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The usefulness of a biosensor controlled perfusion cell culture for the investigation of new drugs demonstrated with the marine fungus *Kirschsteiniothelia maritima*

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The investigation of new biological active substances from limited sources for example from marine organisms needs sensitive and evident test systems. Such a system is the glucose biosensor controlled perfusion cell culture. The glucose consumption of cells is a very sensitive parameter which allows the continuous measurement of external effects of test substances on the cells under *in vivo*-like conditions. Cytotoxic concentrations of active substances as well as a virus infection lowers the glucose consumption of continuously perfused cells. This effect can be monitored using a glucose biosensor. The influence of drugs and the virus infection can be observed simultaneously in the same system continuously over several days. With two substances isolated from the marine fungus *Kirschsteiniothelia maritima* investigations for cytotoxic and for antiviral effects are demonstrated.

1. Introduction

Marine micro-organisms are a prolific resource of new drugs with therapeutic relevance for human and veterinary medicine. Because of the specific circumstances of their life (aquatic environment, pH, pressure, light, many competitive and symbiontic organisms) they produce secondary metabolites with properties and biological activities different from those of terrestric organisms. In their natural environment they are active in high dilution and over long distances. But there are many difficulties in cultivation of marine micro-organisms and in the isolation of sufficient quantities of active substances. Therefore the rational investigation of these resources needs appropriate methods which are sensible, specific and economic. Biosensor based test systems perform these conditions in an ideal mode.

Our search for new biological active compounds from marine fungi led to the investigation of the metabolites from *Kirschsteiniothelia maritima* (Linder) D. Hawksw., a marine ascomycete living on decaying wood. Organic extracts of the culture filtrate showed antimicrobial activity. By chemical characterisation of these extracts the two related compounds ascochitin and ascochital could be isolated. Both substances show antibacterial activity against several Gram positive and Gram negative bacteria in usual test systems like agar diffusion assay and micro dilution assay [1, 2]. By proteome-based methods an influence of ascochitin and ascochital on the protein biosynthesis of *B. subtilis* could be shown [3].

A perfusion cell culture is characterised by a continuous addition of fresh nutrient medium to cultivated cells and the withdrawal of an equal volume of used medium containing products of cell metabolism [4, 5]. Carbohydrates are the major energy source for cultured cells and the most frequently used sugar in nutrient media is glucose [6]. By combining a perfusion cell culture with an enzyme glucose biosensor the glucose consumption rate of the cell culture can be monitored continuously. The resulting biosensor controlled perfusion cell culture is a complex biomonitoring system rendering it possible to check continuously the metabolic state of a perfusion cell culture noninvasively over several days. With this test system the cell growth and viability can be monitored and external effects on the cell metabolism can be tested at early stages under *in vivo*-like conditions [7].

The present paper reports the usefulness of that new biomonitoring system for further biological investigation of the metabolites from *K. maritima*.

2. Investigations and results

2.1. Glucose consumption of continuously perfused FL cells

After a 24 h pre-cultivation phase cell supports grown over with human amniotic epithelial cells (FL cells) were transferred into a perfusion chamber and perfused continuously by nutrient medium. The glucose concentration in the medium after passing the cells was measured by a biosensor and related to the glucose concentration in the fresh medium. After starting the perfusion a period of increasing glucose consumption was registered, i.e. the glucose concentration in the used medium behind the perfusion chamber decreased continuously (Fig. 1 and 2, control curves). Following this dynamic phase after 18 to 24 h perfusion time the glucose consumption of the cell culture became more or less stabilised reaching a maximum level of glucose consumption (100% relative glucose consumption) which corresponded to an absolute consumption of between 150 and 200 mg/l glucose related to

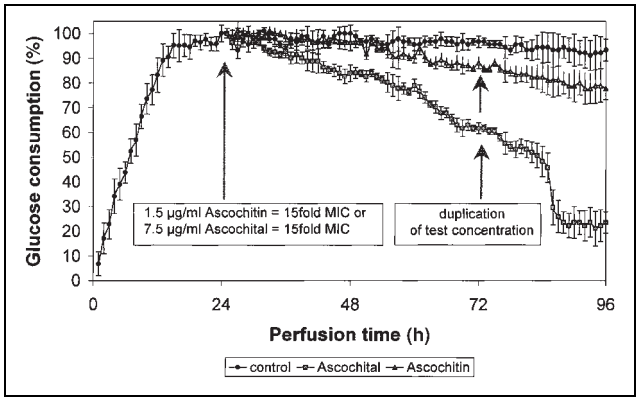

Fig. 1: Glucose consumption of perfused FL cell cultures during the exposition to ascochitin (n = 2) and ascochital (n = 2) in comparison to untreated controls (n = 9; mean ± SD)

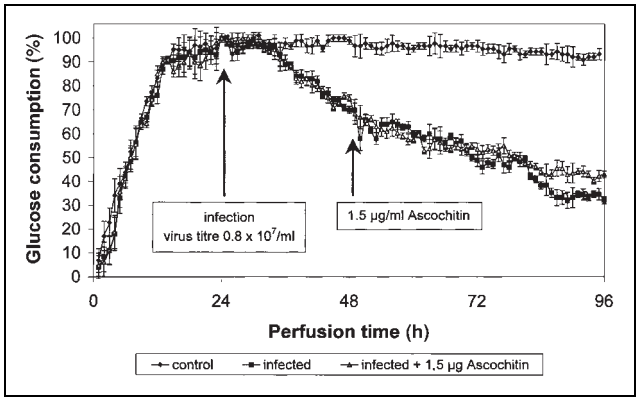

Fig. 2: Glucose consumption of perfused FL cell cultures infected with poliovirus (virus titre $0.8 \cdot 10^7$/ml, n = 2) as well as treated with ascochitin (n = 2) in comparison to non-infected controls (n = 9; mean ± SD)

a glucose content of 1000 mg/l of the fresh nutrient medium. This equilibrium state of glucose consumption could be maintained over several days.

2.2. Antibacterial activities of ascochitin and ascochital

Ascochitin inhibited the growth of *Bacillus subtilis* SBUG 14 with a MIC of 0.1 µg/ml whereas for ascochital the MIC was 0.5 µg/ml. Based on these MIC values the concentrations for the further tests were specified.

2.3. Cytotoxic/cytostatic activities of ascochitin and ascochital

In the conventional neutral red uptake assay a cytotoxic effect of ascochital was first detectable with a concentration which was twentyfold higher than the MIC. A 100% reduction of the cell viability was found with 0.015 mg/ml ascochital corresponding to the thirtyfold MIC concentration (Fig. 3).

Cytostatic effects of ascochitin could only be shown for higher concentrations. 0.015 mg/ml ascochitin (150fold MIC) reduced the cell viability by less than 10% (Fig. 3), 0.05 mg/ml (500fold MIC) by 30%, respectively (data not shown).

Fig. 1 shows the influence of ascochitin and ascochital on the glucose consumption of FL cells in the perfusion cell culture. While in the neutral red assay it was found that at least ascochital had a cytotoxic effect already at a twentyfold MIC concentration, in the more sensitive perfusion cell culture test system only fifteenfold MIC concentrations were necessary. The test substances (1.5 µg/ml ascochitin or 7.5 µg/ml ascochital, respectively) were added to the perfusion medium 24 h after the start of the perfusion. 48 h later the concentration of the test substances in the perfusion medium was doubled. It can be seen that ascochitin had only a weak influence on the glucose consumption of the cells also during a longer incubation time (three days). The duplication of the test concentration had only a slight effect. In contrast to that, ascochital diminished the glucose consumption of the cells significantly. 7.5 µg/ml ascochital reduced the glucose consumption to about 60% of the control. With 15 µg/ml ascochital the glucose consumption was only 20% of the maximum level measured before the addition of the test substances (Fig. 1).

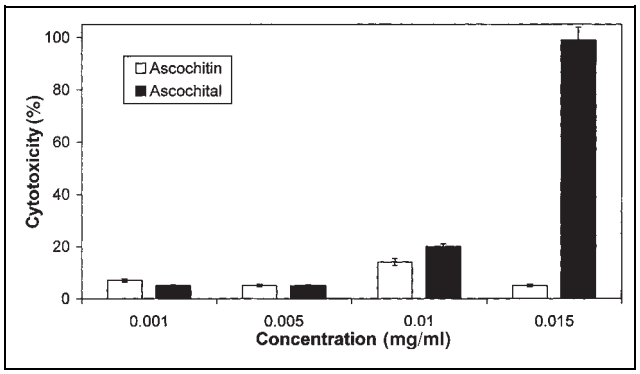

Fig. 3: Influence of ascochitin and ascochital on the viability of FL cells measured by the neutral red uptake assay (mean ± SD, n = 8)

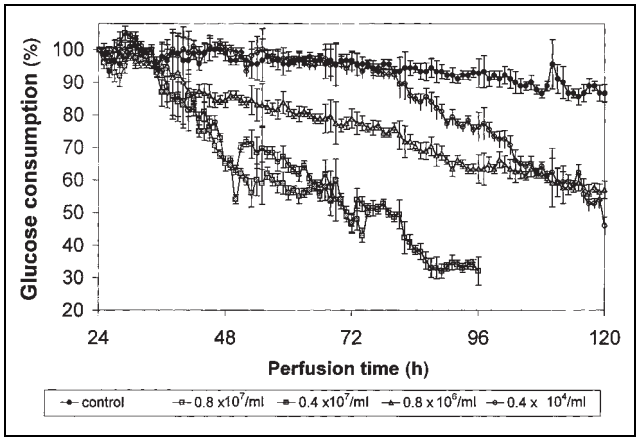

Fig. 4: Glucose consumption of perfused FL cell cultures in the course of infections with poliovirus in comparison to non-infected controls (n = 2 each; mean ± SD)

2.4. Influence of virus infection on perfused FL cells and antiviral activity of ascochitin

A poliovirus infection reduced the glucose consumption of the infected cells by about 40% to 60% (Fig. 4). The beginning as well as the kinetics of the influence on the glucose consumption of the cell culture was dependent on the virus titre of the suspensions used for cell infection. However, the cytopathogenic effects of poliovirus in dependence on the infection dose are demonstrable in the perfusion cell culture at an early stage of infection when the cell viability was not yet fully destroyed by the viruses.

Ascochitin, added 24 h after poliovirus infection as well as start of the cell perfusion, could protect the cells partially from the damaging effect of the virus. In the presence of ascochitin the remaining glucose consumption was about 10% higher compared to the control (Fig. 2).

3. Discussion

Test systems based on cell cultures are used in an increasing extent in biological and medical research, environmental toxicology or biotechnology. In contrary to the well established cell cultivation under stationary conditions, in a perfusion cell culture the medium is added continuously to the cells and any metabolic products are removed continuously, too. As a result of this method the cells can be cultivated under conditions which approximate the *in vivo* situation as closely as possible [4, 5].

Usually, the growth of cultured cells is registered as the change of the cell number in dependence on the cultiva-

tion time. Established cell number determination methods are based on visual counting of cells or nuclei under the microscope, densitometric analyses of fixed and stained cultures, determinations of the DNA content, colorimetric assays based on the cellular content of a specific enzyme or substrate, or the uptake of a dye [8]. All these methods are time consuming and/or invasively, i.e. the integrity of the cell culture will be disturbed by the determination procedure. Moreover, such methods seem not to be useful for continuous monitoring of the cell viability.

In the last years several methods were recommended to monitor the viability of cultivated cells by non-invasive methods as continuous detection of pH and oxygen partial pressure or impedance measurements [9–12]. Special characteristics of cell metabolism are considered as to be valid for a more specific control of the cell viability. In our case the metabolic state of the cell culture reflected by its glucose consumption is taken as a representative parameter for its growth state.

The continuous measurement of the glucose concentration in a flow-through system by biosensors is a well-established principle [13]. The main advantage of applying enzyme biosensors is the possibility to detect specifically biochemical species in an original sample mixture without the necessity of large-scale sample preparation. The instrumentation of an open perfusion cell culture system by an enzyme glucose biosensor as it was realised in this study allows to monitor the glucose consumption of the cultivated cells continuously. The glucose biosensors used here consisted of an electrochemical Clark type electrode, a reaction layer containing immobilised glucose oxidase and an outer covering membrane from regenerated cellulose. This hydrophilic membrane has a protective function to exclude substances with higher molecular weight as proteins and to fix the enzyme layer onto the electrode surface. With the simple construction of the sensor a measuring tool was realised with a very short response as well as a high sensitivity to glucose concentration changes [7].

The integrity of the cultivated cells in the perfusion chamber could be shown by electron microscopic investigations [14]. In previous studies this biosensor controlled perfusion cell culture test system was used to characterise the cytotoxicity of active substances for sterilisation and disinfection purposes [14, 15].

In the course of the comprehensive investigation of new antibacterial as well as antiviral drugs which were isolated in very small amounts from the marine fungus *K. maritima* the biosensor controlled perfusion cell culture was used again for cytotoxicity test purposes. But furthermore with this biomonitoring system a virus infection of cells was monitored continuously and the antiviral activity of a natural product was characterised.

The results of the cytotoxicity test using the conventional neutral red assay could be confirmed by the perfusion cell culture but this method was much more sensitive. Conventional cytotoxicity tests using cell cultures are often endpoint methods based on the detection of acute lethal effects. With the biosensor controlled perfusion cell culture it will be possible to detect influences on the viability of the cells in an early sublethal stage. The reduction of the consumption of glucose which is the main energy source of the cells can be taken as a meaningful indicator for any influences on the cell metabolism. Moreover, changes in the glucose consumption of the perfused cell cultures were found with test substance concentrations which were fifteenfold as well as thirtyfold higher compared to the MIC. Possibly, it could be rewarding to investigate further if this model may be used as a tool to estimate the margin of therapeutic safety of active substances.

Besides this it could be shown that also in the course of a virus infection the glucose consumption of the cells decreased. Normally for poliovirus diagnostic purposes a monolayer batch FL cell culture is incubated over at least 5 days with a test sample containing the virus and inspected microscopically to register characteristic morphological changes of the cells. In our experiment the cells were transferred in the perfusion system immediately after poliovirus infection. Following an initial increase in glucose consumption and a short-term plateau phase at the maximum glucose consumption level a continuous decrease of the glucose consumption could be registered dependent on the virus infection dose. That means that an influence on cell metabolism was registered by this test system at a stage where a detection of the infection is not yet possible by conventional methods. This opens the way for the investigation of potential antiviral drugs with the great advantage that the effects on cells could be registered in the course of an increasing infection to simulate real therapeutic situations in laboratory scale. The protecting effect of ascochitin against the polio virus infection as demonstrated here was weak but significant. Further investigations with other viruses as well as other antiviral substances should follow to verify these effects.

Summarising all, the results demonstrate the usefulness of the biosensor controlled perfusion cell culture for the investigation of new drugs in principle. Even slight changes of the cell metabolism can be detected in a sensitive manner. Advantages and disadvantages of this model compared with conventional test systems are compiled in the Table. In dependence of the used cell type and/or of the used test substances it should be possible to demonstrate further biological effects. For instance, lymphocyte cultures should be suitable for the detection of immunomodulating effects. Using alternative cell cultures, perfusion patterns and cell support materials as well as other biosensors for further substances in addition to glucose it is thinkable

Table: Comparison of conventional test systems and the biosensor controlled perfusion cell culture test system regarding new cytostatic, antibacterial and antiviral drugs

Conventional test systems	Biosensor controlled perfusion cell culture test system
• Mostly endpoint methods	• Continuous method
• Detection of irreversible effects	• Reversible effects are detectable
• Lower sensibility	• Higher sensibility
• Time dependence of the effects is not detectable	• Time dependence of the effects is detectable
• Large difference to *in vivo* conditions	• *In vivo*-like conditions
• The influence on the cells through the drugs and through the virus can be explored only independently of each other in different concentration areas	• The influence of drugs can be explored in a concentration area, which not yet lead to the death of cells. That makes possible the simultaneous investigation of drugs and virus influence in the same test system for several days
• The proof of a therapeutic effect at already infected cells is not possible	• The continuous measurement can make visible a therapeutic effect as well as the decrease of a virus expansion in the cell culture

to establish such a system in a broad extent to monitor several effects of new drugs on living systems *in vitro* but under *in vivo*-like test conditions.

4. Experimental

4.1. Fungal material

K. maritima was originally isolated from submerged wood, collected in December 1992 at the coast of the Greifswalder Bodden, a part of the Baltic Sea (Germany). For isolation and structure elucidation of the active compounds ascochital and ascochitin see [1].

4.2. Biosensor-controlled perfusion cell culture

Human amniotic epithelial cells (FL cells) were trypsinated. 100 µl each of a cell suspension (3×10^6 cells/ml) in nutrient medium (Dulbecco MEM with 10% neonatal calf serum, 1% sodium hydrogencarbonate and 1% penicillin/streptomycin, Biochrom KG, Berlin, Germany) was given on supports (13 mm round, Thermanox Nunc Inc., Naperville, IL, USA) using Minusheet cell holder systems (Minucells and Minutissue Vertriebs GmbH, Bad Abbach, Germany). After a 24 h pre-cultivation time at 37 °C and 5% CO_2 atmosphere six supports each grown over with FL cells were transferred into a perfusion chamber (Minucells and Minutissue Vertriebs GmbH, Bad Abbach, Germany). The cell containing chamber was perfused continuously by nutrient medium (Dulbecco MEM with 1% Hepes and 0,1% sodium hydrogencarbonate and 1% N-acetyl-L-alanyl-L-glutamine, Biochrom KG, Berlin, Germany) with a perfusion rate of 1 ml/h at 37 °C [4, 5].
Cylindrical Clark-type amperometric electrodes (SME/S3, ELBAU Elektronische Bauelemente GmbH, Berlin, Germany) consisting of a platinum (Pt) anode and a silver/silver chloride (Ag/AgCl) cathode as a reference were layered by the enzyme glucose oxidase (GOD, EC 1.1.3.4.; Arzneimittelwerk Dresden, Germany) immobilised covalently onto sepharose microspheres (Sepharose® CL-6B, Pharmacia Fine Chemicals, Uppsala, Sweden) using an established procedure. This enzyme layer was covered by a plane membrane made from regenerated cellulose (PT 150 Cuprophane®, Gerätewerk Medingen, Germany). The overall enzyme glucose sensor reaction involves the oxidation of glucose by oxygen as mediated by GOD with H_2O_2 arising as a reaction product. The principle of the amperometric sensor comprises the subsequent electrochemical oxidation of H_2O_2 at a polarisation voltage of +700 mV generating a current which, because of the stoichiometric interrelation, is a measure of the glucose concentration at the sensor [16–19]. With this type of laboratory-scale prepared glucose sensors a linear glucose measurement is possible up to about 1.5 mM maximum glucose concentration. After covering the amperometric electrodes with the enzyme membrane preparation the sensors were connected to a constant polarisation voltage of +700 mV (Pt anode vs. Ag/AgCl reference) and stored at room temperature in glucose-free imidazol buffer pH 7.0 (Imidazol puriss p.a., Fluka AG, Buchs, Switzerland). After an electrochemical adaptation time over at least 24 h [20] the sensors were calibrated using solutions of glucose in imidazol buffer pH 7.0 at 37 °C. To supply the constant polarisation voltage as well as for data registration a self-designed multi-channel amplifier (Institute of Pathophysiology, Karlsburg, Germany) connected with a data logger (HC-LOG, TIS Technische Informations-Systeme GmbH, Bocholt, Germany) was used.
The glucose biosensor was arranged in the nutrient medium downstream after passing the cell culture [21]. Before measuring the glucose concentration the nutrient medium was diluted 1 : 6 by imidazole buffer pH 7.0 using a bypass arrangement. This dilution step was necessary to increase the maximum glucose concentration to be measured by the glucose sensor. Additionally, with this dilution a higher flow rate of the medium at the sensor was reached serving for a stabilisation of the continuous glucose measurement. The glucose consumption of the perfused cell culture was evaluated as the reduction of the glucose concentration measured in the used medium related to the constant glucose concentration in the fresh medium [7]. In the study presented here the relative glucose consumption was valued as per cent [%] related to the regular measured maximum glucose consumption of the cells which was reached between 18 and 24 h after starting the perfusion.

4.3. Antibacterial tests

The minimal inhibitory concentration (MIC) against *Bacillus subtilis* (SBUG 14) was determined by the standard serial broth microdilution assay. The test strain was grown in nutrition medium II (SIFIN GmbH, Berlin, Germany) and incubated for 16 h at 37 °C. The MIC was used as a basis for the calculation of test concentrations in the other tests.

4.4. Determination of cytotoxicity by neutral red assay

As conventional test system a dye uptake assay was used. Living cells are able to take up neutral red whereas dead cells are not. FL cells were cultivated in a 96 well microtitre plate (10^5 cells/ml Eagle MEM, SIFIN GmbH, Berlin, Germany, 150 µl/well) at 37 °C in a humidified 5% carbon dioxide atmosphere. The Eagle MEM was completed by L-glutamin (0.10 g/l), Hepes (2.38 g/l), penicillin G (10^5 IE/l), streptomycin sulfate (0.10 g/l) and fetal bovine serum (80.0 ml/l, Gibco, Invitrogen GmbH, Karlsruhe, Germany). 24 h later 50 µl of test solution (test) or medium (control) were added. After a further incubation for 72 h the cells were washed three times with phosphate buffered saline solution (PBS). 100 µl neutral red solution (0.3% in Eagle MEM, SERVA, Heidelberg, Germany) were added per well. The cells were incubated for 3 h at 37 °C. Then they were washed again with PBS and 100 µl of an ethanol/acetic acid mixture (50% ethanol and 1% acetic acid in distilled water) were added. The optical density was measured at 540 nm. The cytotoxicity was calculated in percentage of control [22].

4.5. Determination of cytotoxicity using the perfusion cell culture

The FL cells containing perfusion chamber was perfused for 96 h with nutrient medium which contained different concentrations (15fold and 30fold MIC) of ascochitin or ascochital, respectively. The test substances were added to the perfusion medium 24 h after starting the culture in the perfusion chamber. The glucose concentration was measured in the nutrient medium downstream after passing the cell culture The relative glucose consumption was calculated as percentage of the glucose consumption immediately before the active substances were added which was set as 100% glucose consumption.

4.6. Antiviral tests in perfusion cell culture

The virus used was poliovirus type I, Mahoney strain. Multiple inoculation of the poliovirus into FL cells yielded high titre virus suspensions. The culture medium contained 66% Hank's solution, 30% Eagles MEM, 2% neonatal bovine serum (all Biochrom KG, Berlin, Germany) and 2% supplements such as antibiotics and buffer substance. The suspension medium had the same composition, with the exception of bovine serum. The virus suspensions were stored in 1 ml cryotubes at −20 °C. The virus concentration of the suspensions was determined quantitatively by the final dilution method, the virus titre (ID_{50} [ml^{-1}]) was calculated according to the method of Spearman and Kärber [23].
Following the 24 h pre-cultivation phase the cells were incubated over one additional hour by different poliovirus suspensions with titres of 0.8×10^7 ml^{-1}, 0.4×10^7 ml^{-1}, 0.8×10^6 ml^{-1} and 0.4×10^4 ml^{-1}, respectively, before the perfusion was started. Antiviral test substances were added to the perfusion medium 24 h after starting the perfusion culture of the infected cells. The glucose consumption was calculated in percentage of the maximum glucose consumption which was reached between 18 and 24 h after the start of the perfusion. This maximum glucose consumption was set as 100% glucose consumption.

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