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How *in vitro* alterations in cellular energy pathways can overcome obstacles in drug research

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The basic mission of the pharmaceutical industry is to understand disease and to bring safe and effective drugs to patients. Starting with the drug discovery process, for any particular disease a first step involves selecting a disease-specific target, then finding a suitable assay to determine the activity of molecules in relation to the selected target. This path is difficult and, lacking proper technology, is similar to the proverbial search for the needle in a haystack. However, once a molecule emerges as a successful candidate in the drug discovery process, it enters into drug development. The drug development process provides safety data prior to "first-in-man" trials. Drug discovery and development are extended, expensive, and inefficient processes with a success rate of only 0.01%. Placing these difficulties into perspective, it remains the desire of the pharmaceutical industry to develop novel, economical, reliable *in vitro* technologies to meet the above challenges.

In this PhD thesis, a multiparametric cytosensor system was used for real-time identification of physiological parameters in living cells, and to meet the above-mentioned challenges in drug research. This technology allows us to simultaneously monitor extracellular acidification (pH changes), cellular respiration (oxygen consumption), and cellular morphology and adhesion (impedance measurements). All work done by a cell and all of the activities of life in general necessitate energy, commonly in the form of adenosine triphosphate (ATP). The regeneration of the consumed ATP leads to increased oxygen consumption and an excretion of acidifying side products (lactate and carbon dioxide), which can be monitored and used to deduce changes in physiological pathways.

The first project of this thesis explored the influence of the P-glycoprotein (ABCB1) transporter on drug-drug-interactions, drug resistance, as well as drug absorption and distribution, all of which are important factors to be considered during the development of new drugs. Thus, the early identification and the exclusion of compounds that show a high affinity to P-glycoprotein can help to select drug candidates. The aim of this first project was to use the multiparametric cytosensor system for the label-free identification of P-glycoprotein substrates in living cells by monitoring extracellular acidification and cellular respiration upon stimulation with substrates of P-glycoprotein. Using L-MDR1 cells, a human P-glycoprotein-expressing cell line, the influence of P-glycoprotein activity was determined for seven different compounds, demonstrating the applicability of the system for P-glycoprotein substrate identification. Effects were concentration dependent, as shown for the P-glycoprotein substrate verapamil, and were associated with cellular acidification and respiration. P-glycoprotein ATPase activation by verapamil was able to be described by a Michaelis-Menten type kinetics profile showing saturation at high substrate concentrations. Control experiments using a P-glycoprotein inhibitor indicated that the observed effects were related to P-glycoprotein ATPase activity. In contrast, wild-type LLC-PK1 cells that did not express P-glycoprotein were not responsive to stimulation with different P-glycoprotein substrates. Summarising these findings, the microsensor system used is a generic system suitable for the identification of P-glycoprotein substrates. In contrast to other biochemical P-glycoprotein assays, activation of the drug efflux pump can be monitored on-line and label-free in living cells in order to identify P-glycoprotein substrates and to study the molecular mechanisms of ATP-dependent active transport.

The second project of this thesis explored the pharmacology of the purinergic P2X7 receptor and its influence on changes in metabolic activity after ATP treatment. The purinergic P2X7 receptor plays a prominent role in cell metabolism and possibly determines downstream effects based on its

interactions with extracellular ATP. Adenosine triphosphate, a key agent in physiology, provides energy in numerous reactions and acts as a neurotransmitter. Extracellular ATP concentrations are known to rise under pathological conditions, thereby triggering immune system responses that lead to pro-inflammatory states and immune modulation, to the extent of initiating cell death. These adverse effects have been linked to the purinergic P2X7 receptor, which triggers downstream signalling when levels of extracellular ATP are high. The purinergic P2X7 receptor is also involved in modulating cellular responses that include membrane depolarisation, secondary messenger activation, Ca^{2+} influx, and activation of the mitogen-activated protein kinase pathway. Moreover, it features a unique ability to form a large, non-selective pore, allowing molecules up to 900 Daltons to enter the cell, with potentially deleterious consequences. In addition, the P2X7 receptor purportedly regulates many metabolic processes inside the cell, while little is known of how extracellular ATP triggers these P2X7-mediated metabolic effects. In this study, the stimulatory effects of exogenously applied ATP on metabolic activity and the associated morphological changes in cells in relation to the P2X7 receptor were explored using the multiparametric cytosensor system for a deeper view inside the cell.

Analysis of cell physiological parameters revealed that ATP-induced metabolic stimulation was detectable. Furthermore, based on signal patterns of the multiparametric cytosensor system, it was possible to detect ATP-induced oxidative stress to cells. Experiments with rodent brain cells that express P2X7 receptors demonstrated similar activation effects. Exploring and elucidating the evident relationship between the P2X7 receptor and extracellular ATP concentrations leads to the hypothesis that high levels of ATP reflect a pathological state and lead to an increase in metabolic activity in the cell.

The third project of this thesis explored liver toxicology and used the multiparametric cytosensor system to detect these adverse effects. The liver plays a pivotal role in the biotransformation and detoxification of drugs and is consequently vulnerable to potential injury as a consequence of significant drug exposure. Drug-induced liver injury (DILI) is of considerable concern in drug discovery and development, placing emphasis on the need for predictive *in vitro* technologies that identify potential hepatotoxic side effects of drugs. A label-free, real-time, multiparametric cytosensor system has therefore been established for *in vitro* assessment of drug-induced toxicity. The system is based on monitoring cellular respiration, metabolic activity, cell morphology, and adhesion of human hepatocarcinoma-derived HepG2 cells. The read-out derived from the multiparametric cytosensor system has been optimised and permits sensitive, reliable, and simultaneous recording of cell physiological signals, such as metabolic activity, cellular respiration and morphological changes, and cell adhesion upon exposure to a drug.

Analysis of eight prototypic reference drugs revealed distinct patterns of drug-induced physiological signals. Effects proved to be rigidly concentration-dependent. Based on signal patterns and reversibility of the observed effects, compounds were able to be classified as triggering mechanisms of oxidative or metabolic stress or as leading to cell death (necrosis-like and apoptosis-like). A test-flag-risk mitigation strategy is proposed to address potential risks for drug-induced hepatotoxicity.

Concluding all three projects, the general concept of monitoring the physiological parameters of cells with an *in vitro* technology to overcome obstacles in drug research has clearly been shown to be viable. It was possible to develop a successful, novel assay for reliable, real-time, label-free identification of potential drug-drug interactions based on the identification of P-glycoprotein transporter substrates. Furthermore, the pharmacology of the purinergic P2X7 receptor after application of extracellular ATP was characterised and, finally and importantly, drug-induced liver effects were detected by on-line monitoring of liver HepG2 cells exposed to drugs.