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Hamed Alborzinia

Real-Time Monitoring of Cancer Cell Metabolism for Drug Testing

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With a foreword by Prof. Dr. Stefan Wölfl

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To my parents, in love and appreciation!

Foreword by the Supervisor

Laboratory *in-vitro* models that mimic *in-vivo* conditions to follow the response of cells to changes in their micro-environment, including treatment with drugs and toxic compounds, is still a challenging and demanding aim for scientists. In particular for drug research it is very important to know all the effects that can be triggered in mammalian cells when exposed to a bioactive, potential drug or toxic compound. Such a system will be very useful in the field of medicinal chemistry as well as for pharmaceutical companies. Newly synthesized compounds designed for therapeutic applications may not only act on their designated targets, but rather interfere with various biochemical pathways, which could have negative and beneficial impact on their application. These effects have to be carefully monitored before drugs can be used in therapeutic applications in the clinic.

For this, all drug candidates and other chemical compounds are analyzed in great detail to understand their biological activities and modes of action. Nevertheless, many commonly used laboratory model systems are very limited. A major problem of most laboratory assays as well as animal studies is that the results are analyzed after a given treatment time in a so-called end-point assay. This means that after treatment is initiated the response to the treatment is only analyzed at predefined time points. Although, these assays provide very informative information, this is limited to these predefined time points. With the biosensor assay system implemented here for the analysis of drugs and other chemical compounds, the cellular response is monitored continuously and biochemical activity is recorded in real time.

Using a continuous flow system, important limitations of traditional tissue culture requiring high nutrient concentrations such as glucose or amino acids can be avoided and conditions closer to the physiological *in-vivo* nutrient condition can be used.

Thus, a system is needed, in which we will be able the monitor biological effects in real time and keep cells in a continuous-flow perfusion enabling to feed cells with physiological levels of nutrients.

In his research project, **Dr. Hamed Alborzinia** nicely presents the advantages of both time-resolved analysis of cells using online sensor measurement and continuous feeding in a flow-perfusion system. In his work he developed cell-based biosensor culture conditions with specific tissue properties that can be used for detailed analysis of cell metabolism. To learn different methods of sensor technology, he spent several months in the research lab of a small company, where he got introduced to biosensor chip technology and learned basic properties of electronic chip sensors. The initial experiments he performed there have been so successful that we obtained funding from the German Ministry for Research (BMBF) to set up this technology in our lab in Heidelberg. His work with this sensor technology led to a large number of important publications studying the activity of potential anticancer drugs, which could not have been described properly otherwise. Dr. Hamed Alborzinia established this technology as a highly efficient research platform and combined biosensor analysis with biochemical and other cell-based assays for more detailed molecular analysis to follow biological changes at key time points observed in the metabolic measurements. He also demonstrated that the biosensor system can be used to investigate the cellular response to growth factors, general toxic challenges, and to monitor the impact of important regulatory proteins such as SIRT3 and N-MYC on cancer cell metabolism. The impact of his thesis work is nicely visible in several peer-reviewed publications in medicinal chemistry and basic cancer biology and is very likely to attract significantly more attention in the future.

Prof. Dr. Stefan Wölfl

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The aid and support of many people has made this possible after five long years of my Ph.D.

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Hamed Alborzinia

Abbreviations

ADP — **A**denosine **dip**hosphate
ATCC — **A**merican **T**ype **C**ulture **C**ollection
APAF — **A**poptosis **p**roteases **a**ctivating **f**actor
ATP — **A**denosine-5′-**trip**hosphate
CCCP — **C**arbonyl **c**yanide *m*-**c**hloro**p**henyl **h**ydrazone
Caspase — **C**ysteine/**as**partate-specific **p**rote**ases**
***t*BHP** — *tert*-**B**utyl **h**ydro**p**eroxide
CDDP — Cisplatinum or Cisplatin
cDNA — **c**omplementary **DNA**
CREB — **c**AMP **r**esponse **e**lement **r**epressor
DAVID — **D**atabase for **A**nnotation, **V**isualization, and **I**ntegrated **D**iscovery
DMEM — **D**ulbecco's **m**odified **E**agle's **m**edium
DNA — **D**eoxyribo**n**ucleic **a**cid
D-PBS — **D**ulbecco's **p**hosphate **b**uffered **s**aline
EDTA — **E**thylene **d**iamine **t**etraacetic **a**cid
ERK — **E**xtracellular signal-**r**egulated **k**inase
FACS — **F**luorescence-**a**ctivated **c**ell **s**orting
FBS — **F**etal **b**ovine **s**erum
FCS — **F**etal **c**alf **s**erum
FDA — **F**ood and **D**rug **A**dministration (USA)
5-FU — 5-**F**luoro**u**racil
GLUT — **Glu**cose **t**ransporter
GSK-3ß — **G**lycogen **s**ynthase **k**inase-3beta
HEPES — 4-(2-**H**ydroxy**e**thyl)-1-**p**iperazine **e**thane **s**ulfonic acid
IC_{50} — Half maximal **i**nhibitory **c**oncentration
IDES — **I**nter**d**igitated **e**lectrode **s**tructure
ISFET — **I**on-**s**ensitive **f**ield **e**ffect **t**ransistor
MMS — **M**ethyl **m**ethane**s**ulfonate
mtDNA — **mit**ochondrial **DNA**
NaF — Sodium fluoride
NAMI-A — **N**ew **a**nti-tumor **m**etastasis **i**nhibitor-**A**
NIAID (NIH) — **N**ational **I**nstitute of **A**llergy and **I**nfectious **D**iseases (National Institutes of Health)
p53 — Tumor **p**rotein **53**
PCR — **P**olymerase **c**hain **r**eaction
PI3K — **P**hosphatidyl**i**nositol **3**-**k**inase
PKB/Akt — **P**rotein **k**inase **B**/**t**hymoma of the **AK**R mouse strain
PMSF — **P**henyl**m**ethyl**s**ulfonyl **f**luoride
RM — **R**unning **m**edium
RNA — **R**ibo**n**ucleic **a**cid
ROS — **R**eactive **o**xygen **s**pecies
RT-PCR — **R**eal-**t**ime **PCR** (or reverse transcriptase real-time PCR)
SDS — **S**odium **d**odecyl **s**ulfate
SGLT — **S**odium **gl**ucose co**t**ransporters
SIRT3 — **Sirt**uin**3**
TrxR — **T**hio**r**edo**x**in **r**eductase

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Summary

Analysis and identification of biological activities of drug and drug candidates is one of the most challenging tasks in modern drug research. To avoid unnecessary and costly tests in animal models it is very important that *in-vitro* test systems are available to provide detailed information regarding the biological activity and toxicity of potential drug candidates. Currently, most cell-based *in-vitro* bioanalytical methods used in pharmaceutical research are end-point measurements. This means that in each experimental assay only information for one particular time point is obtained, i.e.: *i*) cells are treated with compounds, *ii*) then at a preselected defined time point of interest the cells are fixed, lysed, or labeled, and *iii*) the resulting effects of the compound are monitored. By doing so, important information about the time dependence of biological activities are lost, or many repeated experiments have to be performed to cover all time points of interest.

To overcome this problem, novel biosensor chip analysis systems are enabling the continuous monitoring of cell metabolism and cell morphology in real-time, without any labeling or further disturbance of the system. The Bionas 2500 biosensor chip system used in this work allows the continuous monitoring of three important metabolic and morphological parameters: *i*) **oxygen consumption** using Clark-type electrodes, *ii*) **pH change** of the extracellular environment using ion-sensitive field effect transistors, and *iii*) the **impedance** between two interdigitated electrode structures to register the impedance under and across the cell layer on the chip surface. It also can be used with any adherent cell type, allowing further elucidation of specific drug properties.

In this thesis the biosensor chip was used to monitor the metabolic and morphological changes in five cancer cell lines in real-time in response to:
(1) **cisplatin** (CDDP) treatment, one of the most widely used anticancer drugs; (2) overexpression of **sirtuin deacetylase SIRT3**, a key regulatory enzyme of cellular metabolism; and (3) a choice of several **organometallic compounds**, potential new anticancer drug candidates.
To ensure that the observed parameters are of pharmacological relevance and not just an experimental artifact, further experimental

analysis was performed to confirm the validity of the measured parameters. This included the role of the experimental conditions, like glucose concentration and uptake, but also detailed downstream analysis of molecular changes for the molecular interpretation of the observed results.

In the specific analysis of drug activity and molecular manipulation of the cells the following major results were obtained:

(1) All cell lines treated with **cisplatin** showed a first effect on respiration, which was followed by interference with glycolysis in four of the five cell lines, HT-29, HCT-116, HepG2, and MCF-7 but not in the cisplatin-resistant MDA-MB-231. Most strikingly, the cisplatin-sensitive cell lines start cell death within 10–11 h of treatment, indicating a clear timeline from first exposure to the drug, to cisplatin-induced lesions, and to cell fate decision. Further analysis at time points of most significant changes upon cisplatin treatment in the breast cancer cell line MCF-7 revealed important molecular changes underlying these activities. For this purpose, the phosphorylation of selected signal transduction mediators connected with cellular proliferation, as well as changes in gene expression, were analyzed in samples obtained directly from sensor chips at the time points when changes in glycolysis and impedance occurred. The reported online biosensor measurements reveal details in the timeline of metabolic responses to cisplatin treatment leading up to the onset of cell death.

(2) Overexpression of the metabolic regulator **SIRT3** led to an increase in cellular respiration of up to 35%. To ensure that this can indeed be attributed to the concentration of SIRT3 protein in the cells, the changes in protein levels were confirmed by Western blot directly from cells grown on the biosensor chips.

(3) The biological activity of potential **organometallic** drug candidates, containing the covalently bound (or chelated) metals, iron, rhodium, ruthenium, or gold, revealed not only antitumor activity but also unexpected striking biological activities. While most ruthenium complexes strongly reduced cell impedance but only slightly affected respiration and glycolysis, others immediately caused significant effects

on respiration or glycolysis. Cell-line and drug-specific responses were identified, confirming the versatility of these biosensor chip measurements.

In essence, this work provides *i*) real-time measurements of basic cancer cell metabolism of different cancer cell lines; *ii*) a detailed timeline of the metabolic response to cisplatin treatment and clear detection of the time span between start of cisplatin treatment and onset of cell death, which reflects the time required for the underlying molecular mechanisms of cell fate decision; *iii*) direct functional measurement of the biological activity of a key regulatory protein of cellular metabolism following the kinetic change in respiration upon SIRT3 overexpression; and *iv*) the time-resolved impact of several organometallic compounds on cell metabolism and cell morphology, including unexpected and not yet understood highly significant and specific effects on cell-cell interaction and adhesion.

Zusammenfassung

Die Analyse und Identifizierung der biologischen Wirksamkeit von zugelassenen und in der Testphase befindlichen Arzneistoffen ist eine der größten Herausforderung in der modernen Arzneimittelforschung. Die Verwendung von *in-vitro*-Systemen ermöglicht unnötige und aufwendige Untersuchungen in Tiermodellen zu vermeiden wobei detaillierte Informationen bezüglich der biologischen Wirksamkeit und der möglichen Toxizität potenzieller Arzneimittelkandidaten gewonnen werden können. Die meisten derzeit in der Pharmaforschung verwendeten zell-basierten bioanalytischen *in-vitro*-Methoden beruhen auf Endpunkt- Messungen. Das heißt, dass in jeder experimentellen Untersuchung nur Informationen für einen bestimmten Zeitpunkt gewonnen werden können: *i*) Zellen werden zunächst mit der Wirksubstanz behandelt, *ii*) daraufhin werden die Zellen zu einem vorbestimmten Zeitpunkt fixiert, lysiert oder markiert, und *iii*) die eingetreten Wirkung der Substanz festgestellt. Auf diese Weise gehen wertvolle Informationen zur zeitabhängigen Wirkung verloren, oder man muss viele solcher Messungen in Serie wiederholen um den gesamten Zeitraum zu erfassen.

Zur Vermeidung dieses Problems, wurden in letzter Zeit neuartige Biosensor-Chip Analysesysteme entwickelt, die eine kontinuierliche Messung von Stoffwechselvorgängen und Zellstrukturveränderungen in Echtzeit ermöglichen, ohne die Notwendigkeit von Markierungen oder andersartig störenden Eingriffen in das System. Das in dieser Arbeit verwendete Bionas 2500 Biosensor-Chip System ermöglicht kontinuierliche Messungen dreier wichtiger Stoffwechsel- und morphologischer Parameter: *i*) **Sauerstoffverbrauch** durch Clark-Elektroden, *ii*) **pH-Änderungen** des außerzellulären Milieus anhand von ionenempfindlichen Feldeffekt-Transistoren und *iii*) **Widerstand** zwischen zwei interdigitierten Elektroden, welches entsprechende Messergebnisse von unterhalb und entlang der auf der Chipoberfläche befindlichen Zellschicht liefert. Das System kann auch für jedwede Art von adhärenten Zelltypen verwendet werden, womit verschiedene spezifische Eigenschaften von Arzneistoffen untersucht werden können.