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Establishment of a high content assay for the identification and characterisation of bioactivities in crude bacterial extracts that interfere with the eukaryotic cell cycle

Nickels A. Jensen[a], Klaus Gerth[b], Tim Grotjohann[a], Dieter Kapp[a], Matthias Keck[a], Karsten Niehaus[a,*]

[a] Department of Proteome and Metabolome Research, Faculty of Biology, Bielefeld University, PO Box 100131, 33501 Bielefeld, Germany
[b] Helmholtz Centre for Infection Research (formerly GBF- German Research Centre for Biotechnology), Inhoffenstraße 7, 38124 Braunschweig, Germany

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ABSTRACT

High content microscopy as a screening tool to identify bioactive agents has provided researchers with the ability to characterise biological activities at the level of single cells. Here, we describe the development and the application of a high content screening assay for the identification and characterisation of cytostatic bioactivities from Myxobacteria extracts.

In an automated microscopy assay Sf9 insect cells were visualised utilising the stains bisbenzimide Hoechst 33342, calcein AM, and propidium iodide. Imaging data were processed by the ScanR Analysis-software to determine the ploidy and vitality of each cell and to quantify cell populations. More than 98% of the Sf9 cells were viable and the culture consisted of diploid (~30%), tetraploid (~60%), polyploidic (<10%) and apoptotic (<5%) cells. Treatment with the reference substances blasticidin, colchicine, paclitaxel, and cytochalasin D induced changes in ploidy and vitality, which were characteristic for the respective bioactive substance. Furthermore, crude extracts from the chivosazole producing Myxobacterium *Sorangium cellulosum* So ce56 induced an increase of polyploid cells and a decrease in total cell count, while a mutant producing nearly no chivosazole triggered none of these effects. Purified chivosazole induced the same effects as the wild type extract. Similar effects have been observed for the reference compound cytochalasin D. On the basis of this assay, crude extracts of ten different Myxobacteria cultures were screened. Three extracts exhibited strong cytotoxic activities, further five extracts induced weak changes in the ploidy distribution, and two extracts showed no detectable effect within the assay. Therefore, this robust assay provides the ability to discover and characterise cytotoxic and cytostatic bioactivities in crude bacterial extracts.

1. Introduction

The detection and the characterisation of new biologically active substances like antibiotics, cytostatics, or inhibitors is a major goal of modern biotechnology and drug discovery. Increasing occurrence of multiresistant pathogens and cancer cells over the last decades represents a major driving force for the search for new drugs. A rich source of such potential drugs is the enormous number of naturally produced secondary metabolites (Clark, 1996; Demain, 1999; Gordoliza, 2007). Nevertheless, limited availability of rare source material and chemically complex structures may restrict the wider use of natural compounds. The anti-cancer agent taxol represents a good example for a powerful drug that's long development period and wider use was limited by its availability from natural sources (Miller et al., 2008). Microorganisms that produce bioactive metabolites may solve both problems. Since microorganisms can often be cultivated in bioreactors, they are of particular importance for biotechnological application as producers of novel secondary metabolites with biological effects.

In the last decades efforts were made to find new, efficient producers of novel secondary metabolites with diverse bioactivities. Besides the long known and well-defined groups among these bacteria, like Actinomycetales or Bacilli, another group of bacteria, the Myxobacteria, were identified as promising candidates for the production of bioactive secondary metabolites (Reichenbach, 2001). Myxobacteria are motile, Gram-negative bacteria, that live in places rich of organic matter and microbial life, e.g., in soil or on rotting material. Furthermore, these bacteria are known to live in cellular communities or swarms with complex intercellular communication systems. These swarms have the ability to aggregate under starvation conditions and to develop fruit bodies containing durable myxospores (Reichenbach, 1999). Due to this mode of life and their

* Corresponding author. Tel.: +49 521 1065631; fax: +49 521 1065626.
E-mail address: karsten.niehaus@genetik.uni-bielefeld.de (K. Niehaus).

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natural habitat they produce many antibiotics and cytotoxic substances to defend their ecological niche against competitors, like fungi or other bacteria.

The genera of Myxocccus and Sorangium are of particular interest, because they produce high numbers of novel secondary metabolites (Gerth et al., 2003). The recently sequenced Myxobacterium *Sorangium cellulosum* So ce56, e.g., synthesizes at least three interesting secondary metabolites: the cytotoxic chivosazoles, the antibacterial etnangiens and the siderophore myxochelin (Schneiker et al., 2007). Further interesting bioactive substances are synthesized by other Myxobacteria, like the tubulin destabiliser disorazol (Kopp et al., 2005), the polymerase inhibitor etnangien (Irschik et al., 2007), or the microtubule effector epothilon (Gerth et al., 1996). Epothilon B and its analogues are currently undergoing various clinical phases for treatment of breast cancer. As a result of the high number of available Myxobacteria strains, different fermentation conditions (Müller and Gerth, 2006), the number of secondary metabolites and the great amount of possible bioactivities, efficient and fast multiparametric assays for identification of bioactivities of secondary metabolites of Myxobacteria are needed.

High throughput screening (HTS) and high content screenings (HCS) are possible approaches to analyse this huge number of new compounds. High throughput approaches are applicable to a high number of samples, but only a few, or even one parameter can be measured simultaneously. Although this allows fast screening of many extracts, it only permits the analysis of a small amount of possible effects. To analyse a higher number of different possible effects at once, high content assays are more appropriate. The high content screening approach is a cell culture based method with high implication for the drug discovery industry. It combines methods from cytology, microscopy, and automated image analysis to gain specific and sensitive information about properties of cells in a semi-high throughput fashion (Lang et al., 2006; Taylor, 2007). High content assays are capable of measuring many different parameters in a large number of cells treated with thousands of chemical compounds (Korn and Krausz, 2007). Phenotypic changes in response to various stimuli are visualised with specific cellular markers, as for example GFP fusion proteins, fluorescently labelled antibodies, or specific dyes, utilising sophisticated automated fluorescence microscopy. Automated image analysis enables a quantification based on defined marker properties. High content microscopy was successfully used previously to identify akt pathway inhibitors (Lundholt et al., 2005), neurite outgrowth factors (Laketa et al., 2007), proteosome inhibitors (Rickardson et al., 2007) and a molecule that induces mitotic arrest (Wilson et al., 2006).

Such high content assays could be very useful to identify bioactivities in bacterial extracts and to gain information on the possible effects on eukaryotic cells. Of particular interest as potential antitumoral drugs are substances like epothilon, which disturb the cell cycle and thereby have an influence on the ploidy state of cells. Therefore, the aim of this study was to establish a high content assay for the qualitative and quantitative analysis of cytotoxic and cytostatic effects of extracts from Myxobacteria cultures on eukaryotic cells by the visualisation of their ploidy and vitality.

2. Materials and methods

2.1. Chemicals

The stains calcein AM, propidium iodide and bisbenzimide Hoechst 33342 and the drugs paclitaxel, colchicine, cytochalasin D and blasticidin were purchased from Sigma. The substances were dissolved as 1000× stock solutions in water, DMSO or methanol, the inserted volume never exceeded 1% of the culture volume. Cells were incubated with 1 μM paclitaxel, 100 μg per ml blasticidin, 100 μM colchicine and 4 μM cytochalasin D. Chivosazole was purified as described by Jansen et al. (1997).

2.2. Bacterial extracts

We analysed extracts from the known Myxobacteria strains *S. cellulosum* So ce56 (Pradella et al., 2002) and a streptomycin resistant mutant strain *S. cellulosum* So ce56 S-1, which produces nearly no chivosazole (K. Gerth, HZI Braunschweig). Furthermore, extracts from ten unnamed Myxobacteria strains (from the stock culture collection HZI, Braunschweig) were screened. Cultivation of Myxobacteria and extraction of secondary metabolites from culture supernatants was carried out as described previously (Gerth et al., 1996).

2.3. Cell culture and treatment of cells

Sf9 cultures were purchased from Novagen and maintained in Bac Vector Insect Cell Medium (Novagen). They were grown to a density of about 2×10^6 cells per ml in suspension shaker flasks and passaged every 3 days. For the assays, 10,000 cells were seeded in 100 μl BacVector insect cell medium containing 10% FBS per well of a 96-well glass bottom plate (Greiner). After 2 h incubation at 25 °C for adherence of the cells, the test substances were added to the cells. Then the cells were incubated at 25 °C. After 48 h the cells were stained with 1 μM calcein AM, 20 μM bisbenzimide Hoechst 33342 and 5 μM propidium iodide, incubated at 25 °C for 30 min and then directly microscopied without washing steps.

2.4. Automated image acquisition

The Olympus ScanR screening platform was used for automated image acquisition. The ScanR platform is a modular epifluorescence microscope based imaging platform designed for fully automated image acquisition. The highly constant illumination system MT 20 with a stabilised xenon burner facilitates homogenous, quantifiable imaging.

Images were taken with a 20× objective (UPlanApo, n.a. 0.7) and standard filter sets for calcein AM (U-41025, ex. 450–490 nm, em. 500–530 nm), bisbenzimide Hoechst 33342 (U-MNUA2, ex. 360–370 nm, em. 410–470 nm) and propidium iodide (U-MWIG2, ex. 520–550 nm, em. >580 nm).

A gradient based software auto focus with 20 coarse steps of 3 μm width and 11 fine steps of 0.3 μm width was used to determine the focus plane in the bisbenzimide Hoechst 33342 channel. Images were taken in this focus plane in each channel without an offset. For each treatment at least 4 wells, and in each well 16 positions were analysed. With these specifications it took up to 3 h to screen a 96-well plate.

2.5. Image analysis and assay parameters

The images were analysed by the ScanR Analysis software. ScanR Analysis is flexible software for development of complex assays for image analysis and advanced data evaluation. The user can define objects and several sub objects in different channels and measure various parameters of these objects. The parameters of detected objects are visualised with a cytometry orientated multiparameter data schemes. With gating and classification populations of cells can be easily distinguished and quantified. For every gate or region of interest a reference gallery of objects of the selected area can be selected and, in turn, this gallery can also be controlled for accurate gate settings or incorrect object recognitions.