Charles University
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Summary of Ph.D. thesis

High-throughput screening for the discovery of small molecules modulating cell fate

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Abstrakt

Objevy sloučenin ovlivňujících buněčnou proliferaci, diferenciaci a smrt mohou vést nejen ke vzniku nových léčiv sloužících k léčbě či prevenci onemocnění, ale lze je také využít ke studiu molekulárních drah zapojených v těchto procesech. Pro testování tisíců těchto malých molekul v buněčných esejích je nezbytná přístrojová automatizace a miniaturizace.

V této práci jsou popsány postupy testování s vysokou propustností (HTS). Jako hlavní předměty studia byly vybrány dráhy hypoxie a Wnt zapojené v růstu kmenových a nádorových buněk, diferenciace hematopoetických, neurálních a mesenchymálních kmenových buněk, a dráha TRAIL vedoucí k selektivní likvidaci nádorových buněk. Tímto postupem bylo možné testovat efekt malých molekul v eukaryotických buňkách a jednobuněčných organismech, jak je ilustrováno na příkladu hledání sloučenin účinkujících proti parazitickým prvokům rodu Leishmania.

Několik sloučenin se ukázalo být aktivními modulátory osudu buňky. Jednalo se především o monensin, který inhibuje dráhu Wnt a zabraňuje růstu nádorů v myším modelu kolorektálního karcinomu, hemoharringtonin, který ve spolupráci s proteinem TRAIL indukuje smrt rakovinných buněk implantovaných do imunodeficientní myši, a diphenyleneiodonium chlorid, jenž zabíjí Leishmanie v buňkách. Žádná z těchto látek není v terapeutické oblasti toxická pro zdravé buňky nebo organismy. Pro studium buněčné diferenciace neposkytuje jednokrokový homogenní test dostatek informací, je tedy třeba využít tzv. high-content screenu, což je vícekroková a víceparametrová verze HTS.

Abstract

The discovery of chemical compounds able to modify the way cells proliferate, differentiate or die can lead not only to the formulation of new drugs for disease treatment or prevention but also to their use as biological probes in the study of the molecular pathways involved in these processes. In order to test thousands of these small molecules in cellular assays, instrument automation and assay miniaturization are necessary.

In this thesis, applications of High-Throughput Screening campaigns are described. The Hypoxia and Wnt pathways involved in stem and cancer cell proliferation; the differentiation of hematopoietic, neural and mesenchymal stem cells; and the TRAIL pathway leading to selective cancer cells death were the main subjects chosen. With this approach, it was possible to test the effect of small molecules in eukaryotic cells and in unicellular organisms as exemplified by the search of compounds leading to the death of the protozoan parasite Leishmania.

Several chemical compounds were identified as active in modulating cell fate. Of remark were: Monensin that inhibits the Wnt pathway and prevents the growth of tumors in a mouse model of colorectal cancer; Homoharringtonine that, only in combination with TRAIL, induces the death of cancer cells implanted in immunodeficient mice; and diphenyleneiodonium chloride that kills intracellular Leishmania. None of these compounds display toxicity to healthy cells or organisms within the therapeutic window. To be able to study cell differentiation, homogeneous assays do not provide sufficient information and High-Content Analysis, a multistep and multiparameter version of HTS, is required.

Introduction

Previously performed only at large pharmaceutical and biotechnology companies, high-throughput assays for the discovery of active small chemical compounds in biological assays are nowadays also established in academic laboratories. Although with usually much smaller compound libraries, academic laboratories may have the unique advantages of their compounds resulting from collaborations with academic chemists and also that the targets of their screens do not necessarily need to be financially relevant as opposed to the profit-driven companies.

In this thesis, we reveal our efforts in establishing high-throughput and high-content screenings for the discovery of compounds active in modulating cell fate: self-renewal, differentiation and death.

The Wnt pathway is involved in stem cell maintenance and its aberrant activation is the cause of several cancer types[1].

The Hypoxia pathway, also contributing to the stem cell phenotype, may be responsible for cancer metastases[2].

The discovery of compounds inducing the self-renewal or a controlled differentiation of hematopoietic stem cells (or other adult stem cells) may be useful in transplantation therapies[3].

The TRAIL pathway, leading to the specific apoptotic death of cancer cells, is a promising therapeutic target[4]. However, cancer cells rapidly acquire resistance to TRAIL treatment[5]. Therefore, compounds that would resensitize cancer cells to TRAIL-induced apoptosis could be therapeutically interesting.

Cell-based assays can be extended to unicellular organisms as demonstrated by screening of compounds able to kill the protozoan parasite *Leishmania*, responsible for one of the most neglected tropical diseases[6].

Aims of the study

The main aim of this study was the identification of small chemical compounds able to modulate cell fate and that could be used as molecular probes to further understanding the processes they modulate.

Materials and Methods

The workflow described in this thesis can be divided in three parts: compound management, screening campaign and hit validation.

The compounds, originally from different sources both academic and commercial, were reformatted into a uniform system in 384-well plates. Compounds were then transferred into 384-well assay plates with cells via pintool coupled to the Janus liquid handler, either in a stand-alone instrument or as part of an automated workstation dedicated to high-throughput and high-content screening.

The Wnt and Hypoxia modulators screens were performed using cells transfected with firefly luciferase reporter vectors controlled by TCF/LEF and hypoxia responsive elements, respectively. The results were then assessed with the ONE-Glo™ Luciferase Assay System (Promega).

The hematopoietic stem cells differentiation screen was performed using $CD34^+$ cells obtained from human umbilical cord blood after immunomagnetic cell sorting. These cells were treated with compounds for five days, stained with fluorophore-conjugated antibodies and analyzed by flow cytometry.

The TRAIL pathway sensitizers and the Leishmania killers screens were performed using the CellTiter-Blue® Cell Viability Assay (Promega).

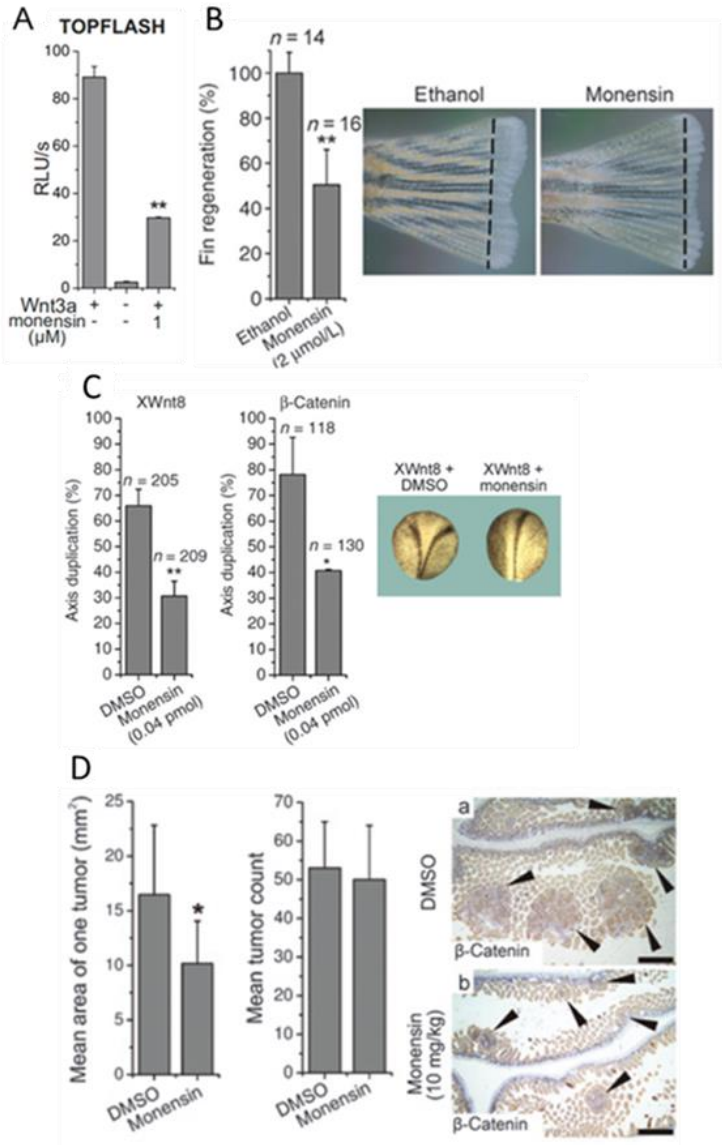
Hit validation involved assays designed for the determination of the potency, cytotoxicity and specificity of the hit compounds. The search for the molecular target in the pathway modulated by the compound involved the application of homogeneous assays and of standard molecular biology techniques such as qRT-PCR, immunostaining and blotting, flow cytometry and fluorescent microscopy. At the end, in vivo tests using established animal models were used.

Published Results - Part 1: Monensin inhibits the Wnt pathway.

The Wnt pathway is involved in the control of embryonic development and mutations in the pathway lead to cancer. To find chemical compounds inhibitors of the pathway, HEK cells stably transfected with the TCF/LEF-Luc reporter, plated at a density of 2500 cells/ml in 384-well plates, were incubated with or without recombinant WNT1 protein for 24 hours in the presence of compounds before measuring luminescence. From the approximately 23000 compounds tested, the polyether antibiotic monensin, isolated from *Streptomyces cinnamonensis*, was found to be a potent blocker of the Wnt-induced transcription in cells stimulated with Wnt ligand (Fig. 1A). Monensin inhibits the translocation of β-catenin to the nucleus, consequently reducing the expression of Wnt target genes. To produce this effect, monensin inhibits LRP phosphorylation and β-catenin production. The use of different animal models further proved the inhibitory effect of monensin on the Wnt pathway. The ability of zebrafish to regenerate their tail fin after amputation is dependent on the Wnt pathway[7] and is inhibited by monensin (Fig. 1B). Injection of Wnt or β-catenin induces dorsalization of Xenopus embryos[8], which is also inhibited by monensin (Fig. 1C). Finally, monensin treatment of $APC^{+/Min}$ mice (carrying a mutation rendering the Wnt pathway constitutively active resulting primarily in colorectal tumor formation[9]) reduced not the number but the size of tumors in the intestine (Fig. 1D). Our data imply that monensin might be used as an anticancer drug, especially in neoplasia displaying aberrant Wnt signaling.

Figure 1. Monensin inhibits *in vitro* and *in vivo* processes dependent of the Wnt pathway: A) inhibits the reporter activation by Wnt ligand; B) inhibits the regeneration of zebrafish caudal fin after amputation; C) inhibits the dorsalization of Xenopus embryos after Wnt or β-catenin injections; and D) inhibits the growth of tumors in $APC^{+/Min}$ mice.

Figure 1



Published Results - Part 2: Homoharringtonine sensitizes resistant cancer cells to TRAIL-induced apoptosis.

Most primary human tumors are resistant to monotherapy with TRAIL apoptogens. The potential applicability of TRAIL in anti-tumor therapy will ultimately depend on its rational combination with drugs targeting these resistances. From the approximately 23000 compounds screened in a viability assay, homoharringtonine (HHT), an alkaloid originally isolated from *Cephalotaxus harringtonia*, was identified as a potent sensitizer of the resistant human colorectal cancer cells to TRAIL-induced apoptosis. Annexin staining and Caspase-Glo® 3/7 Assay (Promega) indicated that the total eradication of the cancer cells, only in the combined treatment with HHT and TRAIL, involved the activation of the apoptotic machinery with an EC_{50} of 15 nM for HHT (Fig. 2A). The sensitization of colorectal cancer cells to TRAIL-induced apoptosis is probably due to the drop in the cellular levels of mcl-1 and cFLIP and to the enhanced activation of JNK and p38 kinases induced by HHT. Combining HHT with TRAIL was not only effective *in vitro*, but also suppressed the growth of xenotransplanted TRAIL-resistant HT-29 cells in immunodeficient mice, proving its efficacy and safety within the therapeutic window (Fig. 2B).

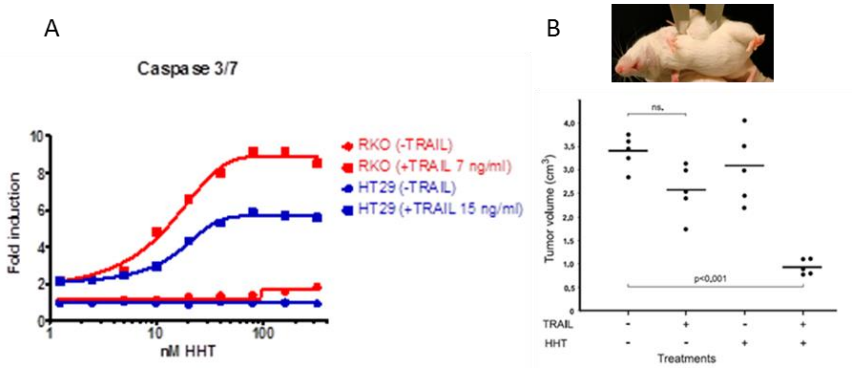


Figure 2. Homoharringtonine assists TRAIL in suppressing the growth of colon cancer cells *in vitro* (A) and implanted in immunodeficient mice (B).

Unpublished Results - Part 1: Discovery of two chemical compounds inducers of the hypoxia pathway.

Proper cellular response to changes in oxygen tension during normal development or pathological processes is ultimately regulated by the transcription factor hypoxia-inducible factor (HIF). The 10000 compounds from the Chembridge DIVERSet library were screened, in a luciferase reporter assay, for their ability to activate the hypoxia-responsive element (HRE). Two compounds, CCG20420 and CCG25177, able to activate the HRE, also induced an increased expression of the hypoxia pathway target gene VEGF and accumulation of non-hydroxylated HIF-1α protein in the nucleus. Co-treatment of cells with ferric citrate abolished the HIF-1α stabilization in response to the compounds. Altogether this data suggests that these compounds are inhibiting prolyl-hydroxylases by blocking their access to iron either by chelating it or through binding to the iron site of the enzymes (Fig. 3).

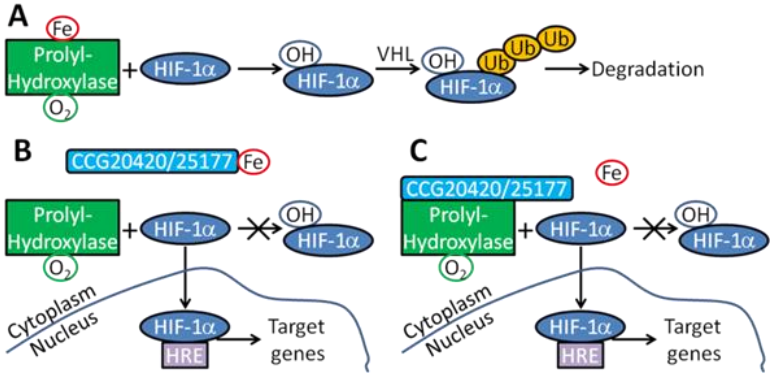


Figure 3. Model of activity. A) In normoxic conditions, HIF-1α protein is hydroxylated signaling for its ubiquitination by the VHL complex and consequent degradation by the proteasome. When either of the compounds is present, it blocks the access of the hydroxylase to iron either by chelating it (B) or through binding to the hydroxylase itself (C). HIF-1α can then translocate to the nucleus, bind the HRE and induce the expression of target genes.

Unpublished Results – Part 2: Discovery of a chemical compound inducing the differentiation of hematopoietic stem cells to the megakaryocytic lineage.

The ability to generate sufficient quantities of progenitor or differentiated, functional cells has long been a focus of regenerative medicine. Starting with a population of $CD34^+$ cells purified from human umbilical cord blood, approximately 2500 compounds were screened for their ability to expand this population or to direct its differentiation into a specific blood lineage. For this, after compound treatment, the cells were stained with a multiplex of seven antibodies, each recognizing a protein present only in the membrane of a specific blood cell subtype, and analyzed by flow cytometry.

No compound directing the differentiation specifically into T cells was found while one compound induced the expression of both B and T cells markers, possibly indicating a more immature phenotype but already directed to the lymphoid lineage. Similarly, for the myeloid lineage, one compound was found that induced the expression of markers of macrophages, granulocytes, platelets and erythrocytes. Other compounds were found to induce the expression of cell lineage markers or to increase the number of $CD34^+$ cells. To validate these hits, groups of 2-4 antibodies directed to the same lineage were multiplexed and flow cytometry analysis of $CD34^+$ cells would indicate if the same cell would express them after compound treatment. Although there is an inherent variability in assays dealing with primary cells, at least one compound repeatedly showed to induce the expression of both CD41 and CD42, indicating an induction of differentiation of hematopoietic stem cells into platelets (Fig. 4).

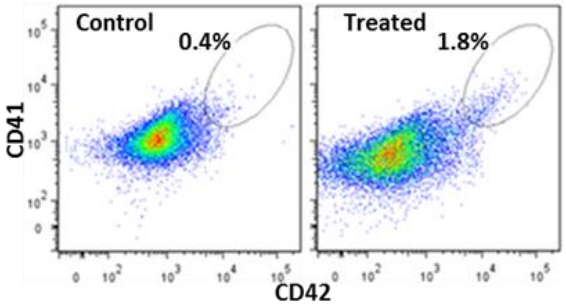


Figure 4. Flow-cytometry analysis of CD34+ cells treated with a hit compound inducing the differentiation into platelets.

Unpublished Results – Part 3: Diphenyleneiodonium chloride has a potent leishmanicidal activity.

Leishmaniasis is a severe disease caused by protozoan parasites belonging to the genus *Leishmania*, transmitted by sand flies. Available treatments for leishmaniasis are either expensive, difficult to apply, with adverse effects, with low efficiency or with decreased efficiency due to resistance acquired by the parasites. Thus there is a continuing need for new chemical substances with leishmanicidal effect to be used in mono or combination therapy.

A viability screen of approximately 23000 compounds identified diphenyleneiodonium chloride (DPI) as a nanomolar killer of leishmania promastigotes, the extracellular stage of the parasites life cycle. After proving its safety against healthy human cells, DPI was added to primary cultures of mouse macrophages infected with green fluorescent *Leishmania*. Flow cytometry analysis showed that DPI also completely eradicated *Leishmania* amastigotes without affecting the viability of macrophages (Fig. 5).

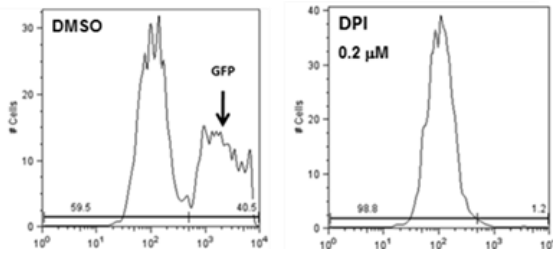


Figure 5. Flow-cytometry analysis shows the disappearance of GFP-*Leishmania* population after treatment with DPI without affecting the number of viable macrophages.

It was possible to inhibit the effect of DPI through co-incubation with bromosulfophthalein, a specific inhibitor of glutathione transporters. Glutathione depletion and the following cell cycle arrest could explain the leishmanicidal effect of DPI (Fig 6).

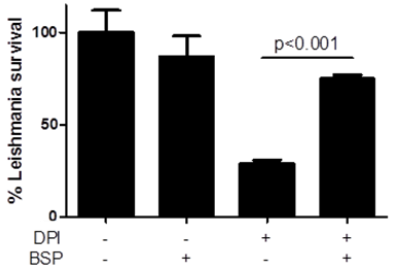


Figure 6. Co-treatment with BSP inhibited the antipromastigote effect of DPI.

Conclusions

Small chemical compounds that change the way cells behave are commonly used in academic laboratories as tools to help dissecting the molecular mechanisms and signaling pathways responsible for such changes. They are also used as drugs to treat the most diverse diseases.

Several approaches can be used for the discovery of active small molecules. For this thesis, high-throughput screening (HTS) was applied to test libraries with thousands of compounds, in the attempt to find some with novel and interesting activities. Assays were developed aiming at the discovery of compounds modulating the three stages of the cell life cycle: self-renewal, differentiation and death.

Maintenance of cell self-renewal is regulated by different signaling pathways, such as Wnt and Hypoxia. Homogeneous, luminescent-based HTS reporter assays led to the identification of monensin as an inhibitor of the Wnt pathway and of two compounds activators of the hypoxia pathway. Monensin inhibits the Wnt pathway both *in vitro* and *in vivo* and showed some promise that it might be used as an anticancer drug. While trying to find the molecular target for monensin, a variety of secondary assays were developed and a pipeline suitable to the study of small molecules modulating the Wnt pathway has been created. Similarly, a pipeline is being developed for the Hypoxia pathway based on the two agonists found. So far, these agonists seem to stabilize HIF-1α through inhibition of prolyl-hydroxylase activity, preventing the access of the enzyme to iron. After full *in vitro* characterization of the compounds activity, *in vivo* assays would provide the necessary information about the specificity and toxicity of the compounds to organisms. A mouse model of diabetes is now being used to test the ability of the hypoxia pathway activators to accelerate wound healing, a process dependent on HIF-1α and its target VEGF that are inhibited in diabetes[10]. In the near future we would like to extend our screenings to other pathways involved in cell self-renewal, e.g. the Hedgehog pathway.

The study of small molecules influencing cellular differentiation is more complex than the reporter-based assays and the data acquisition requires special instruments such as an automated fluorescent microscope or flow cytometer. These instruments provide multiparametric readouts and screenings using them are termed high-content screenings. Primary *ex vivo* cells would be the most reliable source for differentiation studies. These cells are often difficult to obtain and the differentiation requires a multi-step procedure, cells pass through different maturation stages and terminally differentiate into the functional cell types. Therefore, assay development for the neural and mesenchymal differentiation

screens are still ongoing. The hematopoietic stem cell differentiation screen is more advanced and hit validation from a pilot screen is underway. So far, one compound was shown to be able to direct the differentiation towards the platelet phenotype, inducing the expression of both CD41 and CD42 and secondary assays are being designed to evaluate its mechanism of action.

Finally, for the discovery of compounds inducing death in a cell-type-specific manner, two viability homogeneous screens were performed and, for each of them, one compound was found with activities in the nanomolar range of concentrations. From the first screen, Homoharringtonine was found to efficiently sensitize resistant cancer cells to their TRAIL-mediated elimination both *in vitro* and *in vivo* while not being harmful to normal cells or the organism within the therapeutic window. From the second screen, DPI was identified as a potent killer of *Leishmania* both in their extracellular life stage (promastigotes) and in their intracellular stage (amastigotes) without harming the macrophage hosts. A mouse model of infection is now being tested for the ability of DPI to kill *Leishmania in vivo*. To extend our studies about compounds involved in pathways controlling cell death, assay development for both high-throughput and high-content screens of compounds influencing the DNA repair machinery are now underway.

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Curriculum vitae

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Selected Publications: (* equal contribution from the authors)

Lucie Tumova*, **António R. Pombinho***, Martina Vojtechova, Jitka Stancikova, Dietmar Gradl, Michaela Krausova, Eva Sloncova, Monika Horazna, Vitezslav Kriz, Olga Machonova, Jindrich Jindrich, Zbynek Zdrahal, Petr Bartunek, Vladimir Korinek (2014). Monensin Inhibits Canonical Wnt Signaling in Human Colorectal Cancer Cells and Suppresses Tumor Growth in Multiple Intestinal Neoplasia Mice. Mol Cancer Ther 18: 812-22.

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Patent: (two other patent applications)

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