

A Unique Natural Product Platform Applied to the Discovery of Correctors of Mutant Cystic Fibrosis Transmembrane Conductance Regulator

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Introduction

Natural products

Natural products have been a successful source for drugs in the past, accounting for approximately 50% of the drugs introduced to the market in the last 20 years. Nevertheless their use for hit discovery campaigns dramatically decreased in the 1990's due to the advent of high throughput screening and combinatorial chemistry combined with issues experienced in natural product work, e.g. long timelines, lack of reproducibility, loss of activity upon fractionation and false positives due to the use of complex samples. The pressing need for novel compounds combined with technological advances in natural product chemistry nourished the renaissance of natural products in drug discovery. Here we will present BioFocus DPI's approach.

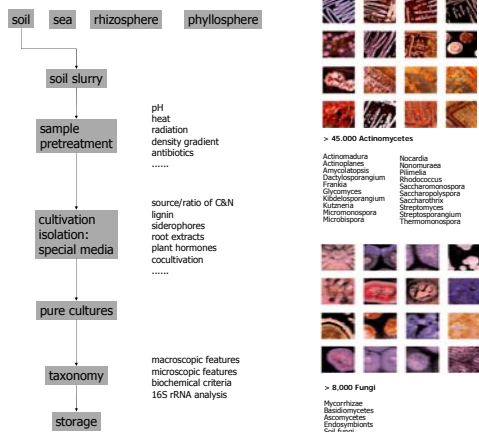
Cystic fibrosis

The hallmark feature of cystic fibrosis is an impaired chloride secretory response due to mutations in the cystic fibrosis transmembrane conductance regulator (CFTR). The most common mutation found in CFTR is a deletion of phenylalanine 508 (Δ F508) which leads to both a defect in trafficking of the mutant protein and compromises channel gating. At this time, there is no way to stop or reverse the course of CF. Restoring expression and/or function of mutated CFTR provides a means to treat CF.

Aim

BioFocus DPI – a Galapagos Company – in collaboration with the Cystic Fibrosis Foundation Therapeutics (CFFT) has established a drug discovery program that will enable the identification of small molecules derived from natural sources that could restore the function of defective CFTR. This collaboration combines two key strengths of BioFocus DPI, expertise in natural product drug discovery and phenotypic screening in cells derived from patients or in primary human cells.

Microbial strain collection



Materials and methods

Natural products library

The natural products library employed in this project consists of 140,000 pre-purified samples "subfractions" which were prepared as described below. Each subfraction contains on average 10 to 20 different compounds, yielding a library with a huge chemical diversity.

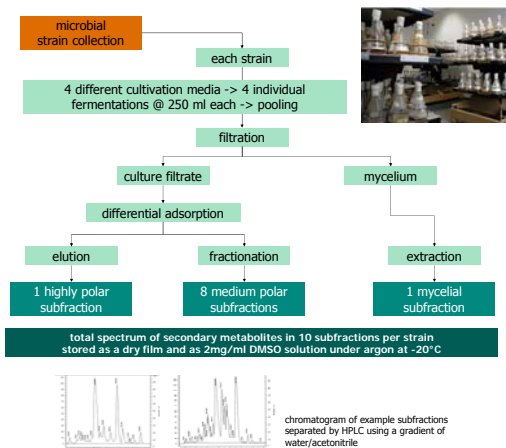
Cells

CFBE410- Δ F508 cells are human lung epithelial cells derived from a homozygous Δ F508-CFTR patient. They are SV40 transformed and express CFTR- Δ F508 by episomal vector. Cells are cultured on a matrix of collagen/fibronectin. They are grown in MEM supplemented with FBS, L-glutamine, penicillin/streptomycin and hygromycin B. CFBE410- were kindly supplied by Dr. Dieter Gruenert.

YFP halide assay

CFBE410- Δ F508 cells are co-transduced with YFP and Δ F508-CFTR adenoviral knock-in vectors. After 2 days, subfractions are added to the cells. The YFP halide assay is performed 24 hr after compounds addition. Cells are pre-incubated for 5 minutes with forskolin (10 μ M) and genistein (50 μ M) in gluconate buffer supplemented with 2mM glucose. Fluorescence at ex485/em530 nm is recorded for one sec, then iodide buffer is added and fluorescence recorded for another 6 seconds. The mean of YFP fluorescent signal before iodide injection is calculated (baseline). The average of the three last data points is determined and calculated relative to baseline (endpoint).

Subfraction generation



Results

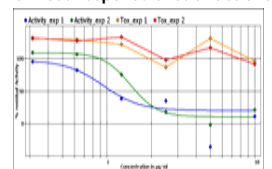
A. Activity distribution of HTS data



B. Selection of active subfractions

1,464 actives: residual activity (Ar) < 35%
 + 182 actives: Ar < 50% and neighbouring subfractions active
 - 403 actives: decreased baseline YFP signal indicating cytotoxicity
 - 150 actives: > 0.7 raw endpoint value indicating false positives
 1,053 actives out of 140,000 subfractions screened

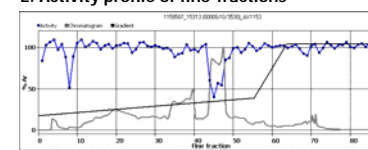
C. Dose response of subfractions



D. Fine fractionation

In order to identify active components 10-20 mg of each subfraction are further fractionated by preparative HPLC on a water/acetonitrile gradient resulting in 88 "fine-fractions".

E. Activity profile of fine-fractions



F. Dereplication

Active fine-fractions and neighbouring inactive fine-fractions are analyzed by LC-MS, UV-absorption and 1H-NMR. Correlation of certain properties (molecular mass, UV-absorption and/or 1H-NMR spectrum) with biological activity defines the active compound. A search in relevant in-house and public databases (Dictionary of Natural Products, Chapman&Hall/CRC and Antibase) allows classification as either known or new compound. Dereplication is currently ongoing.

G. Further steps in the process

Known compounds of interest can be produced in larger quantities by re-fermentation and isolation. For new compounds the producer strain will be re-fermented in a larger scale, usually 10 - 100 liter. The active compound will be isolated using the properties identified during dereplication, e.g. molecular mass and UV-absorption. The resulting compound, usually in mg quantities, will be analyzed by NMR spectroscopy and the structure will be elucidated.

Conclusions

In collaboration with CFFT, BioFocus DPI has performed a high-throughput screen on CFTR activity in CFBE410- Δ F508 cells based on the YFP-halide reporter system using our natural product subfraction collection. The screen resulted in the identification of 1,053 active subfractions, corresponding to 0.8% of the library. The active subfractions were retested in the YFP-halide reporter assay and confirmed hits analyzed in dose-response experiments.

50 subfractions have been identified for follow-up work including validation in secondary assays (western blot, cell surface biotinylation assay, Ussing chamber) and deconvolution starting with the fine-fractionation. The follow-up work is currently ongoing. A small subset of samples already underwent dereplication: interestingly, a subfraction with activity in the Ussing chamber performed with cystic fibrosis human bronchial epithelial cells contains a compound implicated in protein trafficking (data not shown).

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Scientific committee:

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Contribution of materials:

We would like to thank Dr. Dieter Gruenert for CFBE410-cells.

Conflict of interest statement:

The authors work for BioFocus DPI, a Galapagos company.