Combioscreen – Screening net-to-nature libraries of diterpenoids generated by synthetic biology for novel anti-tumor activities

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Metabolite aus der Natur und ihre Abkömmlinge sind ein bedeutender Bestandteil der Pharmazie. Ihr volles Potential kann aber nicht ausgeschöpft werden, da sie in niedrigen Konzentrationen vorkommen, der Zugang zu den produzierenden Organismen schwer ist oder ihre Synthese komplex ist. Allerdings wurden bedeutende Fortschritte im Bereich der Produktion natürlicher Stoffe in Mikroorganismen (z.B. Hefe) gemacht. Unter Verwendung verfügbarer genetischer Informationen über pflanzliche Diterpenbiosynthesen und eines experimentellen Ansatzes basierend auf der Golden-Gate-Klonierung wird eine Genbibliothek von Kandidatenenzymen generiert, die in solchen Stoffwechselwegen beteiligt sind. Ein automatisiertes Massenspektrometrie-Screening soll die Genkombinationen ermitteln, die neue Produkte erzeugen, deren Aktivität dann gegen Krebszelllinien getestet wird. Anschließend wird die chemische Struktur der aktiven Stoffe bestimmt und ihre Funktionsweise in Tumorzellen intensiv untersucht.

I. Topic, open questions and purpose

Next generation sequencing technologies now allow access to the transcriptome and genome of virtually any species. This means huge resources of genes encoding enzymes of secondary metabolite pathways are now available and can be exploited for the production of compounds that are not available or that have not been detected before. Diterpenoids, which occur in plants, bacteria and fungi, have been shown to possess diverse bioactivities emphasizing their potential role as pharmaceuticals, e.g. as anti-cancer drugs (Johnson, 2011). However, their potential cannot be fully exploited because they occur in low concentrations, the synthesis is complex or access to the producing organisms is difficult. We chose yeast as a platform for engineering diterpenoid biosynthesis aiming at expanding the range of this metabolite class. Using all available genetic information about plant, bacterial and fungal diterpenoid biosynthesis and a synthetic biology approach based on Golden Gate cloning, we generate libraries of genes encoding candidate enzymes involved in those pathways. A high-throughput mass spectrometry screening detects which gene

combinations generate new products. After fractionation, the novel metabolites will be tested for activity against cancer cell lines and the structure of compounds with activity will be determined and their mode of action on tumor cells extensively characterized.

II. Theory and methods

In recent years, enormous progress has been made in metabolic engineering of natural products in microorganisms such as *Escherichia coli* or yeast (*Saccharomyces cerevisiae*). For the expression of natural product pathways requiring cytochrome P450 oxygenases (CYPs), yeast is a suitable host due the presence of intracellular membrane compartments. In previous work, we engineered abietane diterpenoids in yeast and identified novel compounds by combining CYPs from related Lamiaceae species (Scheler et al., 2016; Bathe et al., 2019). Therefore, we chose yeast as a platform for engineering diterpenoid biosynthesis in the current project.

There are two criteria which, combined, will lead to the breakthrough in diterpenoid product engineering. The first is the modular character of the diterpenoid biosynthesis which involves first diterpene synthases (diTPS) providing the diterpene skeleton, and second decorating CYPs. The second decisive factor is that CYPs can accept slightly differing substrates. This led to the hypothesis that co-expressed diterpenoid enzymes, which usually do not meet in nature, are capable of producing “new-to-nature” metabolites. This approach is called combinatorial biosynthesis.

Thus far, 94 diterpene synthases and 48 CYPs have been reported to be involved in diterpenoids biosynthesis in plants, bacteria and fungi. Using a synthetic biology approach based on Golden Gate cloning, we generate libraries of genes encoding candidate enzymes involved in those pathways. The libraries are being transformed into yeast, in the process of which every single yeast cell takes up a unique gene combination. Thanks to a high-throughput protocol that includes cultivation of single yeast cultures and mass spectrometry metabolite screening in a 96-well format, gene combinations that produce novel diterpenoids will be rapidly identified among thousands of tested singe yeast colonies.

The candidates' anti-cancer tests are performed immediately after the diterpene mixtures have been isolated and purified. First, the cytotoxic potential of the mixtures and purified compounds is determined and subsequently the apoptosis properties are investigated and a cell cycle analysis of the most active candidates is carried out. In addition, the selectivity index is measured.

III. Results and perspectives

<u>Gene collection and synthesis</u>

Extensive literature research led to a collection of genes which are related to diterpenoid biosynthesis in plants, bacteria and fungi. These includes 15 class II diTPS, 54 class I diTPS, 25 bifunctional diTPS and 48 CYPs. The diTPS have been reported to produce in total 148

polycyclic (bi-, tri- and tetra-cyclic) and 13 macro-cyclic diterpene skeletons which can be modified by CYPs. The collected sequences were processed to be compatible with Golden Gate cloning which means removal of internal cloning sites (BpiI and BsaI restriction sites) but addition of 5' and 3' Golden Gate cloning sites. In addition, all sequences were codon-optimized for expression in yeast to allow optimal protein expression. After an extensive public announcement, GeneArt by Thermo Fisher Scientific were assigned to synthesize 135 genes with a total volume of around 150,000 base pairs. As all gene synthesis products have arrived, they are now available for the generation of diterpenoid gene libraries.

Method development – yeast cultivation

Using 94 diTPS and 48 CYPs, we expect thousands of possible gene combinations. To cover as many as possible, a high- throughput screening of single yeast colonies is essential. We, therefore, developed a protocol for yeast cultivation in a 96-well format and optimized growth conditions to ensure sufficient diterpenoid production. For that, established yeast strains producing diterpene skeletons or oxidized diterpenoids were used. First, we tested several 96- and 24-well plates that allowed yeast cultivation without dropping off of cells. A 96-well plate with angular cavities and a pyramidal bottom turned out to be sufficient. Second, we tested diverse materials of adhesive films and plate lids to secure both, sterile cultivation conditions and sufficient oxygen supply for optimal growth. A breathable adhesive film made of cellulose were chosen for further experiments. Next, different cultivation volumes in a range of 200µl to 400 µl per well were tested for optimal diterpene production. 230µl were proven to produce the highest amount of diterpene per ml cultivation volume. Finally, processing of the yeast cultures was simplified to speed up the handling protocol. Instead of media exchange to induce diterpene production, an induction medium was established that can be easily added to the cultivation medium.
The established cultivation protocol will soon be complemented by using a picking and pipetting robot which will allow an automated process of yeast colony picking and preparation of growth plates.

Method development – high-throughput mass spectrometric screening

In order to detect diterpenoids in a high-throughput process, an automated mass spectrometry screening was established. We use Laser Diode Thermal Desorption (LDTD) coupled to an AB Sciex 5500 QTRAP mass spectrometer which allows screening of 96 samples in 10 min. Two protocols were established and tested on yeast strains which produce known diterpenoids. In the first established protocol, a concentrated yeast cell suspension is transferred from the 96-well cultivation plate to a LDTD measurement plate and analyzed after drying of the samples. This is an easy and fast procedure but can miss metabolites that are produced in low concentrations. Therefore, we established a second protocol in which cell cultures are extracted with *n*-hexane in the 96-well cultivation plate. The extract is then spotted to a LDTD measurement plate and analyzed after drying of the samples. This is a more complex procedure especially with regard to the fact that we aim to

screen as many yeast colonies as possible, but we could show that the signal intensities are way higher compared to the first mentioned protocol whereby diterpenoids in lower concentration will not be missed. Therefore, we will proceed with this protocol in the further library screening.

Proof of concept – using combinatorial biosynthesis to produce new-to-nature diterpenoids that derive from (+) copalyl diphosphate

This project is based on the hypothesis that co-expression of diterpenoid genes from distantly related organism can lead to the production of novel metabolites. To prove this assumption, we chose a small set of 11 diTPS and 4 CYPs which have been reported to produce labdane-related diterpenoids in the normal configuration. Using the Golden Gate cloning system, candidate gene were assembled under consideration of the modular nature of the diterpene biosynthesis. The first gene module consisted of a class II and a class I diTPS or a single bifunctional diTPS which produced the diterpene skeleton of normal configuration in yeast. The second module contained a CYP reductase necessary for proper CYP activity and one of the four CYP candidates. Thereby, 55 gene combinations (including control vectors which contained the diTPS but no CYPs) were assembled in yeast. Not all gene combinations produced novel metabolites. However, ca. 60 oxidized diterpenes could be detected in LC-MS measurements of yeast extracts. As LC-MS is a suitable method to detect higher oxidized diterpenoids, we plan to perform GC-MS measurements to discover single or double oxidized metabolites. Therefore, we expect to increase the number of detected novel diterpenes. Currently, product isolation for structure elucidation by NMR is in progress. Furthermore, the most abundant diterpenoids will be tested for activity against cancer cell lines and their mode of action on tumor cells characterized.

Literature

Scheler et al., Elucidation of the biosynthesis of carnosic acid and its reconstitution in yeast, Nat Commun. 2016

Bathe et al., CYP76 Oxidation Network of Abietane Diterpenes in Lamiaceae Reconstituted in Yeast, J Agric Food Chem. 2019

Johnson, Carnosol: a promising anti-cancer and anti-inflammatory agent, Cancer Lett, 2011