily targeting the intracellular membrane biosynthesis 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^+\text{-ATP}ase enzyme as a 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. Hence, as previously suggested by Monk and coworkers, it is reasonable to assume that some plants have the PM H^+\text{-ATP}ase enzyme as a 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 often time-consuming and suffers from inherent low resolution during the fractionation process. To circumvent this, we have developed a bioassay-guided platform that combines high-resolution mass spectrometry (HRMS) with HPLC-SPE-NMR. In this report, we work crude extract screening of 48 plant extracts for fungal PM H^+\text{-ATP}ase inhibitors followed by high-resolution bioassay and HPLC-SPE-NMR analysis for identification of individual bioactive constituents.

**Results - Crude extract screening**

**From 48 plants to 20 plants**

Extraction was 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-high-resolution screening.

**Results - HR-screening**

**From 2 plants to 2 compounds**

The two plants (Haplocoelum foliolosum and Sauvagesia -ATPase inhibitors) were subjected to high-resolution screening (assay resolution: 5.33 data points per min).

From H. foliolosum two peaks (peak 1 and 2) were correlated with >80% inhibition of the PM H^+\text{-ATP}ase. However, despite the noticeable inhibition in both crude extract screening and semi-high-resolution assay, the peak 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- and crude extract screening.

**Concluding remarks**

- Through investigation of 48 plant extracts for fungal PM H^+\text{-ATP}ase inhibitors, led to identification of two active metabolites, i.e., Chebulic 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^+\text{-ATP}ase inhibition assay allows subsequent HPLC-SPE-NMR analysis to be targeted bioactive constituents only.
- Chymoglycogen probe detection (1.7 mm) allowed characterization of metabolites (with high PM H^+\text{-ATP}ase inhibition) direct from analytical-scale HPLC of crude extract.

**Acknowledgment**

The project FF is granted by the Danish Research Council for Strategic research – Food and Health. HPLC equipment used for high-resolution bioassay profiles was obtained via a grant from The Carlsberg Foundation. The 600 MHz HPLC-HRMS-SPE-NMR system used in this work was acquired through a grant from “Apoteksfondet 1991”, The Carlsberg Foundation, and the Danish Agency for Science, Technology and Innovation via the National Research Infrastructure funds.

**References**