Ph.D. thesis

Development and application of novel small molecule-based methods

Eszter Molnár

Supervisor: László Puskás, Ph.D., D.Sc.

Biological Research Centre

of the Hungarian Academy of Sciences

Laboratory of Functional Genomics

University of Szeged

Biology Ph.D. School

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Introduction

Small molecules, i.e. organic compounds with a molecular weight of some hundred Daltons, serve as basic elements of the living cells and in practice they are known as drugs and molecular biology tools (e.g. fluorescent dyes).

Fluorescent dye discovery

Flurescence is utilized by several disciplines including biology and medicine, which apply it for labeling of cells and cellular elements. Fluorescent proteins and small molecule dyes (fluorochromes) are the most frequent labeling agents. The latter ones possess such advantageous properties like high membrane permeability, less or no toxicity, minimal technical limitations and moderate cost.

Currently rational design and high throughput screening (HTS) techniques are the main sources of novel dyes. Small molecule microarray is one of the HTS methods: chemical libraries containing a large number (even tens of thousands) of compounds are immobilized on a solid surface in order to get in touch with a potential binding partner (usually a protein). Since detection of the interaction is based on the fluorescent labeling of the protein, small molecules showing native fluorescence ought to be excluded from these studies, but they still have the potential to be used as fluorescent probes.

By confocal laser scanning microscopy (CLSM) properties of living cells, organelles and even single molecules can be analyzed. However, time-consuming manual analysis of images makes traditional microscopy an unfeasible method for rapid screening of multitudinous samples.

Combination of the advantages of SMM and CLSM enables the discovery of new fluorescent dyes among tens of thousands of compounds.

Affinity chromatography

Target identification by affinity chromatography

Target identification is a critical step in drug development for understanding the mechanism of action and possible toxic effects of the candidate therapeutic agent. Affinity chromatography is a suitable technique for the determination of these target proteins: drug candidate immobilized on the resin fishes out interacting proteins from a mixture flowing through the resin.

Ligand immobilization on affinity resins has the same controversies (necessity of synthesis, improper orientation of the coupled molecule) like small molecule microarrays do, hence AviLink surface chemistry could be applied on the preparation of affinity matrices also.

Improving the efficiency of affinity chromatography by removing nonspecific proteins

In complex biological samples the nonspecific binding of abundant proteins to small molecule-immobilized matrices makes the detection of low-abundance target proteins fairly challenging. Various approaches have been tried to overcome this problem: competitive elution, parallel application of active and inactive forms of the ligand, serial affinity chromatography, surface modifications, spacer induction or immunoaffinity-based depletion columns used in plasma proteomic studies. In the latter case even specific small molecules could be applied instead of antibodies.

Highly abundant cytoskeletal proteins such as tubulin and actin tend to bind nonspecifically to affinity matrices. It is established that benzimidazoles bind to the tubulin, hence a benzimidazole-coupled resin seems to be useful for pre-purification of cell and tissue extracts.

Aims

1. Combination of SMM and CLSM for the discovery of new fluorescent dyes, and investigation of these dyes.

2. Testing AviLink surface chemistry in the preparation of affinity resin and target identification.

3. Development of a new depletion resin for binding abundant tubulin and facilitating the identification of low-abundance intracellular drug targets.

Methods

1. Preparation of small molecule microarrays
2. Fluorescent signal detection and data analysis
3. Cell culture treatment
4. Confocal laser scanning microsopy
5. Total protein extraction from cultured cells and different tissues
6. Preparation of Ac-2010 affinity resin
7. Affinity chromatography
8. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)
9. Mass spectrometry analysis
10. Measurement of protein disulfide isomerase enzymatic activity
11. Preparation of 2-aminobenzimidazole affinity resin
12. Determination of the carbon content and calculation of the surface coverage
13. Depletion of nonspecifically binding proteins using 2-aminobenzimdazole resin
14. Two-dimensional gel electrophoresis

Results

Combination of small molecule microarray and confocal microscopy techniques for live cell staining fluorescent dye discovery

1. 14585 diverse compounds were immobilized in microarray format and their fluorescent signals were detected by using a laser scanner. In the course of data analysis 278 small molecules showing strong fluorescent emission were selected for microscopic live cell investigation.

2. CLSM was used to eliminate cell impermeable, toxic, low solubility and poorly fluorescent chemicals. The remaining 11 fluorescent dye candidates were further investigated.

3. Intracellular localization of the fluorochromes was determined by colocalization with known fluorescent markers: selected chemicals were *in vivo* localized to lipid droplets, mitochondria, plasma membrane, perinuclear/cytoplasmic region and one of them showed relocalization from mitochondria to cytoplasm/nucleolus due to repetitive laser-excitation.

4. The effect of the 11 molecules on long-term viability of cultured cells was also assessed. During a 24 hours-treatment none of the selected chemicals showed toxicity, hence these dyes are also suitable for long-term experiments.

Affinity chromatographic application of small molecules immobilized on specific surfaces

Preparation of an affinity resin by using AviLink surface chemistry and its application in target identification

1. Ac-2010 compound was covalently attached to activated surface by using the AviLink technology.

2. The Ac-2010 resin was applied on total protein extract in affinity chromatography, then specific binding proteins were identified by mass spectrometry resulting 30 hits.

3. Based on previous results protein disulfide isomerase (PDI) was selected for further investigation: the effect of Ac-2010 on PDI activity was tested by insulin turbidimetry. Ac-2010 inhibited the enzyme at a concentration of 1 μM. IC_{50} was 3.158 μM.

Removal of nonspecific binding proteins from cell and tissue extracts using 2-aminobenzimidazole-tethered affinity resin

1. A 2-aminobenzimidazole-tethered depletion resin was prepared: reactive silanized surface was synthesized for the basis of the resin, then isophtalic acid was built in as a spacer for the benzimidazole group.

2. By analyzing the carbon content of the products from each reaction step we can conclude that half of the active groups of the silanized surface was successfully derivatized with 2-aminobenzimidazole.

3. In order to evaluate the efficiency of the resin, it was used to deplete total cell and tissue protein extracts, respectively. Following two-dimensional gelelectrophoresis proteins bounded by the resin were identified by mass spectrometry (20 from A549 cell and 16 from brain sample). Mainly cytoskeletal components (tubulins and actins), members of the 14-3-3 family and heat shock proteins were detected.

4. In both depleted samples appearing of new proteins as well as an increase in the amount of some proteins were observed in the flow-through fraction, which are ascribed to the depletion of some nonspecific binders.

Conclusions

1. Combination of SMM and CLSM proved to be a suitable method for the discovery of new, live cell-staining fluorescent probes and yielded 11 novel fluorochromes.

2. AviLink technolgy is suitable for the preparation of small molecule affinity resins and the identification of small molecule interacting proteins accordingly.

3. Protein disulfide isomerase binds to Ac-2010, and this binding affects its enzyme activity.

4. The 2-aminobenzimidazole resin applied on crude protein samples binds tubulin and some other abundant proteins, therefore it can facilitate the identification of intracellular drug targets.

Publications

*Publications directly related to the thesis.

Articles published in refereed journals:

1. Molnár E, Hackler L, Jankovics T, Ürge L, Darvas F, Fehér LZ, Lőrincz Z, Dormán G, Puskás LG. Application of small molecule microarrays in comparative chemical proteomics. QSAR Comb Sci. 2006 Nov;25(11):1020-6.

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2. Ménesi D, Kitajka K, **Molnár E**, Kis Z, Belleger J, Narce M, Kang JX, Puskás LG, Das UN. Gene and protein expression profiling of the fat-1 mouse brain. Prostaglandins Leukot Essent Fatty Acids. 2009 Jan;80(1):33-42.

IF: 2.530

3. Puskás LG, Fehér LZ, Vizler C, Ayaydin F, Rásó E, **Molnár E**, Magyary I, Kanizsai I, Gyuris M, Madácsi R, Fábián G, Farkas K, Hegyi P, Baska F, Ozsvári B, Kitajka K. Polyunsaturated fatty acids synergize with lipid droplet binding thalidomide analogs to induce oxidative stress in cancer cells. Lipids Health Dis. 2010 Jun 2;9:56.

IF: 2.14

***4. Molnár E**, Fábián G, Klem J, Darula Z, Hunyadi-Gulyás E, Medgyesi A, Medzihradszky KF, Puskás LG. Removal of nonspecific binding proteins from cell and tissue extracts using 2-aminobenzimidazole-tethered affinity resin. Pharmazie. 2011 Sep;66(9):662-5.

IF: 1.006

***5. Molnár E,** Kuntam S, Cingaram PKR, Peksel B, Suresh B, Fábián G, Fehér LZ, Bokros A, Medgyesi Á, Ayaydin F, Puskás LG. Combination

of small molecule microarray and confocal microscopy techniques for live cell staining fluorescent dye discovery. Molecules. (submitted in 2013, co-first author)

***6.** Nagy LI, **Molnár E**, Kanizsai I, Ózsvári B, Fehér LZ, Fábián G, Marton A, Vizler C, Ayaydin F, Kitajka K, Hackler L, Mátés L, Deák F, Kiss I, Puskás LG. Lipid droplet binding thalidomide analogs activate endoplasmic reticulum stress and suppress hepatocellular carcinoma in a chemically induced transgenic mouse model. Curr Pharm Design. (submitted in 2013)

Patents:

1. Title: Active support, method for preparation of the active support and application of the active support. ID No.: P0600668 Submitted: 2006.08.22.

2. Title: Compounds for labeling of lipid droplets, compositions for labeling of lipid droplets, and method for visualization of cells and/or cellular elements. ID No.: P0700432 Submitted: 2007.06.21.

3. Title: Compounds affecting formation and/or function of cellular vesicular systems, particularly lipid droplets, therapeutical compositions containing these compounds, and application of these compounds for treatment of disease states. ID No.: P0700433 Submitted: 2007.06.21.

4. Title: New affinity surface for purification of proteins, preparation and application. ID No.: P1000515 Submitted: 2010.09.23.