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A Novel Strategy to Identify Drugs that Interfere with Endosomal Lipids

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Abstract: Lipids are major components of the cell and, like proteins, exhibit much diversity and are highly regulated. And yet, our knowledge of lipids remains limited primarily because their study is difficult. We will use novel Systems Biology approaches, and in particular high content screening techniques, to investigate the mechanisms that regulate the cellular lipid content and function. Our project is to carry out a small compound screen using lipid imaging techniques to identify conditions that interfere with cellular levels and distribution of cholesterol, lysobisphosphatic acid and phosphoinositol-3-phosphate. This forward chemical genetic screen approach should reveal new molecular tools to investigate the molecular mechanism involved in the regulation of these lipids. The aim is to apply chemical proteomic techniques to identify the molecular target(s) of compounds able to affect the intracellular cholesterol regulation and to assess if these are novel druggable targets. This will be the ideal complementary study to the RNAi screen, currently run in our group, as the effect of the inhibition caused by a small molecule can be rapidly reversed when this is removed. Such a small molecule can be administered to a cell or an animal for a very short time to study the function of the target protein and to look at biological mechanisms in a short time-frame. This project is highly interdisciplinary, and will benefit from the help of the screening core facility, currently developed with the support of the NCCR.

Keywords: Chemical screen · Chemo-informatic · Cholesterol · Endosome · LBPA · Lipids

The Central Role of Cholesterol and Associated Lipids

Lipids are essential components of any life form. They are a main constituent of the cell and the intracellular organelle membranes. They are involved in many cellular and physiological processes like nutrient uptake, cell adhesion, membrane transport, secretion, and hormonal communication. Among all the lipids, cholesterol is one of the central lipids of mammalian cells. It is an essential structural component of cell membranes. It is required to establish proper membrane permeability and fluidity, and it is also an important precursor of steroid hormones and fat-soluble vitamins. This lipid has a central role in mammalian cells and its homeostasis is highly regulated - a cholesterol unbalance is at the core of many pathologies. Yet, the mechanisms of intracellular cholesterol transport are not well understood.[1]

Cholesterol can have several origins. It can be synthesized *de novo* in the endoplasmic reticulum (ER) or be acquired exogenously through uptake. Cholesterol can be transported between organelles through membrane carriers or by lipid chaperone proteins.[2] A mis-regulation of cholesterol transport is associated with the Niemann-Pick disease type C (NPC), in which a lysosomal cholesterol accumulation can be observed due to defects in either the NPC1 or the NPC2 genes.[3] Work from our group also implicates the unconventional phospholipid lysobisphosphatidic acid (LBPA) in cholesterol transport.[4] LBPA is a major component of multivesicular late endosomes, where it is abundant in the intralumenal membranes, and may be involved in multivesicular endosome organization and function.[5] It has been found that interfering with LBPA functions phenocopies the cholesterol storage disorder NPC at the cellular level. Moreover, knockdown of Alix, an ESCRT-associated protein that acts as an LBPA effector, also affects endosomal cholesterol accumulation.[6] Despite several promising new discoveries, it is still difficult to decipher the pathways and roles of the players involved in cholesterol uptake and transport. Therefore, we are planning to follow a systems biology approach to identify new, presumably missing, players and to characterize their functions.

Endocytic Pathway: Current Knowledge

Endosomes play a central role in the vacuolar apparatus of animal cells, at the crossroads between reutilization of components *via* recycling pathways and degradation in lysosomes. Understanding their functions, organization, and dynamics will contribute to the elucidation of the fundamental cellular processes involved in nutrient uptake, immunity, signalling, adhesion, cellular membrane turnover, development and defense against toxins and pathogens. Indeed, endocytosed proteins and lipids first appear in early endosomes, from where they can be recycled to the cell surface or to the trans-Golgi network, or transported towards late endosomes and lysosomes for degradation. Alternatively, endosomal components can also be retrieved and targeted to other destinations, including the extracellular medium, as is the case with lysosome-related organelles and exosomes, or the plasma membrane, *e.g.* class II molecules in antigen-presenting cells. One of our major research interests has been to study the mechanisms that control membrane dynamics in the endocytic pathway, and previous work has uncovered some of the principles responsible for the biogenesis and dynamic properties of endosomes. Lipids play a major role in this process and their regulation is driving the endocytic pathway. Therefore a better understanding of lipid homeostasis regulation is fundamental to decipher this biological process.

A Genetic Screen on Cholesterol and LBPA

To understand the cellular factors directing cholesterol and LBPA homeostasis within the cell, we initiated a genome-wide screen for candidate gene products involved

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in this process. We are using high-content imaging methods to visualize the amounts and distribution of these lipids after protein knock-down by siRNA. This has required the development of significant automated liquid handling capabilities and image processing algorithms needed to acquire the data set, and to subsequently extract the relevant features from the images. We are presently analyzing these measurements through two distinct approaches. First, we are pursuing a candidate-based analysis to identify interesting proteins for further study. Thus, the cellular functions of these candidates are being tested in several assays already established in our laboratory. We hope this method will rapidly yield new insight into the molecular functions of regulators of lipid homeostasis. Simultaneously however, we are approaching the data set from the other direction and are exploring methods to integrate the complete data set into a coherent description of cholesterol and LBPA trafficking and metabolism in the cell. This 'top-down' systems approach attempts to fit the experimental data with relevant data from public databases in order to construct testable models of lipid metabolism and of the endocytic system. We hope that these models will give valuable insight into the complex network of interactions and activities that regulate these dynamic and important processes within the cell.

Importantly, the accuracy and precision of these system-level models is determined by the amount and type of data available. Therefore, it is desirable to have as much relevant data as possible in order to refine and expand our system models. We have therefore recently initiated a screen of small chemical compounds to identify those that impact lipid distribution within the cell.

Finding Small Compounds Able to Perturb Lipids Homeostasis: Project Definition

Our objective is to run a small compound screen and use high-throughput imaging screening techniques to identify new molecules able to affect the basal phenotype. This forward chemical genetic screen approach should reveal new biochemical tools to investigate the molecular mechanism involved in the regulation of intracellular cholesterol. So the aim is to apply chemical proteomic techniques to identify the molecular target(s) of compounds[7] able to affect intracellular cholesterol regulation and to assess if these are novel druggable targets. One of the main advantages of such biochemical tools is that the effect of the inhibition caused by a small molecule can be rapidly reversed when it is removed. Such small molecules can be administered to a cell or an animal for a very short time to study the function of the target protein and to look at biological mechanisms in short time frame.

To facilitate the identification of the proteomic targets of a selected active compound, the screen will be complemented by the genomic study, currently run by our group. Indeed, the particular strength of this project resides in the combination of the genetic and chemical approach to decipher LBPA/cholesterol intracellular regulation. The mechanisms of intracellular cholesterol transport are not clear. Neither is it clear how LDL-derived cholesterol is redistributed intracellularly. From a systems biology viewpoint, this project is likely to provide information on the mechanism that regulates lipid homeostasis, in particular LBPA and cholesterol – two lipids that are linked functionally. Conversely, we also expect to discover compounds that will help characterize the key steps not only in lipid metabolism or transport but also organelle dynamics, and that will lead to the discovery of novel key players in the pathway.

Thanks to this screen and the identification of LBPA/cholesterol perturbed phenotypes, we will try to understand to what extent mammalian cells are actively regulating the homeostasis and the transport of those lipids. The treatment with thousands of compounds displaying a large variety of scaffolds, followed by automated image analysis able to extract hundreds of features for each cell, should allow the identification of the finest perturbation of LBPA/cholesterol localisation and/or quantity. The following bio-informatics analysis will be an essential step to select the most interesting compounds and to initiate the second part of the project. Indeed, the second step of this global analysis will be to identify new players involved and to decipher their roles, the pathways and the mechanisms involved. Moreover, by perturbing this lipid homeostasis we also expect to highlight their role in different biological processes, like general membrane trafficking, endocytosis, secretion, virus or toxin entry, compartment genesis, segregation and maintenance. From that, we also expect to find some new potential biomedical molecular targets and their chemical modulators.

Practically, we expect to identify new phenotypes of affected LBPA/cholesterol localisation and/or quantity in the cell, and find chemical tools able to rapidly perturb the general homeostasis of these lipids. Indeed by using bio-informatics analysis we will try to cluster the phenotypes identified from the two screens (genomic/chemical), to focus on some particularly interesting phenotype showing some biological relevance. This bio-informatics primary analysis should then facilitate the follow-up step of protein target identification and mechanism description, by identifying potential proteomic targets of the compounds. This clustering approach could also allow the discovery of several compounds affecting the same pathway at different levels, allowing then a more complete understanding of the mechanism involved. Such large system biology analysis has never been done on mammalian lipids, despite the fact that we know that these cellular components are central for any life form. We know that the lipids are the main component of the cellular and organelle boundaries, but their regulation remains poorly understood. It is clear today that a full understanding of cells cannot be restricted to protein interaction and gene expression description, and that we will have to penetrate deeper into understanding the mechanisms of lipid regulation. Until now technical limitations have allowed only limited lipid studies, but the new approach we propose here should overcome some of those restraints. For all these reasons; we believed that running such an original study to increase our knowledge in lipids homeostasis regulation will be of great interest for the entire cell biology and biomedical community.

The Compound Screen Methodology and Objectives

High-content screening (HCS) uses microscopy and mostly immunofluorescence techniques. New automated microscopes allow rapid acquisition of a large number of images of experiments run in microtiter plate format. The objective is to identify phenotypes in the distribution or amounts of the molecules of interest within individual cells in correlation to the drug treatment. For this project we will be using the image-based assay platform originally created by SystemsX.ch and the EPFL, and used within the framework of LipidX for our siRNA (genomic) screens. This platform is now extended and further developed with the support of the Chemical Biology NCCR for our compound screens. We will use two specific markers able to recognize LBPA (monoclonal antibody) and cholesterol (filipin) in addition to a nuclear and a cytoplasmic stain to allow cell segmentation. We will also use the running pipeline for image acquisition and analysis, developed for siRNA screens by LipidX (Fig. 1). Several in-house and commercial drug libraries will be tested on HeLa cells. All experiments will be run in the same conditions, followed by image acquisition using optimized and constant parameters. An extensive image analysis will be performed to identify drugs able to affect the LBPA and/or cholesterol amounts and distribution. Currently we can already use a control compound, called U18666A, able to mimic the NPC disease phenotype (Fig. 2A). With this compound we can optimise all the screen assay parameters and the image analysis (Fig. 2B).

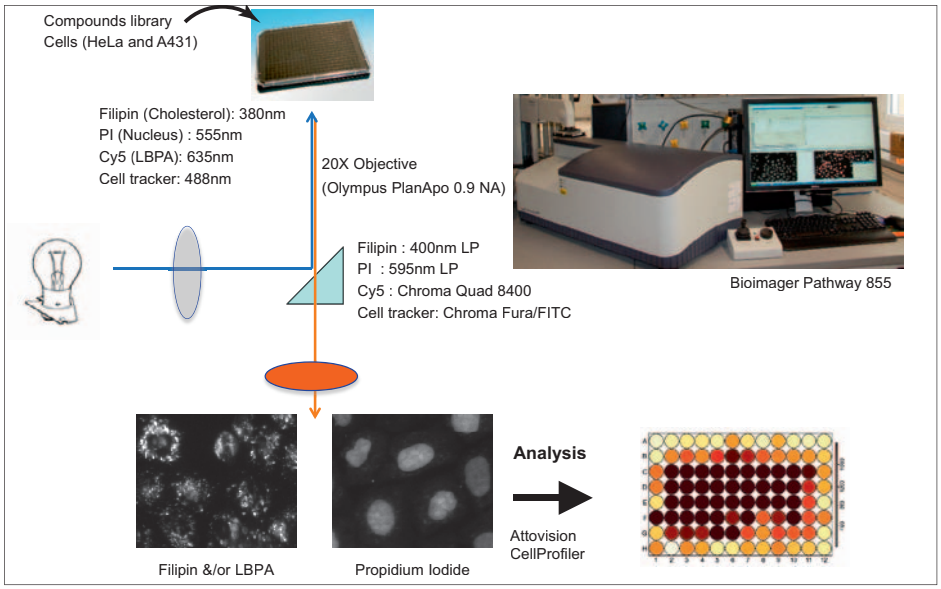

Fig. 1. Pipeline of the screening procedure and the image acquisition and analysis.

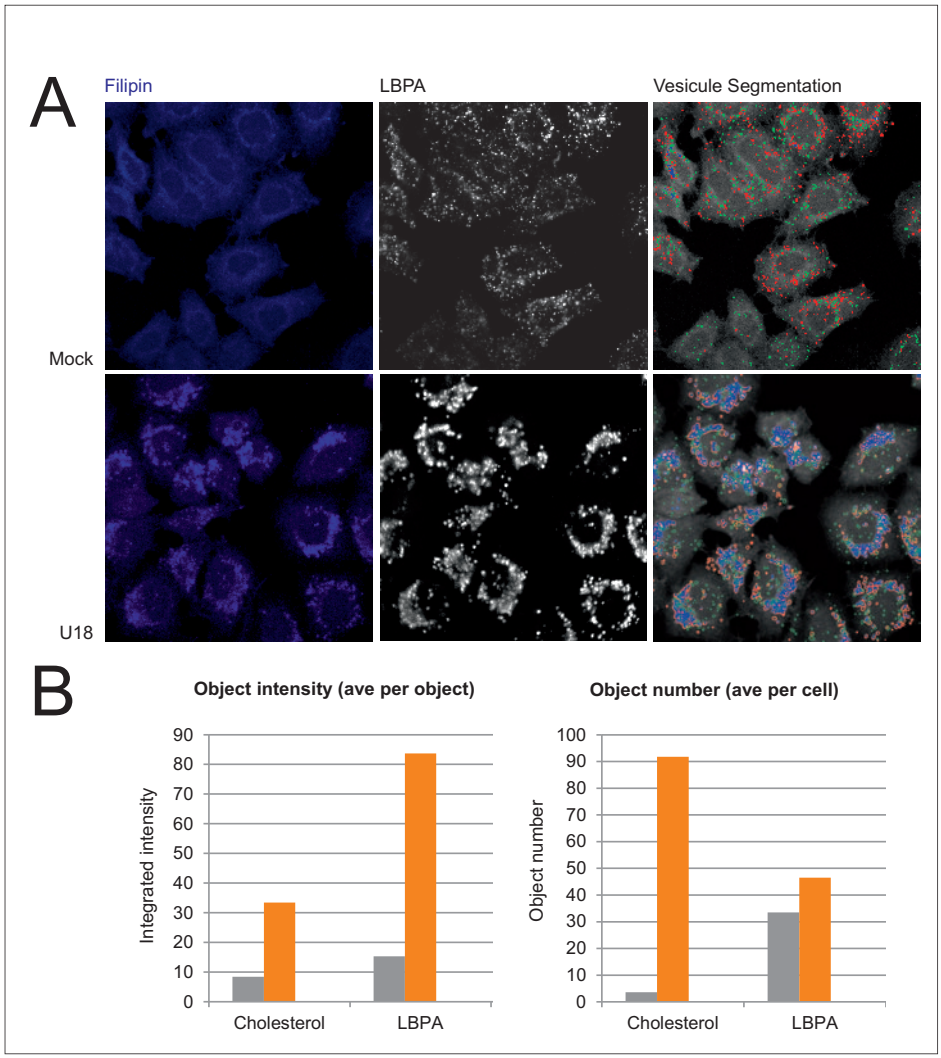

Fig. 2. (A) Immunofluorescence images of human HeLa-MZ cells treated or not with the U18666A drug, stained with filipin (cholesterol) in blue and with LBPA antibody in gray. The U18 treated cells show a clear accumulation of cholesterol and LBPA in the late endosome compartment. (B) Results of the image analysis performed on the images, using Cell Profiler™. The histograms show an increase in the object intensity and number upon U18 treatment.

Several phenotypes will have to be identified to allow a clustering of drugs in relation to their effect on the targeted lipids. The identification and definition of the affected phenotype, using an analysis method based on the high number of features extracted from the images by the analysis software, will be the most challenging part of the screen. The development of dedicated algorithms will be required to allow a deep description of the phenotypes collected from the high-throughput experiment. Based on the bio-informatics analysis of the images we will validate the phenotypic clustering, by correlating the data with meaningful biological hypotheses.

This approach appears currently to be the most powerful and rapid way to decipher biological pathways. Indeed more and more groups are developing systems biology, and the high-throughput screening approach to reveal new component and mechanism of their pathways of interest.

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