B10. Omics and systems biology

Lectures

L18.1

On the complexity of life

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Life has evolved from elementary particles and atoms through simple chemical molecules and macromolecules to the basic entity (unit) of life — the cell. Cell functions are mediated through dynamic and complex system of molecules. Cell exhibits the ability to interpret potential information contained in the genome, and uses it accordingly with actual needs and in "context". A complex system is composed of parts but it is not formed by a simple linear summation of parts, and the parts are not functioning in a linear mode, but rather acting according to complex algorithm rules. In the process of complexity formation new functional and structural properties emerge (termed "emergents") that are not present in parts. Therefore, deterministic prediction of the properties and behaviour of complex "whole" is not possible. Emergence, a characteristic trait of complex systems creates a serious limitation for explaining the behaviour of the complex "whole" by reductionistic approach. The system as "the whole" exercises some constraint on its parts. Biological functions result rather from the activity and interaction of macromolecules (proteins, DNA, RNA, metabolites, etc.) than from activity of individual molecules. Interacting molecules (e.g. proteins) form in a cell highly complex dynamic networks. Some properties, rules, and behavior of such networks will be discussed. Network formation is connected with self-organization properties of chemical molecules, while self-assembly is observed mainly in the course of formation of structural parts of the cell like microfilaments, membranes, ribosomes, etc. All living organisms reveal distinct hierarchical organization from basic level (atoms, molecules) to cells and multicellular individuals. Complexity is increasing with the increased hierarchical levels. However phenomena of life on higher level of hierarchy can not be explained or reduced to characteristics and properties of chemical molecules but by interaction between them and by integration of various molecular processes at lower levels of hierarchy. More detailed view on hierarchical organization of the various levels of hierarchy in humans and the emergence of highest human abilities belonging to the broad level of culture are discussed.

L18.2

Mass spectrometry imaging of lipids

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Mass spectrometry imaging (MSI) has been recognized as emerging biomolecular visualization tool particularly useful for biomarker discovery. For both small and/or higher-mass molecular weight compound distribution studies no labelling is needed and even more importantly, each pixel contains qualitative structural data. The attractiveness of MSI is well documented by frequency of published papers which recently reaches 1 manuscript per day. Although the method itself is quite old (it was first presented in SIMS-MSI format by a French group in 1962), it has been rediscovered in the mid of 1990s in MALDI-MSI format. Due to dynamic range issues protein mass spectrometry imaging has not achieved the companies' expectations and promises in protein biomarker diseases diagnosing (in fact, there are just three protein cases discovered by MSI approach that have consequently been validated in clinical studies). On the contrary, successful small molecular weight compound analysis is demonstrated in this talk on the distribution analysis of glycerophospholipids and sphingolipids in porcine eye lens. In addition, the distribution of globosylceramides in a kidney of knock-out mice suffering from Fabry disease is reported. In addition to basic principles of mass spectrometry imaging, both commercial (MALDI, DESI) and experimental (NALDI, DAPPI) ionizing tools are compared and selected tricks in sample preparation protocols disclosed. The identification of lipid species was carried by using in-house developed software tool mMass (www.mmass.org). Catalytic properties of nanostructures in NALDI analyses are reported and the importance of cationization, dynamic range and polarity issues are emphasized.

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L18.3

Comprehensive human proteomics by high resolution peptide isoelectric focusing prefractionation and mass spectrometry (hrIEF-LC-MS/MS)

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To find disease relevant proteome changes, the analytical depth has to reach sufficient level to detect and quantify key regulatory proteins. Up to date many human proteomics studies both on cell lines and clinical material have failed to reach that level of proteome coverage leading to lack of clinically relevant results. By coupling a high resolution peptide level isoelectric focusing fractionation to high resolution mass spectrometry, we demonstrate comprehensive human cancer proteomics. This data is related to pathways driving tumour growth and metastasis in breast and lung cancer.
The present work, we use of high resolution peptide isoelectric focusing using IPG strips optimized for peptide fractionation for mass spectrometry on narrow pH regions. Semi-automated workflow is set up where the fractionated peptides are eluted from IPG strip using in-house built robotics to 96-well plates, freeze dried and re-suspended prior LC-MS/MS analysis using Orbitrap Velos MS. Six cancer cell lines and tumour samples from lung, breast, vulvar and adrenal cancer samples were profiled using this workflow.
The compatibility of peptide IEF together with isobaric labelling for quantitative clinical proteomics was demonstrated [1]. The method was successfully applied to membrane proteomics [2], to study off-target effect of gene delivery systems [4] and plasma and pleural effusion proteomics in lung cancer samples [3]. Here, we demonstrate greatly improved proteome coverage on cell lines and tumour samples using high resolution peptide IEF fractionation. Application of the method on pathway regulation of oncogenic pathways related to tumor response to endocrine therapy in breast cancer is demonstrated.
Conclusion: Using high resolution peptide isoelectric focusing as pre-fractionation prior LC-MS/MS, we can reach proteome coverage enabling detailed cancer pathway studies both on cell lines and on clinical tumour material.

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L18.4

From data to knowledge

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Chemistry appears in the center of natural sciences and analytical chemistry as the key discipline providing an insight in the qualitative and quantitative composition of the matter. This insight can be gained indirectly from a wide number of instrumental analytical techniques. Consequently, an immediate outcome of all these approaches assumes the form of instrumental signals. These signals can be vectors containing thousands of data points or matrices containing millions of elements.
Instrumental signals can play the role of fingerprints and be used for a comparison of numerous samples. For instance, in supervised settings they can be used for construction of calibration or classification models.
At this point statistics and chemometrics enter our story. Without these tools navigation in the sea of data would not be possible. Their role will be demonstrated upon the example taken from medical diagnosis and based on proteomic study. All steps of data analysis will be discussed and the author will point out to similarities and differences in statistical and chemometrical standpoints.

Oral presentations

O18.1

Peptide arrays as detection devices for proteolytic enzymes

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Recently, assays using peptide arrays, where peptides are immobilized on the solid support, became popular as research tools. Arrays of peptides allow for simultaneous screening of thousands binding events in biological and pharmaceutical areas. Because many proteins and enzyme activities are directed towards peptides, such preparation of arrays offers an unprecedented opportunity for identification of proteins and their interactions [1].
Several methods have been reported for the preparation of peptide arrays. Such systems can be obtained by assembling peptides on the surface or by covalent immobilization onto the chip. Covalently immobilized peptides can be attached directly to the surface or through polymeric linker. It was shown that the presence of polymer between surface and peptide provides many advantages [2]. It minimizes non-specific binding of biomolecules on the surface, provides low background in fluorescence measurement, allows avoidance of steric hindrance, and thus makes linked peptide fully accessible for biomolecules.
Proteases are a large group of enzymes, which regulate protein functions in the body. These enzymes are involved in a multitude of physiological reactions from simple digestion of food proteins to highly regulated cascades [3]. Aberrations in protease expression or function are, therefore implicated in many pathological conditions, such as cancer, arthritis, and Alzheimer's disease. Peptide arrays constructed towards particular protease and MS detection of specific enzyme-peptide reaction products allowed for enzyme identification in biological samples.
In this work, conjugate of peptide and poly(ethylene oxide) were synthesised on the solid support using Fmoc chemistry, and then covalently immobilized by grafting-to method on the silica surface. Silica plates before grafting were modified by 3-aminopropyltriethoxysilane and characterised by AFM and contact angel measurement. The obtained peptide arrays were incubated with enzymes and products of these reactions were analysed by ESI MS.

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O18.2

S-glycosylated (poly)peptides a novel target for proteomics

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N-and O-glycosylation is widely distributed, major modification of proteins. Much less common C-glycosylation is well-documented while S-glycosylation of natural (poly) peptides had been controversial. Only few works reported on this unique and ambiguous modification (Lote *et al.*, 1971; Weiss & Lote, 1971; Olsen *et al.*, 1998), yet finally in January 2011 its existence in Nature was announced.
Complete structural studies of two well-known antibacterial peptides — sublancin from *B. subtilis* 168 and bacteriocin from *L. plantarum* — revealed that both of the peptides possess the sugar moiety (respectively β-D-Glc and β-D-GlcNAc) covalently attached to the sulfur atom of cysteine residue (Oman *et al.*, 2011; Stepper *et al.*, 2011; Venugopal *et al.*, 2011). These small glycans are essential for their antimicrobial activity. Moreover, a novel cysteine-specific glycosyltransferase has been discovered and characterized directing further bioinformatics research. Its result suggests that the existence of thioglycosides in natural proteins and peptides might be more common than previously thought. To verify this conclusion an efficient proteomic method for S-linked glycan detection is needed. Here we present selected results of the project (Buchowiecka, 2010) aimed to develop such a method. Hen egg white lysozyme was chemically transformed into a very complex set of its randomly S-β-D-glucosylated derivatives which were characterized by LC-ESI MS. After tryptic digestion the model compounds yielded a mixture containing structurally diverse model S-glucosylated peptides. Their fragmentation feature was characterized by CID, HCD and ETD high-resolution tandem MS. S-glycosidic bond is moderately stable under standard CID/HCD ionisation and can be detected and localized both by hexose loss, as well as by analysis of backbone fragmentation pattern. Such well-characterized model mixture served as a starting point to elaboration of the S-glycosylation detection method that is based on a sequence of new robust chemical transformations. The method is targeted to high-throughput proteomic application and will be published soon.

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O18.3

Lipidomics of the dietary fatty acids induced formation of lipid droplets (LD) in human preadipocytes (SVF) and endothelial cells

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Insulin resistance — induced hyperglycemia and excess of circulating free fatty acids (FFA) is the hall-mark of glucolipotoxicity and lipid droplet (LD) formation in cells not devoted to metabolic substrate storage. Mitochondrial substrate overload induces free radical formation, endoplasmic reticulum (ER)-stress, changes in mitochondrial membrane potential ($\Delta\Psi$), and LD formation. The fluctuating changes in mitochondrial membrane potential (MMP) induces autophagy promoting lysosomal degradation of modified proteins and lipids — the important mechanisms for cell survival and protection from apoptosis and cellular death. Dietary FFAs (PA, OA, except EPA) induced formation of different to SVF (TG-rich) type of LD than in endothelial cells (phospholipid-rich).
The bioinformatic analysis of microarray (Agilent) and lipidomic (MS/MS) results revealed an different (in comparison with the other FA) ability of EPA to induce the ER-stress, apoptosis, angiogenesis related gene clusters. All used FFAs, activated autophagy — related genes (ie charperones, LAMP2) expression. Additionally AA induced the lipodystrophy gene Lpin what may explain the decreased accumulation of LD in SVF in presence of this FA. Thus EPA protects endothelial cells against the FFA-induced lipotoxicity by accelerating glucose uptake and metabolism, protects against mitochondrial impairment, ER stress and in consequences against the stress-induced LD phospholipidosis, cellular dysfunction and death. The effect of changes in sphingomyelin, ceramide and phospholipid composition (length in FFA) is noticed.

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O18.4

New intracellular FGF1 and FGF2 binding proteins identified by proteomic approach

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Fibroblast growth factors 1 and 2 (FGF1/FGF2) are secreted proteins stimulating specific cell-surface receptors. In contrast to other growth factors they also possess a unique ability to translocate into the cytosol and nucleus. Despite many years of research the intracellular role of these growth factors remains unknown and only a few binding partners of both growth factors were identified. Here, we present a proteomic approach to identify novel FGF1 and FGF2 intracellular interacting proteins.
Purified recombinant FGF1 and FGF2 proteins with different tags were used to pull down binding partners from HEK 293 and NIH 3T3 cell lysates. "Fished out" proteins were separated by SDS/PAGE and identified by mass spectrometry. Subsequently we confirmed the interactions of identified proteins with FGF1 and FGF2 by pulldown experiments followed by Western Blotting. Several proteins were also checked for direct interaction with FGF1 or FGF2 by using purified recombinant binding partner for pulldown assay and surface plasmon resonance. Using proteomic approach accompanied with different methods we identified novel FGF1 and FGF2 binding proteins including Hsp90, SFPQ and MYL9.

Posters

P18.1

The dityrosine cross-link as an intrinsic donor for assembling a donor-acceptor pair in the study of protein structures

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Förster resonance energy transfer (FRET) is a distance-dependent interaction between the electronic excited states of two dye molecules in which excitation is transferred from a donor (D) molecule to an acceptor (A) molecule without the emission of a photon. FRET can be detected by quenching of the fluorescence of the donor. A single tryptophan residue, which is relatively rare in proteins, is the dominant intrinsic protein fluorophore in the D-A pair. Juvenile hormone binding protein (JHBP) has been identified in insect hemolymph as a specific carrier of juvenile hormone (JH), a key hormone that regulates the life cycles of the insects. The amino acid composition of mature JHBP showed, however, no tryptophanyl residues. A direct comparison of the spectroscopic properties of *G. mellonella* JHBP with the properties of simple proteins which lack Trp residues suggested that JHBP contains some unusual chromophore molecules, because it exhibits additional fluorescence with a maximum near 420 nm, after excitation at 315 nm. UV spectroscopy studies, as well as immunoblotting, revealed that the changes which occurred in the JHBP fluorescence spectrum were associated with the formation of Tyr-Tyr (dityrosine; DT) bridges. Using MS analyses, we found that DT formation occurs intramolecularly between Tyr residues Tyr128 and Tyr130 located in the putative JH-binding pocket. The question arises whether DT can be used as an intrinsic donor in FRET studies. Toward this aim, mutational analyses and FRET measurements were performed. It was shown that labeling JHBP molecules with *Lucifer Yellow* (LY) iodoacetamide created a D-A pair in which the fluorescence of DT was quenched.
Several of our observations suggest that there is a conformational transition of the JHBP molecule upon JH binding. Such a conformational change was also observed using a DT-LY pair. Our experiments provide new evidence about changes in the JHBP structure upon ligand binding. Our study also demonstrated the feasibility of the FRET technique using DT cross-links as the intrinsic donor for the FRET pairs. No studies on the usage of DT as a FRET donor to monitor conformational changes in proteins have been reported until now.

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P18.2

Genetic characterization of novel plasmid from *Corynebacterium tuberculostearicum*

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Corynebacteria is a wide group of bacteria which are gram-positive, asporogenous, pleiomorphic and have G+C content ranging from 50% to 71%. Within this group we can distinguish nonpathogenic bacteria such as: *Corynebacterium glutamicum*, *Corynebacterium callunae*, pathogenic bacteria: *Corynebacterium diphteriae*, *Corynebacterium pseudotuberculostearicum*, *Corynebacterium ulcerans* pathogenic for plants: *Corynebacterium nebraskense*, *Corynebacterium michiganense* and wide group of opportunistic pathogens: *Corynebacterium jejkeium, Corynebacterium urealyticum*, *Corynebacterium striatum*.
In our work we described a small 4.2 kb, medium copy cryptic plasmid derived for the first time from *Corynebacterium tuberculostearicum*. Computer analysis and found patterns of RepA protein allow us to claim that this plasmid replicates by rolling–circle mechanism. Neither of predicted by GeneMark ORFs nor additionally calculated small ORFs had properties to be antimicrobial peptide. Quantitative RT-PCR made possible to detect RNA product in the range of each predicted ORF, which implies presence of unknown genes. One from predicted ORF can be identify as FtsK-like protein. Conserved domain search of this ORF provides additional two transmembrane domains and signal peptide at the beginning. The exact function of this FtsK-like protein remains unclear. The role of small cryptic plasmids in *Corynebacterium* sp. is still unknown. Plasmid p1B146 can also be used as framework for shuttle vector construction for further genetics research within *Corynebacterium tuberculostearicum*.

P18.3

Nucleolin, major vault protein and PAX3 — new interacting partners for FGF1 and FGF2

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FGF1 and FGF2 are the members of fibroblast growth factor family and are involved in different biological processes. They play role in proliferation, cell growth, cell survival and morphogenesis by binding to and activating specific cell surface receptor. In addition they possess the ability to translocate across cellular membrane into the cytosol and nucleus. Despite many years of research, their intracellular functions remain elusive.
To better understand the role of FGF1 and FGF2 inside the cell and to identify potential binding partners of FGF1 and FGF2, "pull down" experiments followed by mass spectrometry analysis were performed. Based on proteomic approach we selected three "fished out" proteins for further analysis: nucleolin, MVP and PAX3. We verified their potential interaction with FGF1 and FGF2 by "pull down" experiment from BJ and HEK 293 cell lysates, by *in vitro* binding of recombinant proteins and by surface plasmon resonance method. These three techniques provide coherent results indicating that nucleolin, MVP and PAX3 are the novel binding partners of FGF1 and FGF2, shedding a new light on intracellular function of growth factors.

P18.4

From germ cell cysts to primordial follicles in the mammalian ovaries. Ultrastructural analysis of early germ line cells of the bat, *Carollia perspicillata*

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In the 19th century, Brandt described two types of ovaries: panoistic and meroistic. In the panoistic ovary, all of the oogonia become the oocytes. Among vertebrates, the most detailed descriptions of the formation of the germline cyst and of the differentiation of the oocyte exist for *Xenopus laevis* (Kloc *et al*., 2004) and mouse. (Pepling *et al*., 1998). In *Xenopus*, four synchronous divisions of the cystoblast result in the 16-cell germ-line cyst. Each cyst is eventually partitioned into 16 identical oocytes. In both species the cytoplasmic projections of somatic cells of the gonad penetrate between the cystocytes, which causes the breakage of the intercellular bridges and the separation of the individual oocytes. As a result, the individual oocytes are surrounded by a single layer of somatic cells, and these structures constitute the primordial ovarian follicles. The aim of my studies was to describe the morphology and ultrastructure of early germ line cells in ovaries of bat, *Carollia perspicillata*. The ovaries of early embryos (40 dpc) contain numerous germ-line cysts which are composed of 10 to 12 sister germ cells (cystocytes). The serial section analysis showed that cystocytes are interconnected by atypical, strongly elongated and short lived intercellular bridges rich in microtubule bundles and microfilaments. It seems very likely that these cytoskeletal components are the remnants of the midbody. During later stages of embryonic development (44-46 dpc) the somatic cells penetrate the cyst, and their cytoplasmic projections separate individual oocytes. Separated oocytes surrounded by the single layer of somatic cells constitute the primary ovarian follicles. The oocytes of *C. perspicillata,* similar to mouse oocytes, are asymmetric (Kloc *et al*., 2008). In both species, this asymmetry is clearly recognizable in the localization of the Golgi complexes which were located in one zone of the ooplasm. The presence of germ-line cysts and intercellular bridges (even atypical) in fetal ovaries of *C. perspicillata* indicate that the formation of germ-line cysts is the evolutionarily conserved phase in the development of the female gamete throughout the animal kingdom.

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P18.5

Comparison of binding of 44 transcription factors to putative regulatory regions identified by histone modifications, open chromatin and conservation

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We designed and implemented a database, named TRAM (Transcription Regulatory Areas and Motifs database), unifying representation of the data from Ensembl schemas relevant for cis-regulation (funcgen, compara and core) to the analysis layer. Among the key features of TRAM are pre-computed data on pairwise overlap on the genomic sequence of putative regulatory areas of different types, in several different cell types.
Here, we report analysis of pairwise overlap among 41 histone modifications, 44 transcription factors (TFs), two markers of open chromatin, and regions of sequence conservation from two algorithms. We focused on the overlap between the chip-seq data for the 44 transcription factors represented in funcgen v.60 and the remaining putative regulatory regions. We found that the binding of TFs is highest in regions of histone modifications associated with active promoters (H3K4me2-3, H3K9ac, Pol II), high in the regions of modifications associated with active enhancers (H3K27ac) and in cis-RED promoters; and low in the regions associated with poised enhancers (H3K27me1-3). We report a notable difference between the high binding of TFs to the regions of open chromatin identified by DNase-seq and lower binding to the regions identified by FAIRE. TF binding to the regions identified by sequence conservation was lower than to the regions identified experimentally. Interestingly, it was also low for the 400 VISTA enhancers verified experimentally, confirming that enhancer occupancy by TFs is cell and developmental stage specific.
The highest conservation of the same feature localization in different cell types was found for the markers of active promoters (H3K4me2-3) and insulators (CTCF); intermediate conservation for regions identified by Dnase1 (but not FAIRE) and markers of active enhancers (H3K27ac); whereas localization of histone markers of poised enhancers (H3K27me1-3), as well TFs binding was variable among the different cell types. To conclude: TF binding correlates well with regions of regulatory activity computationally predicted from gene expression data. TF binding is higher, and more conserved across cell types, in promoters than in enhancers. Regions of open chromatin identified by Dnase1 are more enriched in TF binding than regions identified by FAIRE.

P18.6

Endosymbiotic microorganisms in aphids (Insecta, Hemiptera: Aphidinea)

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Aphids like other plant sap-sucking hemipterans, possess intracellular symbiotic microorganisms (Buchner, 1965). Endosymbiotic microorganisms are harbored in the cytoplasm of specialized, poliploid cells termed bacteriocytes which are localized in the close neighborhood the ovaries. Bacteriocytes may contain primary endosymbionts (P) or secondary endosymbionts (S) (Buchner, 1965). The presence of primary endosymbionts in aphids is connected with their restricted diet. Aphids feed on phloem sap which is rich of carbohydrates but deficient in essential amino acids. The P- endosymbionts are responsible for synthesis of amino acids. Apart from primary endosymbionts most aphids species have bacteriocytes with secondary endosymbionts. Whereas P-endosymbionts are nessesary for survival and reproduction of aphids, the role played by S-endosymbionts remains still unknown. Probably they may protect aphids against heat stress (Montlor *et al*., 2006) as well as against attack of parasitic hymenopterans (Scarborough *et al*., 2005).
The members of advanced aphid families (except family Hormaphididae) possess primary endosymbionts belonging to the species *Buchnera aphidicola* (γ-Proteobacteria). These bacteria are surrounded by three cell membranes: two own membranes and host-derived membrane (termed perisymbiotic membrane). Endosymbionts are transovarially transmitted from mother to offspring. The begining of the invasion is correlated with the development of female germ cells. In viviparous generations bacteria infest young embryos whereas in oviparous generations mature oocytes are invaded. In viviparous females bacteria enter the embryo and invade the bacteriocyte cytoplasm. In oviparous females bacteria migrate through the follicular epithelium. Next, these microorganisms locate in the cytoplasm of the posterior pole of the oocyte and form a characteristic "symbiont ball".

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P18.7

Ultrastructure and vertical transfer of bacterial endosymbionts in psyllids (Insecta, Hemiptera: Psylloidea)

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Psyllids, like other hemipterans, are plant sap-feeding insects that harbor prokaryotic endosymbionts in their body. The endosymbionts are localized in the cytoplasm of huge, poliploid cells termed bacteriocytes. Bacteriocytes form large aggregates called bacteriomes which are located in the vicinity of gonads. Psyllids contain in the body cavity two types of ultrastructurally distinct bacteria: primary endosymbionts (P-symbionts) and secondary endosymbionts (S-symbionts). Function of primary endosymbionts is the synthesis of essential amino acids which are missing in the diet of insect. Since an association between bacteria and host is obligatory, the primary endosymbionts are present in all the examined psyllids. Secondary endosymbionts are less numerous than primary endosymbionts. Their function remains unknown. Endosymbionts are transmitted from one generation to the next transowarially (vertically), i.e. via the female germ cells. Before infection, bacteria leave the bacteriocyte cytoplasm and start to migrate towards developing oocytes. Next, enter the ovaries. They pass through the cytoplasm of follicular cells and enter the perivitelline space (space between oocyte and follicular epithelium). Simultaneously, oolemma starts to form a deep invagination in which endosymbionts gather. The bacteria do not infect the oocyte until the end of its growth.

P18.8

Comparison study on the biclustering techniques in HTS data analysis

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Background: Recent high-throughput screening (HTS) developments have resulted revolution in biomedical research and have allowed on biological systems exploration in global view. In result quantities of data have increased, which analysis using conventional tools has become difficult. One of the main goals in such datasets analysis is to discover local structures composed by features and conditions subsets. These patterns may help in the process of interpreting and understanding the complex biology mechanisms that are underlying experimental data.
Results: In this work we present nonnegative matrix factorization (NMF), a relatively new, powerful approach to clustering features and conditions highly related in subportions of the data, based on decomposition by parts that can reduce the dimension of large scale data. We shown advantages this method over other conventional clustering techniques, such as hierarchical clustering (HC). We demonstrate also the properties and potential of this methodology analyzing synthetic datasets, microarray Golub dataset and large set of proteomics data. We obtained a good quality of classification in genomics dataset and similar quality proteomics data classification to supervised classification method. The method was able to identify localized patterns related to subsets of features that show consistent expression patterns across subsets of conditions. Moreover we compare two well known groups of NMF algorithms: one group based on multiplicative update rules and second based on gradient methods.
Conclusions: Our study establishes a high potential of nonnegative matrix factorization algorithms to analyze large and heterogeneous datasets. The methods are able to identify complex relationships among features and conditions that are difficult to identify by using standard clustering algorithms.

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P18.9

Limited proteolysis assisted by MS — a new tool to verify protein-protein complex formation

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Human acidic fibroblast growth factor (FGF-1) belongs to a large family of growth factors that binds to transmembrane receptors with a cytoplasmic tyrosine kinase domain. After binding to FGF receptor (FGFR) and its activation, FGF-1 can be translocated across the membrane into the cytosol and further into the nucleus. As FGF-1 is a powerful mitogen of potential medical interest, implicated in morphogenesis, angiogenesis and wound healing processes, it is important to elucidate the role of FGF-1 inside the cell by characterizing its intracellular binding partners. To achieve this, it was necessary to develop a fast and simple method to confirm protein-protein interactions. Here, we present an alternate technique to verify protein-protein complex formation based on limited proteolysis assisted by mass spectrometry, as a supporting tool for protein microarray, fluorescent techniques, pull-down assays or SPR experiments.

Proteolytic degradation requires formation of the transition state of the hydrolytic reaction preceded by an induced-fit mechanism of adaptation of the protein to the active site of the protease. Formation of protein-protein complex results in increased rigidity of proteins structure and lower accessibility of proteolytic cleavage sites in the contact area. Thus, whether two proteins of interest interact, can be easily determined by time-dependent proteolysis followed by MS analysis. By comparing resulting MS spectra after tryptic digestion of FGF-1 in the presence and absence of Hsp90 and CK2 alpha subunit, it was possible to verify protein-protein interactions and, at the same time, to elucidate the protein regions involved in the complex formation.

P18.10

Metabolism of arachidonic acid during ischemia and reperfusion — predictive factors of delayed renal (allo)graft function

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Introduction: Arachidonic acid (AA) metabolites, eicosanoids, are known to play a significant role in the regulation of renal homeostasis, and are synthesised by various enzymes including lipoxygenases (LOX), as well as, cytochrome P450 (CYP450) enzymes. Primary products of LOX pathway of AA metabolism in leukocytes are 5- and 15-hydroxyeicosatetraenoic (HETE) acids, which are synthesised by 5- and 15-LOX respectively. Platelets, which express 12-LOX activity, may only generate 12-HETE. In the CYP450 pathway of AA metabolism 20-HETE and other forms of eicosatetraenoic acids are synthesised. The fact, that various products of AA metabolism may strongly contribute to kidney protection or, paradoxically, destruction, clearly justifies the need to examine the dynamics of HETE changes during early phase of kidney allograft reperfusion.
Material and Methods: Renal recipients (n=64) were retrospectively divided into two groups: DGF(+) with delayed renal graft function and DGF(-) with the kidney functioning immediately after grafting. Blood samples were collected intraoperatively: directly before reperfusion from the iliac vein (0), immediately after reperfusion (I), 2 (II) and 4 (III) minutes after termination of reperfusion. Concentrations of arachidonic acid derivatives were measured using RP-HPLC and ELISA. The results were statistically analyzed using t-Student's test, ANOVA/MANOVA test. Pearson's linear correlation coefficients were calculated. Statistical significance was defined as $p<0.05$.
Results*:* Significantly higher plasma concentrations of 20-HETE, 12-HETE, 15-HETE and 8-iPF$_{2\alpha}$-III were found in DGF(+) patients. Levels of TXB_2, were not significantly differed DGF(-) patients from DGF(+) individuals. In DGF(-) patients 5-HETE levels in platelets were significantly higher. Moreover, high pre-reperfusion 20-HETE levels were strongly associated with DGF.
Conclusion: The most useful predictive factors for delayed renal graft function proved to be plasma AA derivatives: pre-reperfusion 20-HETE levels, and TXB_2 changes during reperfusion.

P18.11

Tandem Affinity Purification (TAP) as a method for identifying binding partners for FGF1

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Tandem affinity purification is a method which allows fast and precise purification of protein complexes formed in natural conditions in the cells. The method requires fusion of the TAP tag with "bait" protein either at its N- or C-terminus. In this study we applied TAP technique to identify novel binding partners of FGF1 — fibroblast growth factor 1 involved in a variety of biological processes, including embryonic development, cell growth, morphogenesis, tissue repair and tumor growth. Our main aim was to identify proteins interacting with FGF1, especially in the translocation of growth factor to cytosol and nucleus.
We prepared three stably transfected cell lines — one overexpressing FGF1 as the C-terminal fusion with TAP tag (consisting of Protein A and CBP tag), one expressing FGF1 as the N-terminal fusion with TAP tag and one expressing only TAP tag to be used as a control for the experiment.
Using IgG Agarose and Calmodulin Agarose columns and multistep purification protocol we identified by mass spectrometry several proteins specific for FGF1 samples but absent in the control experiments. For a few identified proteins the interactions with FGF1 were confirmed by Western blotting. These results show that TAP technique can be successfully applied in parallel to "pull down" proteomic approach to identify novel binding partners for growth factors.

P18.12

Evaluation of the influence of temperature and storage time for sphingosine 1-phosphate concentration. Preliminary report

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Introduction: Sphingosine1-phosphate (S1P) is circulating bioactive lipid mediator that is abundant in the lymph and blood, largely in forms bound to plasma proteins including HDL and albumin. S1P is produced by platelets following activation sphingosine kinase and stored by erythrocytes. S1P play critical roles in a wide pathophysiological functions. According to the determination of the level of S1P in various processes, it seems reasonable to investigate the effect of temperature and storage time on S1P concentration in plasma, serum and erytrocytes.
Materials and Method: Biological material was collected from healthy patients. Plasma, serum and erythrocyte samples were placed at +20°C, +4°C, –20°C, –80°C. After 0 h, 24 h, 48 h and 7 days of storage S1P concentrations were analyzed. S1P was extracted from plasma and serum by a modified one-step method (Hänel *et al.*, 2007) and from erythrocytes using the novel method (unpublished data). S1P was coupled with *o*-phtaladialdehyde (OPA), and the resultant fluorescent derivative was separated by HPLC. S1P-C17 was used as the internal standard. The quantitation was based on peak areas with internal standard calibration.
Results: In all the samples S1P level changes were found High decreases S1P concentration were found in erythrocytes stored at +20°C, +4°C. In plasma samples stored at +20°C, +4°C S1P level changes were minor.
Conclusion: These results may indicate in the tested biological materials the occurrence of factors that protect against degradation S1P caused by temperature and storage time. These data may help to identify not known mechanisms controlling the level sphingolipids in the blood.

P18.13

The novel method of determination sphingosine-1-phosphate in erythrocytes

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Introduction: Sphingosine-1-phosphate (S1P) is a bioactive sphingolipid metabolite in plasma, erythrocytes and platelets. Recent studies indicate that S1P is a chemoattractant for HSPCs. Erythrocytes are a major reservoir of this molecule. Activation of the complement system may result in formation of the membrane attack complex (MAC). MAC induces release of S1P from erythrocytes. We proposed accurately and precisely method of determination S1P content in erythrocytes.
Materials and metod: Blood was collected from healthy individuals. S1P from erythrocytes was extracted with hexane and isopropanol mixture (3:2, v/v). Hexane phase was vacuum-dried in a SpeedVac. Residue evaporation was reconstituted in methanol. S1P was coupled with o-phtaladialdehyde (OPA), and the resultant fluorescent derivative was separated by HPLC. S1P-C17 was used as the internal standard. The quantitation was based on peak areas with internal standard calibration.
Results: The average content of S1P in erythrocytes (the recoveries 75–80%) was 378.1 g/g Hb±128.33 g/gHb. The accuracy of the method was 10,1%. Intra-assay variation (% CV) was 12% and inter-assay variation was 17.1%.
Conclusion: Our method of the determination S1P in erythrocytes is simple and effective.

P18.14

Effect of whole-body cryostimulation on the protein profile of healthy subjects

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Introduction: In clinical practice, cryotherapy is used in the treatment of locomotor and neurological disorders. It is also increasingly often used in competitive sports and recreation as a form of recovery and wellness. Although it is postulated that cryogenic temperatures may mobilize the immune and antioxidative systems, there are not many reports on the effects of low temperature on a healthy person and the physiological mechanisms of a multifaceted action of cryotherapy and cryostimulation are not fully understood and explained. The aim of this study was to assess the impact of whole-body cryostimulation (WBC) on the electrophoretic protein profile in healthy subjects.
Material and Methods: The study group consisted of 15 clinically healthy men aged 21±2 years, who underwent a series of 20 cryotherapy sessions in a cryogenic chamber (120s, –140°C±10°C). Research material was blood serum taken from subjects in the first day of testing before the first entrance to the chamber (A), 30 minutes after leaving the first cryostimulation in the chamber (B), 24 h after the first cryostimulation (C), and one after a series of ten (F) and twenty (I) cryostimulations sessions. In the serum we determined total protein and performed electrophoresis of serum proteins in agarose gel. The results were statistically analyzed using nonparametric Wilcoxon test and the Friedman ANOVA test ($p<0.05$).
Results: The results showed statistically significant changes in the concentrations of albumin ($p=0.013$) and alpha1-globulin ($p=0.010$) determined in the material collected at designated time points (A, B, C, F, I). During the first cryostimulation we observed a significant reduction in serum albumin and alpha1-globulin 30 min after treatment, which significantly increased in the next day and remained at elevated levels over the next 10 treatments. During prolonged exposure to 20 treatments, the concentration of albumin and alpha1-globulin did not change any further.
Conclusion: Our results indicate regular whole-body cryotherapy increases concentrations of albumin and alpha1-globulin, which may confirm the stimulating effect of cryogenic temperatures on the body's immune system.

P18.15

Cloning, expression, purification and molecular characteristics of the nuclease from extreme psychrophilic bacterium *Psychromonas ingrahamii* 37

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Nucleases are important molecular tools belonging to the group of hydrolase-degrading nucleic acids [1]. Currently, only two nucleases degrading every form of nucleic acids are commercially known. They are Cryonase® (Takara, Japan) and Benzonase® (Merck, USA). Cryonase®, which originates from the psychrotrophic strain *Schewanella sp.* AC10, has an optimum activity temperature of 20ºC, while Benzonase® which was isolated from the mesophilic microorganism *Serratia marcescens*, is most active at a temperature of 37ºC. Both nucleases have found applications as tools used in molecular biology and genetic engineering techniques, and as industrial enzymes used in the elimination of nucleic acids from protein solutions, the elimination of genomic DNA, the degradation of DNA templates, the synthesis of DNA libraries, the footprinting method, the reduction of protein extract viscosity and the pretreatment of samples for two-dimensional electrophoresis [2, 3].
We report the identification and characterization of the nuclease from extreme psychrophilic bacterium *Psychromonas ingrahamii* 37 that grows expotentially at –12°C and may well grow at even lower temperatures. Psychrophilic microorganisms, living in extremely cold environments, produce enzymes which are adapted to performing reactions at a low temperature [4]. This is extremely important in the case of nucleases, as it will allow their utilization in the reaction for the elimination of nucleic acids from thermosensitive preparations.
Nuclease from *P. ingrahamii* 37 is protein consisting 256 amino acid residues with a calculated molecular mass of 26.5 kDa. In comparison with the commercially available protein Cryonase®, the endonuclease from the strain *P. ingrahamii* 37 shows a high degree of homology with Cryonase® (35% identity and 49% similarity).

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P18.16

MutS3 a MutS homologue of unknown biological function

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The homologues of MutS proteins are widespread among both Prokaryotes and Eukaryotes. MutS designated as MutS1 is a part of MMR (mismatch repair) system which is responsible for removal of mispaired bases and small insertion/deletion loops in DNA. Initially, the only MutS homologues known were those engaged in mismatch repair and these were later designated as MutS1. Subsequently, the MutS2 homologue was distinguished. MutS2 does not recognize mispaired bases but it acts as the inhibitor of RecA-dependent homologous recombination. The phylogenetic analysis of multiple aminoacid sequences of MutS proteins revealed the existence of three other MutS homologues: MutS3, MutS4 and MutS5. The main feature of the three homologues is the lack of both the N-terminal domain typical of MutS1, which is responsible for mismatch recognition, and the C-terminal endonuclease domain, characteristic of MutS2.
The analysis of representative prokaryotic genomes showed that the MutS3 homologue in a species is always accompanied by MutS1 and MutS2. The retention of MutS3 together with MutS1 and MutS2 suggests that this homologue gives some evolutionary benefit and that MutS3 may play an important role in DNA metabolism. However, until now MutS3 has not been isolated and studied in terms of its biological function.
Staphylococcus aureus is the etiological factor of numerous infections and it is frequently reported to develop antibiotic resistance. As *S. aureus* pathogenicity is directly connected with the ability for adaptation and genetic plasticity, it is of great concern to clarify whether MutS3 is involved in DNA repair processes. Assuming that MutS3 participates in an unknown mechanisms involved in the control of genetic variability, it could be associated with important medical implications.
The gene coding for *S. aureus* MutS3 was PCR amplified and cloned into a T7 expression vector. The efficient expression of 61 kDa his-tagged MutS3 was obtained in *Escherichia coli* cells. Unfortunately, the protein formed inclusion bodies and was insoluble in all buffers tested. The computer prediction based on the amino acid sequence revealed the presence of transmembrane regions. The analysis of MutS3 amino acid sequences from *S. aureus* and other bacteria identified the presence of an ATPase domain typical of MutS homologues but provided no convincing evidence for the presence of a DNA binding domain. The question whether MutS3 is involved in DNA metabolism remains to be answered.

P18.17

Investigation of interaction between xanthene dye rhodamine B and metagenomic-derivated methylthioadenosine phosphorylase RSFP

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In the previous study we found that the expression of the metagenomic — derived methylthioadenosine phosphorylase gene (*rsfp*) in *E. coli* cells and the presence of rhodamine B in the growth medium were crucial for the pink fluorescence of one *E. coli* colony from Antarctic soil metagenomic library (Cieśliński *et al.,* 2009). In this study, *rsfp* gene expression was induced by an arabinose promoter in an LMG194 *E. coli* strain and the RSFP protein was then purified with using fast protein liquid chromatography. Purified, recombinant RSFP enabled us to examine the hypothesis that it is the binding of RB molecules to RSFP proteins that was responsible for the observed fluorescence phenomena. Indeed, it was with this in mind that absorbance and fluorescence measurements were employed, as they have already been successfully used in studies of RB molecule interaction with carrier surfaces such as clay particles, quartz, gold nanoparticles, hydrophilic glass slides and bovine serum albumin. The results of this study revealed that the rhodamine B molecules binding to RSFP protein caused remarkable changes in UV-VIS absorption and fluorescence spectra of rhodamine B and induces conformational changes in the protein structure. What was important to note is that the analysis of the presented results in the light of the literature study reveal: (*i*) the lack of literature data on rhodamine B interaction with any proteins belonging to the class of methylthioadenosine phosphorylase enzymes and/or influence on their enzymatic activities, (*ii*) that the interaction between rhodamine B molecules and proteins are crucial for observed carcinogenic properties of RB, however the knowledge of the interaction mechanism of RB with proteins is very limited, and (*iii*) that the methylthioadenosine phosphorylase enzymes are studied in details due to their roles in biological processes such as methionine salvage methylation reactions, purine salvage, and polyamine biosynthesis.

Reference

Cieśliński H *et al.* (2009) *FEMS Lett* **299**: 232-240.

P18.18

Novel single-stranded DNA-binding protein from psychrophilic bacterium *Psychrobacter arcticus*

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To study the biochemical properties of SSB from *Psychrobacter arcticus* (*Par*SSB), we have cloned the *ssb* genes obtained by PCR and have developed *Escherichia coli* overexpression systems. The gene consists of an open reading frame of 642 nucleotides encoding SSB protein of 213 amino acids with a calculated molecular mass of 22.8 kDa. The amino-acid sequence of *Par*SSB exhibits 49% identity and 57% similarity to *Escherichia coli* SSB. In analysis by gel filtration chromatography we show that *Par*SSB is functional as homooctamer, a structure as yet unlisted in published SSB proteins. Each monomer encodes one single-stranded DNA binding domains (OB-fold). In fluorescence titrations with poly(dT), it binds single-stranded DNA with a binding site size of about 22-34 nt depending on the salt concentration, and fluorescence is quenched by about 95%. Thermostability with half-lives of about 15 min at 100°C makes SSB from *Psychrobacter arcticus* the most thermostable SSB protein among psychrophilic and mesophilic bacteria indentified to date. *Par*SSB is more thermostable even than commonly-used SSB from *T. aquaticus* and *T. thermophilus*. The rare, high thermostability of this cold-adapted protein and the homooctameric structure, which is unique among all known pro- and eucariotic single-stranded DNA binding proteins, could prove to be useful in various molecular biology techniques.

P18.19

Novel primosomal protein B from thermophilic bacterium *Thermoanerobacter tengcongensis*

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We report the identification and characterization of the primosomal protein B (PriB) from thermophilic bacterium *Thermoanerobacter tengcongensis* (*Tte*PriB). It is the largest known bacterial PriB protein consisting 216 amino acid residues with a calculated molecular mass of 25 kDa. Surprisingly, it is functional as monomer containing two single-stranded DNA binding domain (OB-fold) and it is the completely new kind structure of SSB protein. Bacterial SSBs proteins identified to date are homodimers (e.g. PriB from *Escherichia coli*) or monomers (e.g. PriB from *Klebsiella pnuemoniae*).
The ssDNA-binding site for *Tte*PriB is 34±2 nucleotides long as shown by using fluorescence spectroscopy. The half-lives of *Tte*PriB was 10 min at 75°C. These results showed that *Tte*PriB as the first characterized PriB from thermophilic microorganizm is thermostable primosomal protein B with unique structure, offering an attractive alternative for other thermostable proteins with two OB-folds per monomer (*Taq*SSB and *Tth*SSB) in their applications for molecular biology techniques.

P18.20

Calorimetric and conformational studies of the folding of two mutants of the B-domain of staphylococcal protein A differing in stability

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Small proteins with simple fold often serve as models with which to study the details of protein folding and determine the kind of strength of specific interactions responsible for folding. However, such studies are usually performed by adding a chemical denaturant, while the temperature is the principal denaturaing factor in living cells. In this study, we cloned and investigated two mutants of the N-terminal part of the B-domain of staphylococcal protein A, which has a simple three-helix-bundle fold, one (the Z-domain) with increased and another one (the newZ-domain) with decreased conformational stability. The heat-capacity curves were determined by differential scanning calorimetry giving the melting temperature of 59°C for the Z-domain. For this protein, the heat-capacity peak coincided with the midpoint of the melting of secondary structure found from circular dichroism and tyrosine fluorescence measurements. For the newZ-domain, the heat-capacity curve exhibited a broad band with weak peaks at T=35°C and T=55°C. The position of the second peak corresponded to the midpoint of secondary-structure melting suggesting that the first peak corresponds to the melting of the tertiary and the second of the secondary structure. Small x-ray-diffraction (SAXS) studies at various temperatures revealed that the dimensions of the Z-domain only slightly increase with temperature and those of the newZ-domain decrease with temperature even beyond the melting point. Therefore, the hydrophobic collapse seems to hold at temperatures at which both the tertiary and the secondary structure is already destroyed, in contrast to clear increase of the dimensions of a protein upon chemical denaturation. The results of this study were utilized in the calibration of our UNRES united-residue force field for the simulation of protein structure and dynamics.

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P18.21

DNA*traffic* — a new database for systems biology of DNA metabolism and repair

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DNA*traffic* (http://dnatraffic.ibb.waw.pl/) is dedicated to be an unique comprehensive and richly annotated database resource for systems biology of DNA metabolism, nucleic acids organization, histone modification, as well as DNA damage and repair pathways, and diseases correlated to nucleic acids metabolism dysfunction.

The main goal of DNA*traffic* database is to collect data from other well known and commonly used databases (such as: KEGG, Reactome, UniProt, OMIM, NCBI, PDB, DrugBank, and other) and organize them in the following types of information: (i) genes linked to proteins involved in nucleic acids metabolism pathways, (ii) anticancer drugs and environmental mutagenic and cytotoxic agents linked to DNA damage, (iii) DNA damage linked to the recognizing proteins, (iv) diseases correlated with mutations in genes encoding the DNA*traffic* proteins linked to DNA metabolism pathways. Moreover, DNA*traffic* is dedicated to spread information concerning main DNA metabolism pathways: chromatin organization (histone modifications), DNA replication and translesion synthesis (TLS), DNA recombination, DNA transcription, DNA damage signaling, DNA damage repair (DRR, BER, NER, MMR, HRR, NHEJ) and DNA degradation. Till now, the pathway/protein dataset is limited to seven model organisms —*Homo sapiens*, *Escherichia coli* K-12 MG1655 and *Saccharomyces cerevisiae* (budding yeast), *Schizosaccharomyces pombe* (fission yeast), *Caenorhabditis elegans* (nematode), *Mus musculus* (mouse) and *Drosophila melanogaster* (fruit fly).

The final result will be the open access database containing extensive data on related to the DNA metabolism proteins, diseases and drugs used in therapy. DNA*traffic* will also contain systemic information on the nomenclature, chemistry and structure of DNA damage and base modifications. It will also offer external links for tools searching drugs or proteins interaction.

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