

A unique natural product platform applied to the discovery of novel compounds with activity against the malaria parasite, *Plasmodium falciparum*

Michael Kemmler¹, Sandra Duffy², Vicky M. Avery², Robert Witzig¹, Kim Beyer¹, Ian Bathurst³ and Peter Eckard¹

1. BioFocus DPI AG, Gewerbestrasse 16, CH-4123 Allschwil, Switzerland

2. Eskitis Institute for Cell and Molecular Therapies, Eskitis Building 75, Griffith University, Brisbane Innovation Park, Don Young Road, Nathan QLD 4111, Australia

3. Medicines for Malaria Venture, ICC, CP 1826, Route de Pre-Bois 20, CH-1215 Geneva, Switzerland

E-mail: michael.kemmler@glpg.com, www.biofocusdpi.com

A. Introduction

Natural products

Natural products (NP) 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 (HTS) and combinatorial chemistry combined with issues experienced in NP work, such as 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 has nourished the recent renaissance of NP in drug discovery. Here we will present BioFocus DPI's approach.

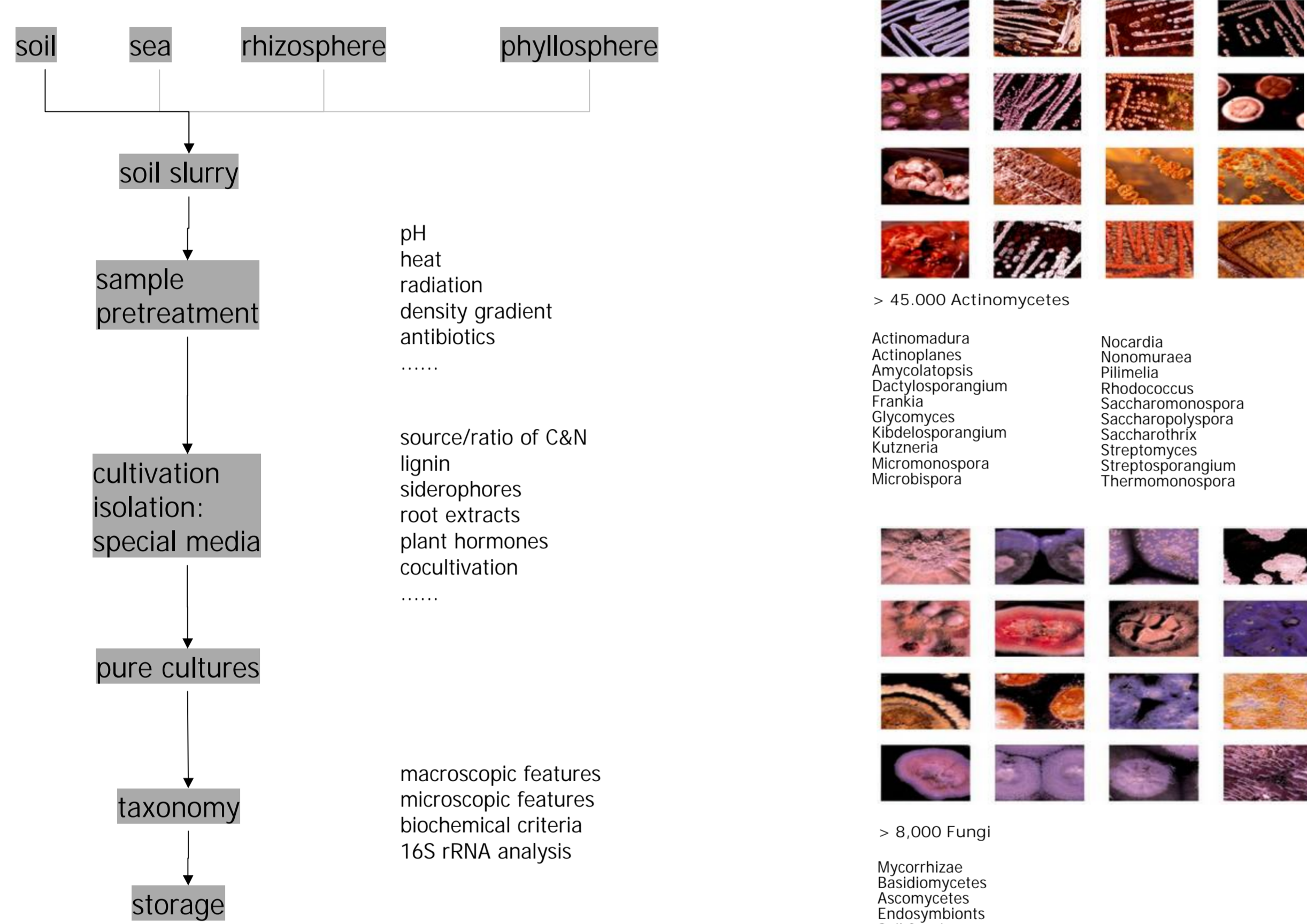
Malaria

The four malaria species that produce human disease are *P. vivax*, *P. falciparum*, *P. malariae* and *P. ovale*. *Plasmodium falciparum*, the most lethal strain, is the most prevalent species throughout the tropics and subtropics. Malaria is usually transmitted when a person is bitten by an infected female Anopheles mosquito. Clinical symptoms of the disease include fever and flu-like illness, such as chills, headache, muscle aches, and tiredness. These symptoms may be accompanied by nausea, vomiting, and diarrhea. Malaria can also cause anemia and jaundice (yellow coloring of the skin and eyes) due to the loss of red blood cells (RBC). Infection with one type of malaria, *Plasmodium falciparum*, if not promptly treated, may cause kidney failure, seizures, mental confusion, coma, and death. Malaria is a major public health problem in more than 90 countries, inhabited by more than 2.4 billion people - 40% of the world's population.

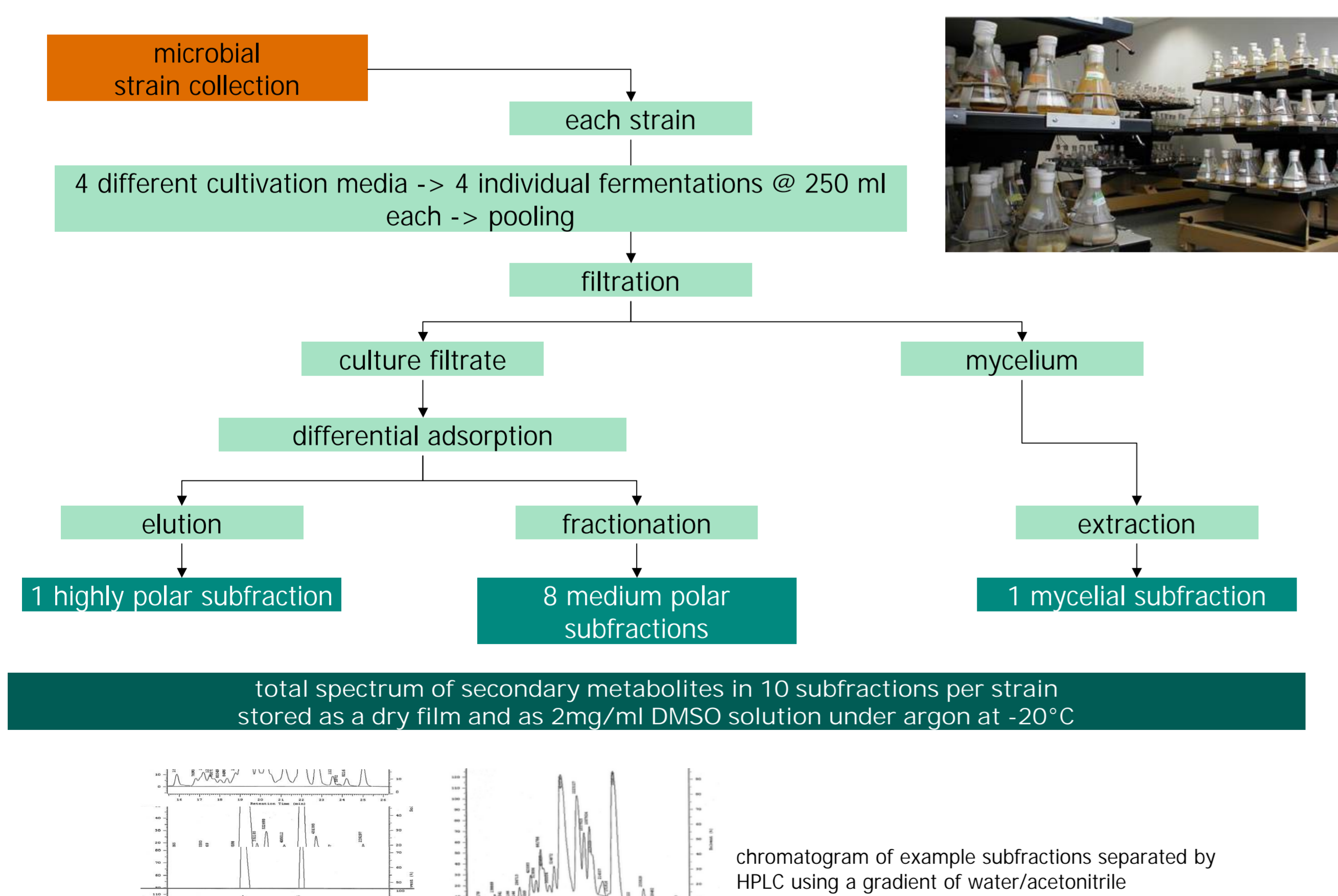
Aim

Medicines for Malaria Venture (MMV) in collaboration with BioFocus DPI and the Eskitis Institute for Cell and Molecular Therapies (Eskitis) has established a drug discovery program that will enable the identification of small molecules derived from natural sources to become new therapies fighting Malaria. This collaboration is based on key strengths of BioFocus DPI and Eskitis, expertise in NP drug discovery and anti-malarial HTS.

B. Microbial strain collection



C. Subfraction generation



D. Materials and methods

All HTS and biological testing was performed at Eskitis. All NP studies including provision of the subfractions was carried out by BioFocus DPI.

Natural products library

The NP library employed in this project consisted of 140,172 pre-purified samples (subfractions) which were prepared as described below. Each subfraction contains on average 10 to 20 different compounds, yielding a HTS compatible library with a huge chemical diversity.

Test organisms

The strains 3D7 (chloroquine sensitive strain of *Plasmodium falciparum*) and Dd2 (chloroquine resistant strain of *Plasmodium falciparum*) were used as the test organisms. For determination of cytotoxicity HEK293 cells (non-cancerous human cell line) were used.

HTS imaging assay

The assay utilizes the DNA-intercalating dye DAPI (4',6-diamidino-2-phenylindole) to monitor changes in parasite number in infected RBC. Images of the stained parasite within the well are analysed utilizing the Accapella spot detection programme associated with Opera™. The HTS imaging assay was developed in 384-well format for use on the Opera™ confocal imaging system with Twister arm, and is capable of screening over 70,000 assay wells (200 plates) per day. The basic assay can also be used with Total Intensity data output from a standard microplate reader such as a VICTOR II. The assay typically yields Z'-factor values of 0.5-0.6, with signal-to noise ratios of 10:1.

Control images for Artemisinin at varying concentrations are illustrated in Fig. 1. Conversion of the image format to numerical output based on the number of spots which have a defined fluorescence is shown in Table 1.

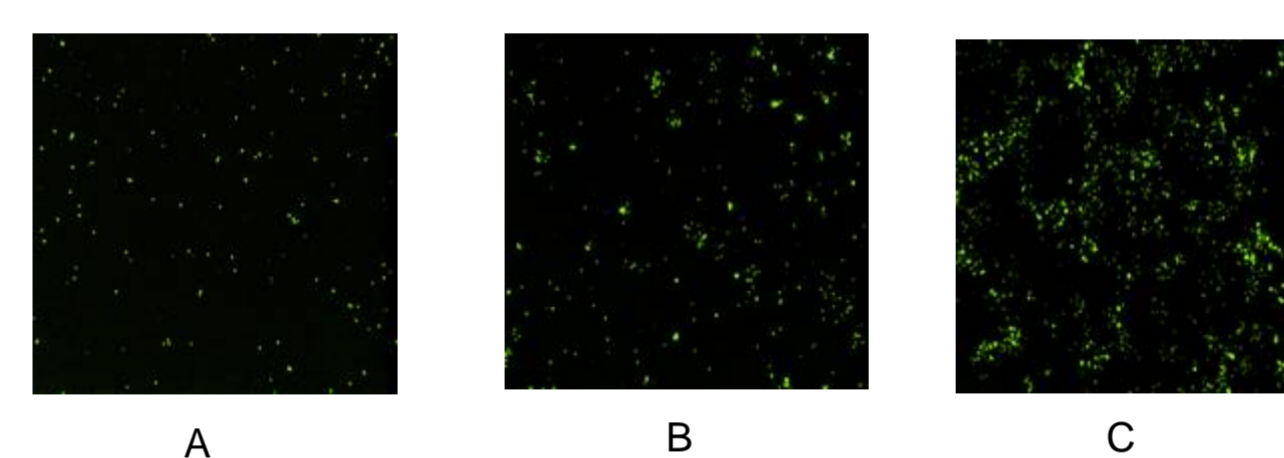


Image	Condition	Spots	% Inhibition
A	500nM Artemisinin	159	100
B	20nM Artemisinin	332	78
C	0.5nM Artemisinin	954	0

Figure 1. Images obtained from *Plasmodium falciparum* treated with A. 500nM, B. 20nM and C. 0.5nM Artemisinin, respectively.

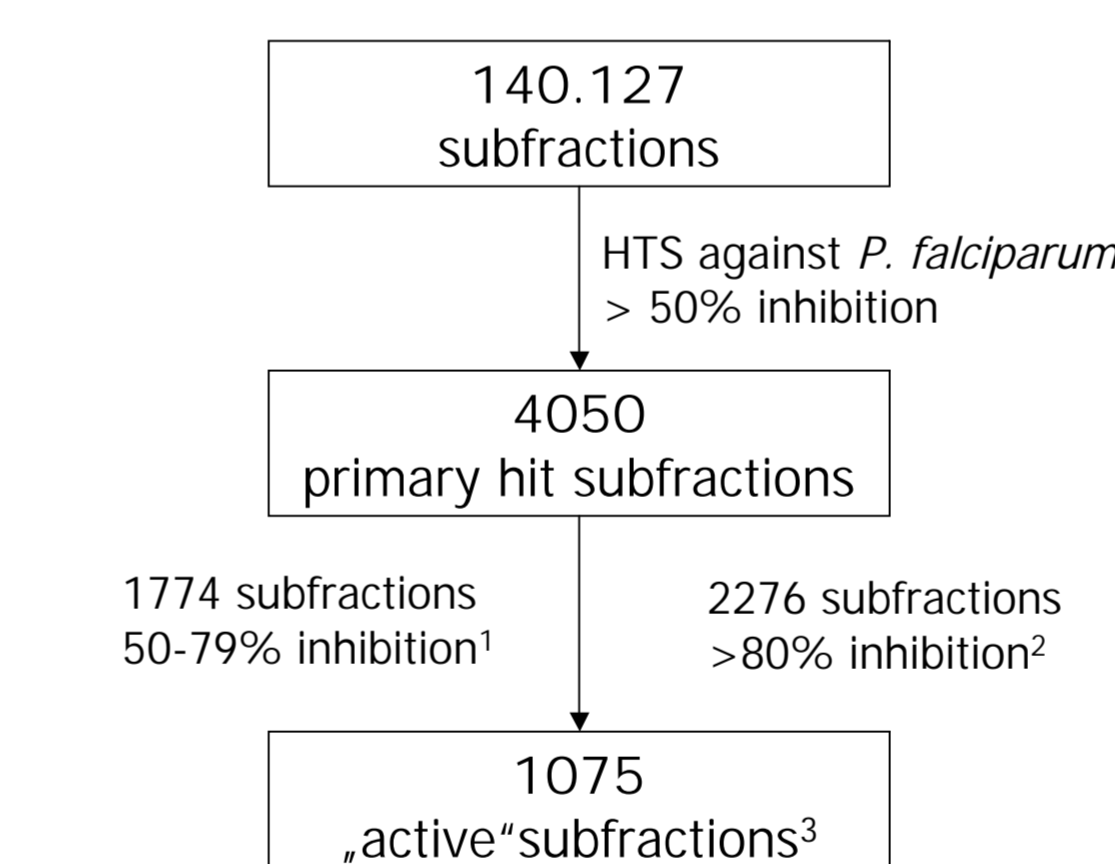
Table 1. Conversion of the data from image format (Fig.1) to a numerical output.

E. Results

HTS data

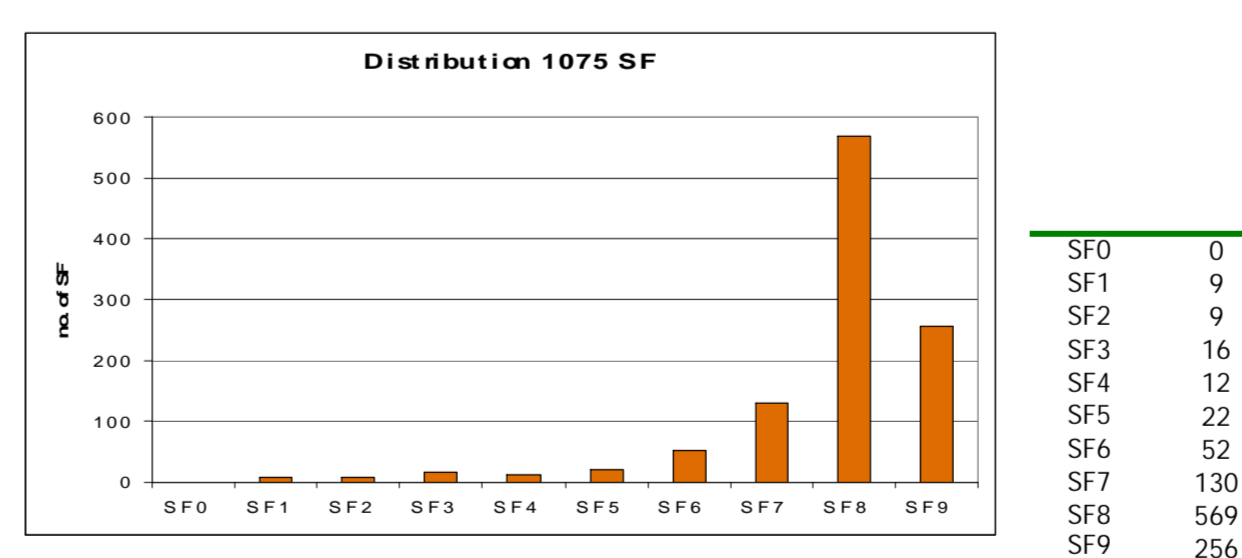
In the primary HTS the 140,172 NP subfractions were screened at a concentration of 0.7µg/ml against *P. falciparum*. The achieved quality was good as the HTS assay has consistently demonstrated Z' values of 0.5-0.8 for this screening campaign.

Selection of active subfractions



¹ retested at 0.7 and 0.35µg/ml against 3D7 and 0.7µg/ml against HEK293
² retested at five concentrations (0.7µg/ml and less) against *P. falciparum* 3D7 and Dd2 and at 0.7µg/ml against HEK293
³ criteria: >50% activity at ≤0.35µg/ml against 3D7 and Dd2 (if tested) with selectivity of >10 over HEK293. Comparable activity against the two strains, 3D7 and Dd2 (if tested).

The distribution of the "active" subfractions was analysed (Fig. 2).



total spectrum of secondary metabolites in 10 subfractions per strain stored as a dry film and as a 2mg/ml DMSO solution under argon at -20°C

Figure 2. Distribution of the 1,075 selected active SF's.

Fine fractionation

When the microbial source organism and the fraction order together with the observed activity and selectivity of the subfractions was analysed, the number of fractions for follow-up could be reduced to 149 without significantly sacrificing diversity. In order to identify active components 10-20 mg of each subfraction were further fractionated by preparative HPLC on a water/acetonitrile gradient resulting in 88 "fine-fractions", per subfraction.

Activity profile of fine-fractions

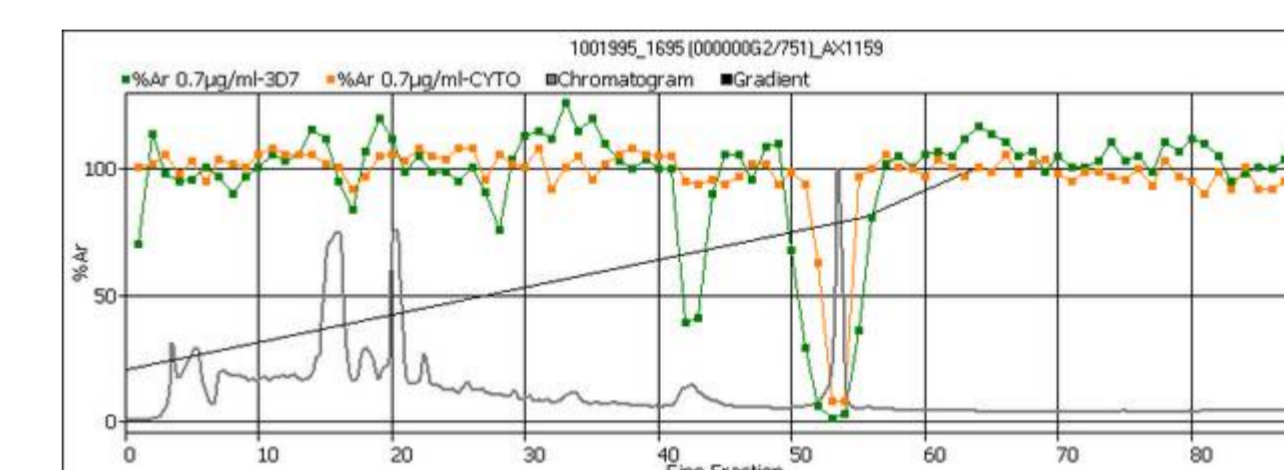


Figure 3. Example of fine fraction testing result (0.7µg/ml, Ar in %, 3D7 (green), HEK293 (orange)). The UV-chromatogram of the semipreparative fractionation is shown in light grey and the water/acetonitrile gradient in black. Compound peaks showing significant activity and selectivity, e.g. fractions 42-43, are subjected to dereplication.

Dereplication

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

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 and is usually obtained in mg quantities which allows for structure elucidation and further biological testing.

F. Conclusions

MMV has in collaboration with Eskitis and BioFocus DPI performed a high-throughput screen against the malaria parasite *Plasmodium falciparum*. At this point in the joint project BioFocus DPI's proprietary subfraction collection has been successfully screened against the 3D7 (CQ sensitive) and Dd2 (CQ resistant) strain of *P. falciparum*.

The screen resulted in the identification of 1,075 active subfractions, corresponding to 0.8% of the library.

149 subfractions with a greater than 10 fold selectivity for *P. falciparum* over HEK 293 have been identified for follow-up work which is currently ongoing.

G. Acknowledgements

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Conflict of interest statement:
Some of the authors work for BioFocus DPI