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Program

Date	Venue	Timings	Title
23/08	LH1	6pm	**OF MUSIC, MATH AND MEASUREMENT** M.W. Linscheid, Humbold Universitaet Berlin, Germany
	Dining Complex	7pm	**Mixer** Concert by Ananth Menon Quartet
	Dining Complex	8pm	**Dinner**
24/08	LH1	9:00am	**Tutorial I** **MASS SPECTROMETRIC ESSENTIALS IN OMICS RESEARCH** D.Schwudke
	LH1	9:30am	**Session I / Proteomics** (Chair D. Schwudke) **MASS SPECTROMETRIC IDENTIFICATION OF A NOVEL TRANS-SPLICING EVENT IN GIARDIA LAMBLIA HEAT SHOCK PROTEIN 90** U.S. Tatu, IISC Bangalore, India **REVISITING PROTEOMICS OF GLIOMAS: DIFFERENTIAL MEMBRANE PROTEINS AND MOLECULAR INSIGHTS** R. Sirdeshmukh, IOB Bangalore, India **NEW HIGH SELECTIVITY WORKFLOWS FOR TARGETED QUANTITATIVE PROTEOMICS** M. Cafazzo, AB Sciex, US **Break** **ABSOLUTE QUANTIFICATION OF PROTEINS AND PEPTIDES - USE OF METAL CODING AND LC/MS WITH ICP AND ELECTROSPRAY** M.W. Linscheid, Humbold Universitaet Berlin, Germany **MATERNAL VITAMIN B12 DEFICIENCY INDUCED ALTERATION IN PROTEIN EXPRESSION IN RAT OFFSPRING** S. Sengupta, IGIB New Dehli, India **CLINICAL PROTEOMICS OF EYE DISEASES** K. Dharmalingam, Madurai Kamaraj University, India
	Dining Complex	1pm	**Lunch**
	Open Deck	2pm	**Poster Session I**

Date	Venue	Timings	Title
24/08	LH1	4pm	**Session II / Lipidomics** (Chair S. Hebbar) **TOWARDS THE COMPLETE STRUCTURE ELUCIDATION OF COMPLEX LIPIDS BY MASS SPECTROMETRY: NOVEL APPROACHES TO ION ACTIVATION** S. Blanksby et al., University of Wollongong, Australia **LIPIDOMICS AT THE HIGH MASS RESOLUTION** A. Shevchenko, MPI-CBG Dresden, Germany **STRATEGIES FOR IMAGING BIOMOLECULES BY TOF-SIMS AND MALDI-TOF/TOF MASS SPECTROMETRY** O. Laprévote, Université Paris Descartes, France **Break** **MEIBUM LIPID COMPOSITION IN ASIANS WITH DRY EYE SYNDROME** M.R. Wenk, NUS, Singapore **INTEGRATION OF OMICS RESEARCH WITH DEVELOPMENTAL BIOLOGY** D. Schwudke, NCBS Bangalore, India **ADIPOSE TISSUE LIPIDOME: BENEFITS AND COSTS OF LIPID REMODELING AS ADAPTATION TO ACQUIRED OBESITY** M. Orešič, VTT Technical Research Centre of Finland NN D. Ghosh, JNU Dehli, India
	Dining Complex	8pm	**Dinner**

Date	Venue	Timings	Title
25/08	LH1	9:00am	**Tutorial II** **SOFT IONIZATION METHODS** K. Dreisewerd
	LH1	9:30am	**Session III / Proteins and Proteomics** (Chair A. Shevchenko) **GAS PHASE STRUCTURAL AND DYNAMICAL BIOLOGY** J.L. Benesch, University of Oxford, UK **IDENTIFICATION OF MULTIPLE FOLDING PATHWAYS OF MONELLIN USING PULSED THIOL LABELING AND MASS SPECTROMETRY** J.B. Udgaonkar et al., NCBS Bangalore, India **DIFFERENTIAL EXPRESSION OF RED CELL PROTEINS IN HEMOGLOBINOPATHY** A. Chakrabarti, SINP Kolkata, India **Break** **COMPARATIVE PROTEOMICS OF EXTRACELLULAR MATRIX DEMONSTARTES THE COORDINATED EXPRESSION OF DEHYDRATION-RESPONSIVE PROTEINS** N. Chakraborty, NIPGR Dehli, India **ON-LINE UPLC/ION MOBILITY SEPARATION/TOF MS FOR QUALITATIVE AND QUANTITATIVE PROTEIN PROFILING IN COMPLEX BIOLOGICAL SAMPLES** M.A. McDowall et al., Waters, Manchester, UK **A DRAFT MAP OF THE HUMAN PROTEOME** H. Gowda, IOB Bangalore, India
	Dining Complex	1pm	**Lunch**
	Open Deck	2pm	**Poster Session II**

Date	Venue	Timings	Title
25/08	LH1	4pm	**Session IV / Metabolites and Small Molecules** (Chair R. Kannan) **G-QUADRUPLEX FORMATION OF GUANOSINE DERIVATIVES IN PRESENCE OF ALKALINE EARTH METAL IONS STUDIED BY ELECTROSPRAY IONIZATION MASS SPECTROMETRY** M. Vairamani, IICT Hyderabad, India **IR-MALDI MASS SPECTROMETRY FOR THE ANALYSIS OF (SMALL) MOLECULES DIRECTLY FROM BIOLOGICAL TISSUE AND FROZEN AQUEOUS SOLUTIONS** K. Dreisewerd et al., University of Münster, Germany **QUANTITATIVE ANALYSIS OF SMALL BIOMOLECULES USING LASER DESORPTION IONIZATION MASS SPECTROMETRY** V. Panchagnula, NCL Pune, India **DISCOVERY OF NOVEL DROSOPHILA LIPID PHEROMONES USING MASS SPECTROMETRY** J. Yew et al., Temasek Life Sciences Laboratory, Singapore **Break** **MULTI-OMICS WORKFLOW FOR STUDYING DRUG TREATMENT ON HUMAN CELL LINE** S. Rajagopalan, Agilent Technologies, India **LIQUID EXTRACTION SURFACE ANALYSIS (LESA) COMBINED WITH NANOESI-MS FOR DIRECT SAMPLING OF SURFACES** R. Almeida, Advion BioSciences, UK **NOVEL APPROACHES BASED ON HIGH RESOLUTION AND ION MOBILITY MASS SPECTROMETRY FOR THE QUAL/QUAN ANALYSIS OF PHARMACEUTICALS AND THEIR METABOLITES** G. Hopfgartner, Université de Genève, Switzerland
	Dining Complex	8:00pm	**Dinner**
26/08	LH1	9:30am	**Short Talk Session (Students Presentations)** (Chair Schwudke Lab)
	LH1	11:00am	**Interaction**
	LH1	12:00am	**Poster Prizes - Certificates**
	LH1	12:30am	**Closing Remarks**

Speakers Abstracts

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MASS SPECTROMETRIC ESSENTIALS IN OMICS RESEARCH

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Many mass spectrometric experiments performed in context of biological research are belonging to the Omics domain. I will comment on the design of high-throughput workflows to measure entire pools of biomolecule classes. In that context I will give an overview on how mass spectrometric parameters like resolution and mass accuracy influence our ability to interpret OMICS data in qualitative and quantitative manner. We will discuss terms like dynamic range, mass resolution and separation power of a mass spectrometric approach. Further we will discuss the ability to decrease false positive and false negative assignments by using accurate masses and isotopic distributions.

MASS SPECTROMETRIC IDENTIFICATION OF A NOVEL TRANS-SPLICING EVENT IN GIARDIA LAMBLIA HEAT SHOCK PROTEIN 90

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Material and Methods

My laboratory is interested in examining in vivo roles of molecular chaperones in protozoa, many of which cause infectious diseases in humans and animals. We have examined Hsp90 gene expression and function from Dictyostelium discoideum, Plasmodium falciparum, Trypanosoma evansi as well as Giardia lamblia.

G. lamblia trophozoites are grown to confluency and total protein was extracted. Proteins were resolved in SDS PAGE. Band corresponding to Hsp90 was subjected to in-gel trypsinization and peptides were enriched. Peptides were separated in nano- reverse phase column which was directed to Nano Spray ESI- QTOF MS. Ionized peptides were analyzed by one full MS scan and four consecutive product ion scans of the four most intense peaks. The acquired data was analyzed using ProteinPilot 4.0 software with combined Giardia database. Peptide confidence greater than 90% was considered as significant hits. Spectra of each peptide used for identification of the proteins were verified manually.

Results

In my talk I will specifically describe our recent results incorporating mass spectrometric analysis of Hsp90 from *G.lamblia*, which revealed a novel *trans*-splicing based expression of GlHsp90 in this minimalist protozoan parasite. We have recently shown that Hsp90 in *Giardia* is arranged as a split gene, HspN and HspC separated by 777 kb intergenic sequence. The full length Hsp90 transcript is a resultant of a novel *trans*-splicing phenomenon which is mediated by spliceosomal complex. The mature full length transcript has all the hallmarks of the canonical Hsp90. The protein band that corresponded to a region of around 80 kDa was identified to be GlHsp90, with peptides matching from two individual exons, HspN and HspC. In addition, during the course of *trans*-splicing there is incorporation of 99 nts at the junction of two exons which was obtained by sequencing of full length cDNA. Since the Giardiadb database does not contain the full-length GlHsp90 sequence, we manually included the protein sequence in our search list by translating the cDNA sequence obtained. Using mass spectrometric based approach we have sequenced and identified the peptides joining these two exons. This junctional peptide houses an ‘Arg’, a catalytical residue conserved in other Hsp90s, required for the functioning of Hsp90 chaperone cycle. We have also identified ‘Val’, a conserved residue across Hsp90s, formed during the junction of the two ORFs. Thus the sequenced peptides scan the entire junction of the full length Hsp90.

Conclusions

Hsp90 is a conserved molecular chaperone required for normal functioning of the cell. Hsp90 also provides survival advantages at time of stress to the baring organisms. Hsp90s indentified so far is 80 – 90 kDa in size and encoded by a single ORF. Interestingly in Giardia Hsp90 is encoded by two different ORFs which can be functional only when brought together by a post transcriptional mechanism, trans-splicing. Here we provide evidence for such a reconstruction mechanism at proteomic level. Sequencing of the junctional peptide by MS/MS provides evidence of functional full length Hsp90 in Giardia, a novel approach to identify trans-splicing event using mass spectrometry.

REVISITING PROTEOMICS OF GLIOMAS: DIFFERENTIAL MEMBRANE PROTEINS AND MOLECULAR INSIGHTS

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membrane proteomics, Glioblastoma, mass spectrometry, iTRAQ

Material and Methods

The talk will cover analysis of the differential membrane proteins from the microsomal fraction of clinical specimens Glioblastoma multiformme (GBM) using high resolution LC-MS/MS mass spectrometry and quantitation by iTRAQ followed by validations of select molecules by immunohistochemistry. Bioinformatic analysis of the differential proteins was carried out to assess their functional relevance and potential as biomarkers or targets.

Results

A total of 1834 proteins were identified with high confidence from the membrane fraction of GBM tissues. Of these, 356 proteins were found to be differentially regulated by 2 fold or more (198 up and 158 down regulated). Each protein identification was supported with at least two peptides and each peptide with two iTRAQ label reporter ions. Differential proteins include proteins groups of regulatory functions and their isoforms. Bioinformatic analysis of the data confirms proteins and genes implicated in GBMs and other cancers in earlier reports as well as reveals entirely novel proteins that are being reported for the first time in GBMs or any other cancer. Differential levels of select proteins was also confirmed by immunohistochemistry or immunoblots. In a prototype analysis using tissue microarray, four differntial proteins exhibited high reproducibility across a cohort of 35 GBM specimens. The dataset provides strong differentials which may be useful as diagnostic markers with tissue biopsies. Cell surface proteins identified may be useful targets for therapeutic application whereas other proteins with TM domains or signal sequences may be pursued in the CSF or plasma from GBM patients for diagnosis or post surgery surveillance.

Conclusions

Gliomas are primary tumors of the central nervous system, Glioblastoma multiformme (GBM) being the most prevalent and aggressive among them. Developing a high confidence discovery portfolio of differential proteins is the first step towards validating these molecules with a targeted approach in a specific clinical background. The analysis represents an attempt towards this objective. It is the first large scale effort to identify changes in the membrane protein profile of GBM by proteomics approach and provides an important resource for targeted study of their translation to clinical applications. In an expanded analysis, comparison with differential membrane proteins from Gr II and III gliomas, further revealed proteins that are unique to a particular grade.

NEW HIGH SELECTIVITY WORKFLOWS FOR TARGETED QUANTITATIVE PROTEOMICS

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HR MRM; SWATH; TripleTOF; MS/MS; differential mobility separation

Material and Methods

Increased focus on biomarker verification/validation strategies holds great promise in the protein biomarker field for advancing more markers towards clinical utilization. Multiple Reaction Monitoring (MRM) capabilities of triple quadrupole-based MS systems have been an area of focus due to known throughput and quantitative robustness advantages. Careful assay development, high selectivity acquisition techniques and sophisticated down-stream data processing strategies can help ensure highest data integrity. As studies push towards higher throughput, reduced sample preparation, lower abundant proteins and larger biomarker panels, methods for higher specificity quantitative detection are being explored to extend targeted quantitative MS applications.

Results

On the TripleTOF™ 5600 system, the sensitivity and specificity of high resolution MRM-like analysis has been demonstrated for the quantification of peptides and proteins. As full scan MS/MS is acquired at high resolution, quantification and confirmation are obtained simultaneously. Upfront method development is simplified and more flexibility of analysis post-acquisition is enabled. The result is a quantitative assay that can provide a higher degree of specificity than traditional triple quadrupole MRM analysis performed at unit resolution, with automatic and simultaneous confirmation of analyte identity.

For more comprehensive sample coverage, the next step up in multiplexing is to perform MS/MS of everything (MS/MS^ALL) using high resolution detection of fragment ions, such that MRM-like data can be extracted post-acquisition on a much larger population of peptide targets. To do this in an LC time frame, a wider isolation window is used and systematically stepped through the mass range of interest (SWATH – Sequential Windowed Acquisition), again collecting high resolution MS/MS spectra. Data highlighting the use of these techniques on a complex proteome will be discussed.

Finally, for the highest selectivity on specific proteins and peptides, additional orthogonal separation strategies can be employed to reduce background and interferences in high throughput assays. Using differential mobility separation in front of a QTRAP® 5500 System provides an added layer of selectivity to enhance assay robustness and improve detection limits. Examples of this workflow will also be discussed.

Conclusions

As the results of protein biomarker experiments make their way toward the clinic, increased effort will be required in the accurate verification and quantitation of biomarkers. Tools that provide higher selectivity and enable higher throughput via a decreased need for sample preparation and fewer experiments will aid this process. The new workflows discussed here, utilizing the TripleTOF™ 5600 System or the QTRAP® 5500 System with SelexION™ Technology, should provide such attributes in high performing, reliable platforms.

ABSOLUTE QUANTIFICATION OF PROTEINS AND PEPTIDES - USE OF METAL CODING AND LC/MS WITH ICP AND ELECTROSPRAY

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Quantitative proteomics, Mass spectrometry, Metal labeling, ICP MS, lanthanide DOTA

Material and Methods

MeCAT – Metal Coded Tagging – of proteins and peptides is a new, but one of the most capable protein methods in the proteomics field for exact protein quantification.
Proteins and peptides are labelled by MeCAT reagents which contain a protein-reactive labelling group and an element tag with lanthanides. By using different lanthanides in the MeCAT reagent such as Lutetium, Holmium, Thulium and Terbium, multiplex experiments can be performed to analyze several protein samples simultaneously in a proteomic study.
After MeCAT labelling proteins are separated by 2D- gel electrophoresis or chromatography and quantified by Inductively Coupled Plasma Mass Spectrometry (ICPMS; instrument: Element XR, ThermoFisher Scientific, Bremen) measuring the amount of MeCAT metal. If required, regulated proteins of interest are identified by MALDI MS or nano-LC/ESI MSMS (Instrument: LTQ FTMS-Ultra, ThermoFisher Scientific, Bremen, Germany).

Results

In this contribution we show results of MeCAT duplex labelling of several model proteins and with E. coli proteomes. A recombinant E.coli fermentation of the fusion protein aprotinin-ß-galactosidase was performed using a heat-inducible promoter system. Samples were obtained before and after heat induction by rising the fermentation temperature from 30°C to 38°C. The E.coli samples were labelled using MeCAT-Lu and MeCAT-Tm. Protein separation was performed in high resolution 2D electrophoresis with dissolvable DPAGE gels and MS compatible gel staining using the FireSilver staining kit.
Then, the protein spots were cut out from 2DE gels for subsequent quantification of the MeCAT-Lu/ -Tm tagged proteins. The protein DPAGE gel spots were dissolved and quantified using flow-injection ICP-MS and identification was carried out by nanoLC-ESI MS/MS.
Several regulated proteins were quantified and identified including the recombinant aprotinin-ß-galactosidase, heat shock proteins, aconitase, oligopeptide binding protein precursor and pyruvate-formiate lyase.
In addition most recent results in the development of a new tagging chemistry using Ionacetamid reagents and the application of isotope dilution combined with metal tagging will be presented.

Conclusions

ICPMS has several advantages in quantification. 1) The detection capability for lanthanides is in the ppt range or lower, which is equivalent to low attomol amounts of protein. Thus the detection limit is 3-6 orders of magnitude lower than for other MS techniques like MALDI MS for the quantification of intact proteins. 2). The unrivalled linear dynamic range of ICPMS exceeds 6-8 orders of magnitude. For comparison ESI MS techniques can cover about three orders of magnitude. 3) Absolute quantification of proteins can be carry out by ICPMS using a protein or peptide independent calibration based simply on metal standards.

MATERNAL VITAMIN B12 DEFICIENCY INDUCED ALTERATION IN PROTEIN EXPRESSION IN RAT OFFSPRING

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Rat,Vitamin B12, Folate, DIGE, iTRAQ

Material and Methods

Micronutrient deficiency (like vitamin B12 and folate) during early developmental stages are known to predict risk of complex disorders. The objective of the study was to ascertain whether maternal micronutrient (Vitamin B12 and folate) deficiency affects developmental reprogramming and thus could form a basis of complex diseases in adults. Using rat as a model, we studied the effects of maternal malnutrition in pups. Mothers were fed vitamin B12 and/or folate deficient diet and the progeny were kept on the same diet. Further, in another set vitamin B12 deficient mothers were supplemented with vitamin B12 at the time of conception and the pups were kept on normal diet thereafter. We performed 2D-differential in gel electrophoresis (DIGE) in the liver tissue and iTRAQ of brain tissue of offspring born to mothers fed with vitamin B12 and/or folate deficient diet to identify proteins that are differentially expressed due to micronutrient deficiency.

Results

2D-differential expression of proteins in liver of offsprings born to mothers fed with vitamin B12 and folate deficient diet led to the modulation of expression of 43 and 18 proteins respectively. While 25 proteins were differentially exprssed in pups born to mothers fed with both folate and vitamin B12 deficient diets. The proteins that were differentially expressed in folate and double deficient group were mainly involved in metabolism of amino acids and lipids while proteins involved in the metabolism of amino acids, lipids, carbohydrates, and nucleotides were differentially expressed in the vitamin B12 group. Interestingly, supplementation of vitamin B12 after conception/parturation restored the expression of proteins in the amino acid, lipid and carbohydrate metabilic pathways thus, indicating that these proteins are specifically altered due to deficiency of vitamin B12 in mothers.

Isobaric tags for relative quantitation (iTRAQ) technique was used to study the differential protein expression in brain of pups born to vitamin B12 deficient mothers. iTRAQ experiments resulted in the identification of 2368, 1606 and 1796 proteins in three independent experiments of which about 1740 proteins were identified in atleast 2 experiments. Of these 56 proteins were differentially expressed. These were involved in MAPK signalling pathway, VEGF signalling pathway, Calcium signalling pathway, and cardiac muscle contraction. Supplementation of vitamin B12 restored the expression of proteins in the MAPK signalling pathways and calcium signalling pathways.

Conclusions

Proteomic profiling of adult liver tissue reveals differential expression of proteins in lipid, carbohydrate and amino acid metabolic pathways while proteins involved in MAPK and calcium signalling pathways that are altered due to vitamin B12 deficiencies. Supplementation of vitamin B12 at conception restores the expression of these proteins.

CLINICAL PROTEOMICS OF EYE DISEASES

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Top down proteomics, ophthalmic diseases, 2D DIGE

Material and Methods

A proper understanding of the disease pathogenesis will help in refining the existing treatment options and also facilitate detection of disease early. In this study we examined the proteomes of relevant tissue materials such as tear, aqueous humor and plasma to examine the protein profile, and its alteration in fungal keratitis and Primary Open Angle Glaucoma. Adapting appropriate sample preparation methods proteins were separated on 2D and 2D DIGE. DeCyder software was used for spot comparison and quantitation. Differentially expressed protein spots were identified using MALDI and Nano LC MS/MS mass spectrometry.

Results

High resolution 2D PAGE and DIGE analysis allowed detection of proteome wide changes in the whole protein profile in the tissues affected in fungal keratitis and Primary Open Angle Glaucoma. Interestingly, we uncovered significant differences in the protein profile of male and female tear fluid in uninfected control cases. These results indicate that one has to be extremely careful in validating the disease specific changes at the proteome level. Proper sample selection and sample collection and tissue selection are all important to arrive at proper conclusions apart from using proper medical profile of the patients.

Conclusions

Differentially regulated proteins, their isoforms and their biological significance are analyzed in detail. It appears in majority of the case the down regulated proteins are known to be involved in local immune function and the up regulated proteins are known to be involved in inflammatory process. The importance of whole protein profiling will be discussed.

TOWARDS THE COMPLETE STRUCTURE ELUCIDATION OF COMPLEX LIPIDS BY MASS SPECTROMETRY: NOVEL APPROACHES TO ION ACTIVATION

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lipid mass spectrometry, lipidomics, ion activation, structure elucidation

Material and Methods

Ozone induced dissociation spectra were obtained on both a linear ion-trap mass spectrometer (LTQ, ThermoFisher) and a tandem linear ion-trap mass spectrometer (QTRAP2000, AB Sciex). Both instruments have been modified in-house to allow mass-selected ions to be isolated in the presence of ozone vapor as previously described [1,2]. The LTQ has been further modified, in a manner similar to that previously reported by others [3], to allow irradiation of trapped ions with a Nd:YAG laser (Minilite II, Continuum).

[1] Thomas, et al. Anal. Chem. 2008, 80, 303.
[2] Poad et al. J. Am. Soc. Mass Spectrom. 2010, 21, 1989.
[3] Ly and Julian, J. Am Chem. Soc. 2008 130, 351.

Results

Overcoming the challenges of lipid structure elucidation by mass spectrometry (as outlined above) requires ion activation methods that are able to bring about cleavage of carbon-carbon bonds. We are using two complementary ion activation approaches to bring about this kind of dissociation, namely (i) ozone induced dissociation (OzID) that selectively targets double bond cleavage and (ii) radical directed dissociation (RDD) that gives rise to more generalized fragmentation.

In OzID analysis lipid ions are mass-selected in an ion-trap mass spectrometer and allowed to react with ozone vapor with the resulting chemical induced fragment ions allowing localization of double bonds. Recent implementation of this technique on a tandem linear ion-trap mass spectrometer (QTRAP) has significantly decreased the acquisition time (thus increasing sensitivity) for OzID analysis. New data from this instrument will be presented showing the use of workflows that include combinations of OzID and CID analyses to elucidate the structure of complex lipids from a range of biological samples on a timescale compatible with liquid chromatography.

RDD was developed by Ly and Julian [3] and involves introducing an iodine on tyrosine as a photo-labile initiator that can be selectively cleaved to promote the radical-driven fragmentation of peptides. We have recently shown that electrospray ionization of lipids in the presence of 4-iodoaniline or 4-iodobenzoic acid can produce adduct ions in positive or negative ion mode, respectively. Once formed, these adduct ions can be mass-selected and subjected to either CID where they give rise to predictable product ions or photodissociation giving rise to a radical ion by cleavage of the carbon-iodine bond. Subsequent CID of the nascent radical ion gives rise to a rich fragmentation chemistry not dissimilar to conventional electron ionization. Analysis of a range of lipids by RDD suggests that differentiation of double bond- and stereo-isomers may also be possible by this approach.

Conclusions

OzID and RDD can differentiate double bond-isomeric lipids and thus identify the presence of lipid diversity

LIPIDOMICS AT THE HIGH MASS RESOLUTION

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lipidomics, high mass resolution, Orbitrap, HCD

Material and Methods

Shotgun analysis of total lipid extracts was performed on LTQ Orbitrap Velos and Q-Exactive hybrid tandem mass spectrometers (Thermo Fisher Scientific). Lipid species were identified and quantified by LipidXplorer software (Herzog et al, 2011). Ecdysterols in Drosophila larvae extracts were profiled by LC-MS/MS in multiplexed SIM mode using the Agilent 1200 LC interfaced to the Q-Exactive mass spectrometer.

Results

The paper demonstrates how high resolution of hybrid tandem mass spectrometers tackles the compositional complexity of total lipid extracts and enables rapid and quantitative characterization of individual molecular species of major classes of eukaryotic lipidomes. Because of its technical simplicity, rational organization of spectra datasets and available cross-platform software, shotgun set up can be adopted by any interested laboratory having no previous experience in lipidomics. We also provide evidence that a powerful combination of the high mass resolution Orbitrap analyzer and selecting quadrupole mass filter in Q-Exactive instruments fosters new lipid discovery strategies that take advantage of accurate mass measurement by multiplexed SIM.

Conclusions

The emerging high mass resolution tandem mass spectrometers are changing the most basic analytical concepts of lipidomics by providing rapid, accurate and quantitative characterization of individual molecular lipid species.

STRATEGIES FOR IMAGING BIOMOLECULES BY TOF-SIMS AND MALDI-TOF/TOF MASS SPECTROMETRY

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Material and Methods

Mass Spectrometry Imaging (MSI) is becoming a more and more widely used method for chemical mapping of organic and inorganic compounds from various surfaces, especially tissue sections. Two main different techniques are available: Matrix-Assisted Laser Desorption/Ionization (MALDI), where the sample, preliminary coated by an organic matrix, is analyzed by a UV laser beam, and Secondary Ion Mass Spectrometry (SIMS), for which the target is directly submitted to a focused primary ion beam.
MALDI is limited to a spatial resolution (defined here as the smallest pixel size, and equal to the beam focus) of ~50 µm, while current TOF-SIMS instruments can reach resolutions of 0.4 2 µm.
Different samples may need different preparation methods. Tissues are easily sectioned and deposited onto flat supports using a cryomicrotome whereas samples of great hardness such as cultural heritage samples or seeds need to be embedded in resin and cut with ultramicrotomes and diamond blades.

Results

Both MALDI and TOF-SIMS techniques revealed excellent performances for mapping biomolecules, mainly lipids, on tissue surfaces. Nevertheless, in TOF-SIMS, the sub-micrometric spatial resolutions are obtained at the expense of the mass resolution, which is mandatory for the structure assignment of the ion species. The identification of the ion species is then a key point, since tandem mass spectrometry is available with modern MALDI mass spectrometers but not on the majority of the TOF-SIMS instruments. In the later case, the structure identifications can be done using PSD (post-source decay), comparison of the mass spectra with reference compounds and colocalisation with fragments.
The analysis of the data needs to be driven with great care. "Manual" analysis, using regions of interest and human eye scrutinizing of spectra and images is a first step which in most cases provides the main features of the chemical content of the tissue under investigation. However, statistical analysis, such as clustering and principal component analysis cannot be neglected.
These different aspects of imaging mass spectrometry will be discussed and illustrated by the results obtained in various analyses of diseased biological tissues. Localized lipidomics carried out by use of imaging mass spectrometry provides new insights in the metabolic pathways involving lipids for some diseases. However, other types of biological compounds can be successfully imaged, such as flavonoids, peptides or primary metabolites. Other applications in the field of xenobiotics will also illustrate the usefulness of imaging mass spectrometry for PK/PD studies of drugs and for environmental exposure to toxicants.

Conclusions

Today, MSI can be performed on most conventional or tandem mass spectrometers. Its future use in biomarker discovery and as a clinical diagnostic tool is very promising, assuming appropriate original and efficient strategies are developed. The contribution of MSI in biology is indisputable. In the field of lipids, for example, no other imaging technique is able to locate structurally well-defined molecules in a biological tissue. The constant improvement of the instrumentation, the simplification of experimental procedures, and the needs expressed by the biology and medicine communities make MSI one of the most interesting approaches of bioanalytical chemistry.

MEIBUM LIPID COMPOSITION IN ASIANS WITH DRY EYE SYNDROME

Sin Man Lam[1], Louis Tong[3,4,5], Siew Sian Yong[4], Bowen Li[2], Shyam S Sunder[4], Guanghou Shui [2,3], Markus R Wenk [1,2]*

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meibum, mass spectrometry, dry eye, Asians

Material and Methods

We examined the meibum lipidome of 27 dry eye patients and 10 control subjects for a total of 256 lipid species from 12 major lipid classes, including cholesteryl ester (CE), wax ester (WE), triacylglyceride (TAG), (O-acyl)-omega-hydroxy-fatty acids (OAHFA), glycerosphingolipids (phosphatidylcholine, PC; phosphatidylethanolamine, PE; phosphatidylinositol, PI; phosphatidylglycerol, PG) and sphingolipids (sphingomyelin, SM; ceramide, Cer; glucosylceramide, GluCer; dihexosylceramide, DihexCer). Neutral lipids were analysed using high-performance liquid-chromatography coupled with mass spectrometry (HPLC/MS) and tandem mass spectrometry (MS/MS) was used for the qualitative and quantitative analysis of polar lipid species. Dry eye patients were classified into three severity groups (i.e. mild, moderate and severe) based on the ocular surface disease index (OSDI). The lipid levels in patients from the three categories were compared to correlate changes in lipid levels with disease progression. The changes in meibum lipid profiles of dry eye patients compared to normal individuals classified based on OSDI score were also investigated.

Results

Results from multiple lipidomic analyses revealed that the non-polar lipid classes of CE, WE and TAG comprise the bulk of human meibum lipids (approximately 96%), while the class of OAHFA constitutes the majority of the polar lipid fraction (approximately 3.5%). At least 20 different species of CE were observed, with essentially long chain and very long chain fatty acid residues (LCFA and VLCFA) ranging from C16 to C32. Also, a number of highly unsaturated TAG species were detected, and their identities had been further confirmed by subsequent accurate mass analysis using the LTQ Orbitrap mass spectrometer. Numerous isobaric OAHFA species were found and their identities were confirmed using precursor ion scans in the negative ion mode as well as fatty acyl-based MRM approach. To our knowledge, this is the first attempt at the quantification of OAHFA species using HPLC-MRM approach. A significantly lower level of TAG ($p<0.05$) was observed in patients under the moderate category compared to the mild category, which might imply the critical structural function exerted by TAG at the preocular tear film. Notably, a number of OAHFA species displayed consistently decreasing levels that correlate with increasing disease severity. Their clear and discernible trend across different disease severity levels might possibly allow them to function as indicators of disease progression. In addition, a number of highly unsaturated TAG species, as well as lyso-PC and ether/plasmalogen-PC species were present in significantly higher levels ($p<0.05$) in patients than control subjects, indicating the possible roles of lipid peroxidation and inflammation in disease onset.

Also, the levels of a number of GluCer species were significantly increased ($p<0.05$) in the meibum of patients compared to control subjects, which might be related to the process of abnormal hyperkeratinization that represents a key causative factor for evaporative dry eye.

Conclusions

This represents the first attempt to provide an insight into lipid species that might be pathologically relevant for dry eye syndrome on scale of the entire lipidome, which can undoubtedly facilitate a more comprehensive understanding of the disease itself. In essence, more drastic quantitative differences were observed in minor lipid species of lower abundance compared to nonpolar species (CEs and WEs) that constitute the bulk of the meibum. Amongst the various lipid classes investigated, OAHFAs represent the only class of lipids with consistently decreasing levels that correlate with increasing disease severity, which renders these lipids suitable indicators of dry eye disease progression.

INTEGRATION OF OMICS RESEARCH WITH DEVELOPMENTAL BIOLOGY

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Lipidomics, Imaging, Data Integration

Material and Methods

I will exemplify how lipidomics experiments performed in the *Drosophila melanogaster* model can be associated with imaging, behavioral assays and biochemical parameters to gain functional insights. Mass spectrometric analysis was performed on a hybrid LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) coupled with a 1200 micro-LC-system (Agilent Technologies, Waldbronn, Germany) using the Nanomate TriVersa (Advion BioSciences Ltd, Ithaca NY). Mass spectrometric data interpretation was performed with proprietary software LipidXplorer and Xcalibur (Thermo Fisher Scientific, Bremen, Germany). Imaging was carried out on an Olympus FV100 Confocal Microscope (Olympus Corporation, Japan). Thin sections were prepared with a Leica CM 1850 cryostat (Leica Microsystems, Wetzlar, Germany). Laser microdissection and imaging was carried out on an customized Zeiss PALM MicroBeam system (Carl Zeiss MicroImaging GmbH, München, Germany).

Results

In recent years Omics approaches have enabled to gain insight into the tremendous complexity of bio-molecules involved in life processes. Depending on the bio-molecule class regulatory and/or structural aspects can be investigated by identifying and quantifying the key molecular player. Often the discovery of the primary molecular target(s) is hindered by the fact that complex networks are perturbed where their responses are difficult to predict and/or not all building blocks of the concerned pathways are known.
We have developed workflows integrating genetic tools, biochemical assays, behavioral assays, Imaging and Omics which allow us to quantify lipids in context of progression of biological processes. As case studies I will discuss investigations on the influence of sphingolipids metabolism on neurodegeneration and how neutral lipid homeostasis is impaired by perturbations of Calcium (Ca^{2+}) signaling.

Conclusions

Omics research provides an enormous descriptive power for biological processes on the molecular level. For the *Drosophila melanogaster* model we present our first functional insights into the role of lipids in developmental processes which were acquired through integration of diverse experimental approaches.

ADIPOSE TISSUE LIPIDOME: BENEFITS AND COSTS OF LIPID REMODELING AS ADAPTATION TO ACQUIRED OBESITY

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lipidomics, membrane phospholipids, allostasis, obesity, diabetes

Material and Methods

We performed lipidomics analyses to further expose AT lipid changes and their relationships with gene expression and insulin sensitivity in acquired obesity. All healthy obesity-discordant MZ pairs (TwinA BMI>30, TwinB BMI = approx 25) from birth cohorts 1975-1979 (MZ n=658) were invited to the study. Of the eligible 18 pairs, 14 participated. AT lipidomics measurements were performed by UPLC/MS, fatty acid (FA) analyses by gas chromatography, and transcriptomics arrays by Affymetrix chips. Insulin sensitivity was assessed by the euglycemic clamp technique.

Results

The obese co-twins had profound alterations in AT lipid and FA composition. Ten most significantly changed lipids belonged to ether phospholipid molecular species including plasmalogens. We performed molecular dynamics simulations of lipid bilayers according to phospholipid composition in obesity discordant twins. The results of simulations suggest that the phospholipid changes in acquired obesity may be due to the need to maintain membrane fluidity in growing adipocytes. Furthermore, obese co-twin's fat was characterized by lipogenic palmitoleic and proinflammatory arachidonic acids, and a depletion of essential linoleic and alpha-linolenic acids. These changes correlated with the inactivation of mitochondrial and the overactivation of inflammatory transcription pathways, as well as whole body insulin resistance.

Conclusions

Our findings suggest that AT membrane lipid changes may be associated in the development of metabolic aberrations in obesity independent of genetic effects. If interventions can modulate this network for both membrane functional maintenance and vulnerability to inflammation, new opportunities may arise for the prevention or treatment of obesity-related metabolic complications and comorbidities.

TUTORIAL II / SOFT IONIZATION METHODS

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One key factor for the sensitive mass spectrometric analysis of non-volatile biomolecules (e.g., peptides and proteins, oligosaccharides, and lipids) is the “soft” transfer of intact compounds from the condensed (liquid or solid) phase into the gas phase. A second factor is the concomitant ionization of the analyte, typically achieved by protonation/deprotonation or by adduct formation with alkali cations. Different techniques have been developed to achieve these goals, with electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) being currently the two most widely used techniques.

This tutorial will provide an overview of basic physico-chemical, laser irradiation, and instrumental parameters that facilitate the intact transfer of labile biomolecular ions into the gas phase and their mass spectrometric detection.

GAS PHASE STRUCTURAL AND DYNAMICAL BIOLOGY

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ion mobility MS; structrural biology; protein folding; molecular chaperones; dynamics

Material and Methods

We employ nanoelectrospray mass spectrometry to examine protein assemblies in the 1MDa range, intact in the gas phase. Time-resolved measurements are performed in real-time and multiplicate using a nanoelectrospray robot (NanoMate; Advion BioSciences). All spectra are obtained on mass spectrometers optimised for the analysis of high mass analytes (QToF 2, Synapt HDMS, Synapt G2 HDMS; Waters).

Results

We have investigated members of the small heat-shock proteins (sHSPs), an important family of molecular chaperones which act to prevent protein aggregation and desposition under conditions of cellular stress. Molecular level details of these proteins have been hard to come by due to their intrinsic heterogeneity and equilbrium fluctuations, properties which likely govern their function in vivo.

We obtain three types of information from our MS-led approach: compositional, structural, and dynamical. By performing tandem MS we separate the oligomers which are adopted by the polydisperse mammalian sHSP alphaB-crystallin, such that we can quantify their relative abundance.

Strucural restraints are obtained from travelling-wave ion mobility MS measurements, in which arrival times can be converted into a physical size in terms of a rotationally averaged collisional cross-section. The values we obtain are directly related electron microscopy measurements, and allow us to filter candidate structures.

Equilibrium dynamics are studyed on the quaternary level by monitoring the exchange of subunits between oligomers by means of MS. These can be correlated with tertiary fluctuations obtained in advanced NMR experiments.

Combining our MS data, along with the complementary information from EM and NMR, allows us to determine structrures for the different oligomers populated by alphaB-crystallin. Furthermore, we extract the thermodynamic and kinetic consequences of known disease-related mutations and post-translational modifications.

Conclusions

Elucidating molecular details of the most challenging protein assemblies can not be performed by one technique alone, but rather requires the integration of multiple methods into a hybrid approach. MS, with its sensitivity, universality, and ability to provide insights into the quaternary structure and dynamics of proteins has a vital role to play in structural and dynamical biology.

IDENTIFICATION OF MULTIPLE FOLDING PATHWAYS OF MONELLIN USING PULSED THIOL LABELING AND MASS SPECTROMETRY

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folding, multiple pathways, pulsed-thiol labeling, monellin, mass spectrometry

Material and Methods

In this study, the complex folding reaction of single-chain monellin has been investigated using a pulsed thiol labeling (SX) methodology in conjunction with mass spectrometry, which measures the kinetics of burial of a cysteine side chain thiol during folding. Because it can directly distinguish between unfolded and folded molecules and can measure the disappearance of the former during folding, the pulsed SX methodology is an ideal method for investigating whether multiple pathways are operative during folding.

Results

The kinetics of burial of the C42 thiol of monellin was observed to follow biexponential kinetics. To determine whether this was because the fast phase leads to the partial protection of the thiol group in all the molecules or to complete protection in only a fraction of themolecules, the duration and intensity of the labeling pulse were varied. The observation that the extent of labeling did not vary with the duration of the pulse cannot be explained by a simple sequential folding mechanism. Two parallel folding pathways are shown to be operative, with one leading to the formation of thiol-protective structure more rapidly than the other.

Conclusions

The use of a pulsed SX methodology coupled to mass spectrometry has made it possible to show that unfolded molecules enter two separate folding pathways. The relative utilization of the three pathways depends upon the conditions used for folding. These results elucidate the roles played by folding intermediates in directing the utilization of alternative folding pathways when many pathways are available.

References:

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DIFFERENTIAL EXPRESSION OF RED CELL PROTEINS IN HEMOGLOBINOPATHY

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Scikle Cell Disease; Thalassemia; Hemoglobin variant; Chaperone; Redox regulators

Material and Methods

We have used 2D-gel electrophoresis and tandem MALDI mass spectrometry based techniques to investigate the differential proteome profiling of membrane and hemoglobin-depleted fraction of cytosolic proteins of erythrocytes isolated from the peripheral blood samples of SCD patients, thalassemics and normal volunteers.

Results

Our work showed that redox regulators like PRDX2, Cu-Zn superoxide dismutase and thioredoxin and chaperones like AHSP and HSP-70 were up-regulated in HbEbeta-thalassemia. We have also observed larger amounts of membrane associated globin chains and indications of disruption of spectrin-based junctional complex in the membrane skeleton of HbEbeta-thalassemic erythrocytes upon detection of low molecular weight fragments of alpha-spectrin and decrease in beta-actin and dematin content. Our group is currently trying to analyze homogygous HbS and HbSbeta-thalassemics looking for differential expression in the red cell proteome. PRDX2 showed significant dimerization in the hemoglobin depleted cytosol of SCD patients, hallmark of severe oxidative stress. We have also observed elevated levels of AHSP, Hsp 70 and thioredoxins in SCD.

Conclusions

Our studies have revealed differential regulation of 7 membrane proteins including proteins like spectrin, actin and band 3, while from the cytosolic part, a total of 12 proteins including redox regulators like peroxiredoxin 2, Cu-Zn-superoxide dismutase, catalase and chaperones like HSP70 and AHSP were differentially regulated. We have also found out a correlation or association of the extent of change of the levels of the cytosolic proteins in the thalssemic patients with varying levels of HbE. This could lead to better understanding of the pathophysiology of the disease and role of HbE in HbEbeta-thalassemia.

COMPARATIVE PROTEOMICS OF EXTRACELLULAR MATRIX DEMONSTARTES THE COORDINATED EXPRESSION OF DEHYDRATION-RESPONSIVE PROTEINS

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extracellular matrix; proteome; differential response; redox homeostasis; dehydration tolerance

Material and Methods

Chickpea (c.v. ICCV-2 and JG-62) seedlings were grown at 25 ± 2 ºC, 50 ± 5% relative humidity under 16 h photoperiod. Dehydration treatment was given to 3-week-old seedlings by withdrawing water, and aerial tissues were harvested at every 24 h up to 192 h. The extracellular matrix (ECM) fraction was isolated from unstressed and stressed seedlings using standard protocol. Isoelectric focusing was carried out with 125 µg protein on 13 cm IPG strips (pI range of 4-7). The strips were then loaded on top of 12.5% polyacrylamide gel for SDS-PAGE. The electrophoresed proteins were detected with silver stain. Dehydration-induced temporal changes in the protein profile were monitored in both the genotypes. The dehydration-responsive proteins (DRPs) that changed their intensities for more than 2.5-fold at least at one time point were excised, in-gel digested with trypsin, and identified using ESI-LC/MS/MS (Applied Biosystems, USA).

Results

Interdependence of the extracellular matrix, plasma membrane, and cytoskeletal network is the major determining factor that dictates cell fate decision in response to different stress conditions. In this process, the extracellular matrix serves as a repository for the extracellular domains for plasma membrane proteins and various signaling molecules. To better understand dehydration adaptation, a proteomic study was performed on the ECM of c.v. ICCV-2 and JG-62, two contrasting genotypes of chickpea. The extracellular matrix was isolated by mechanical disruption and differential centrifugation, and evaluated by transmission electron microscopy. The proteomes were generated with extracellular matrix proteins using classical 2-DE coupled with LC-ESI-MS/MS analysis. The temporal changes in the matrix proteome of c.v. JG-62 and ICCV-2 were monitored under progressive dehydration (0-192 h). The comparison of dehydration-responsive proteome revealed 163 DRPs observed in c.v. JG-62 as compared to 118 in ICCV-2. The comparison of different functional classes of the DRPs in these genotypes showed a significant variation, indicating their differential response to dehydration. The proteomes of c.v. ICCV2 and JG-62 at unstressed condition revealed that only 50% of the protein spots were common between them. While c.v. ICCV-2 and JG-62 showed 326 and 271 protein spots respectively, the number of DRPs were found to be much higher in JG-62. These data suggest that the subtle changes in the genome might lead to significantly distinct proteome, contributing towards variety-specific response to dehydration. This study led to the identification of several potential dehydration-responsive components, which may be utilized to manipulate cell wall composition towards improved adaptation to dehydration through genetic engineering approaches. Our future efforts will focus on determining the molecular mechanism of function of these components in dehydration tolerance in plants.

Conclusions

The differential response of extracellular matrix in c.v. ICCV-2 and JG-62 may be explained by virtue of early- and late-dehydration responses by cell wall restructuring, and activation of targeted proteins involved in cell defense and rescue. The major difference emerged in the redox homeostasis wherein the major ROS-catabolizing enzymes were either downregulated or not effectively activated in c.v. ICCV-2. It is thus proposed that cell wall restructuring, and superior homeostasis particularly the management of reactive oxygen species may render better dehydration-adaptation in plants, which are otherwise sensitive to such stresses.

ON-LINE UPLC/ION MOBILITY SEPARATION/TOF MS FOR QUALITATIVE AND QUANTITATIVE PROTEIN PROFILING IN COMPLEX BIOLOGICAL SAMPLES

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Material and Methods

There is a growing consensus within the proteomics community that, in the analysis of complex digests, exact mass analysis aids the unambiguous matching of tryptic peptide spectra to databanks of known protein structure. Where exact mass measurement of both precursor and product ions contribute significantly to minimizing false discovery in peptide/protein identification.
We have analysed bacterial, yeast and mamalian proteomes by combined multidimensional nanoUPLC (nanoACQUITY, Waters Milford MA, USA) and Ion Mobility Separation-Time of Flight Mass Spectrometry (Synapt G2, Waters Manchester, UK). The adaventage of incorporating the additional dimension of Ion Mobility Separation is ilustrated by comparison with conventional LC/MS/MS analysis.

Results

We will elucidate the challenge of sample complexity in proteomics by summarizing our findings, initially, from a one dimensional reversed phase UPLC separation (120 minute gradient) of a complex protein digest (E. coli) with Electrospray exact mass MS detection. The data show Ca 450,000 unique ions that, following charge state and Isotope deconvolution, reveal Ca 40,000 non-redundant precursor ions for identification and quantification. This complexity is further compounded by the fact that the detected ions are not uniformly distributed in time or m/z range. Typically 50% of all ions detected are observed in <25% of the total run time and >60% of all ions detected are observed in the m/z 400-800 range. Moreover 70% of all ions are two or more orders of magnitude less intense than the most abundant ions detected. In addition precursor ions of similar mass and retention time are frequently observed (<50% of the time) that can give rise to mixed or chimeric spectra that distract the library search algorithms commonly used for protein identification.

We describe a novel approach to address the analytical challenge inherent in such sample complexity embodying the on-line combination three dispersive analytical techniques; 2D UPLC, Ion Mobility Separation (IMS) and Time of Flight (ToF) MS. Additionally the advantage of collecting UPLC-IMS-MS data at high mass resolution and mass accuracy will be summarized. Preliminary results illustrate how UPLC-IMS-MS successfully increases peak capacity by a factor of 7 enabeling more components of complex protein digests to be unambiguously identified and quantified per unit time.

Conclusions

Protein digests can be extremely complex, where the number of unique (precursor)ions may excede100s ions/sec at peak flux . UPLC/MS/MS is a self limiting, serial, process whereas UPLC/IMS/MS is a multiplexed alternative. Chimericy is observed to be a major limitation in conventional UPLC/MS/MS aproaches. However, UPLC/IMS/MS may significantly reduces chimericy due to the fact that IMS can differentiate isobaric (co-eluting) precursor ions.

A DRAFT MAP OF THE HUMAN PROTEOME

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Material and Methods

Autopsy tissue samples from different human organs were obtained after ethical clearance. Protein samples were fractionated using SDS-PAGE/Basic RPLC. LC-MS/MS analysis was carried out on LTQ-Orbitrap Velos mass spectrometer. The database searches were carried out using Sequest search engine through Proteome Discoverer 1.2. False discovery rate (FDR) of 1% was used as a cut-off to filter identified peptides. In addition to human protein database search, we carried out searches against 6 frame translated human genomic regions encompassing intronic and intergenic regions.

Results

The draft sequence of the human genome was published in 2001. It was a concerted effort from different sequencing centers from across the globe. Ever since, there is a constant effort to accurately annotate protein coding genes in the human genome. Annotation of protein coding genes in human genome has largely depended upon mRNA sequences from large scale transcriptome sequencing efforts, ESTs, comparative genomics and gene prediction programs. The longest ORF from these transcripts is often annotated as the coding sequence. These proteins remain hypothetical until experimental evidence is established for the presence of that protein. There are several shortcomings of this approach considering that a significant part of our genome is transcribed but need not necessarily translated. This includes transcripts arising from pseudogenes and other regulatory RNAs that are often considered non-coding. There is no experimental basis to conclude these transcripts do not code for proteins. Addressing these issues requires a systematic effort to characterize human proteome using appropriate technologies that measure proteins.

The past decade has witnessed significant developments in proteomic methodologies. Mass spectrometers with capability to identify thousands of proteins in a given sample are now available. Further, high sensitivity of these mass spectrometers enables identification of proteins over a wide dynamic range. This provides an opportunity to systematically sample various human tissues to generate a map of the human proteome. We have employed high resolution mass spectrometry to systematically map the human proteome from various tissue types. Our efforts have resulted in obtaining mass spectra for most of the proteins annotated in human genome and has also resulted in identification of several novel protein coding regions. Various transcripts that were previously annotated to be non-coding were found to be protein coding. This includes transcripts arising from “pseudogenes”. Further, we have identified various protein isoforms resulting from alternative splicing events.

Conclusions

A thorough understanding of human proteome has significant implications on human biology. Diagnostic and therapeutic value of understanding human proteome necessitates efforts to characterize human proteins along the similar lines that human genome characterization was undertaken. The proteome complement of a cell poses additional challenges due to various modifications and alternative spliced forms that result in numerous versions of a single gene product. In addition to complementing existing genome annotation, this data serves as a basis for proteomics driven annotation of several novel protein coding regions in the human genome.

G-QUADRUPLEX FORMATION OF GUANOSINE DERIVATIVES IN PRESENCE OF ALKALINE EARTH METAL IONS STUDIED BY ELECTROSPRAY IONIZATION MASS SPECTROMETRY

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G-quadruplex, guanosines,alkaline earth metals

Material and Methods

Stock solutions of deoxyguanosine, guanosine, and isopropylidene guanosine and metal salt solutions (0.01M) were prepared in water:methanol (50:50 v/v). The experiments were performed using an LCQ ion trap mass spectrometer (Thermo Fisher), and with Q-STAR (Applied Biosystems) mass spectrometers equipped with ESI sources. The data acquisition was under the control of Xcalibur and Analyst softwares respectively. The data with LCQ instrument was acquired in the positive ionization mode and the typical source conditions were: spray voltage, 4.5 kV; capillary voltage, 15V; heated capillary temperature, 200°C; tube lens offset voltage, 30 V; sheath gas (N2) flow rate, 20 units; and helium was used as damping gas. All the spectra were recorded under identical experimental conditions, and averaged over 30 scans. All the samples were infused into the ESI source at a flow rate of 10 microlitre/min. by using Harvard syringe pump.

Results

Guanine rich nucleotides are known to form planar guanine quartets linked through Hoogsteen hydrogen bonds and are stabilized by metal cations. Guanine quadruplexes (G-quadruplexes) are formed by stacking guanine quartets one above the other. It is reported in the literature that alkali metal ions such as sodium and potassium stabilize the G-quadruplex structures by occupying the middle of the guanine quartet cavity and coordinate with the four guanine oxygen atoms, whereas divalent metal ions such as strontium, barium, and lead intercalate between two G-quartets by coordinating with eight oxygen atoms of guanines present in the two planes. We undertook a detailed investigation on the formation of G-quadruplexes of deoxyguanosine, guanosine, and isopropylidene guanosine with alkali earth metal cations such as magnesium, calcium, strontium, and barium. The electrospray ionization mass spectra were studied for deoxyguanosine, guanosine and its isopropylidene derivative with alkaline earth metal ions Mg2+, Ca2+, Sr2+ and Ba2+. The spectra of the substrates with all the metals studied, except for Mg2+, showed [8dG+M]2+ as a major peak. The [8dG+M]2+ ion is expected to have the quadruplex structure wherein the metal ion is intercalated between two deoxyguanosine quartets. The competitive experiments showed that the stability order for the octamer ion, [8dG+M]2+ is Sr2+>Ba2+>Ca2+>Mg2+. This order reveals that Sr2+ and Ba2+ have higher affinity to stabilize the octamer ion when compared to the other alkaline earth metal ions. The abundance of [8dG+M]2+ ion as a function of collision energy also reflected the same order of stability that we observed in the competitive experiments.

Conclusions

Electrospray ionization mass spectrometry (ESI-MS) has been found to be a convenient method for studying the interaction of various substrates with metal ions. This study has also shown that the formation of the quartet is the inherent property of guanine base and it is not affected by the presence of ribose or deoxyribose in the nucleosides. The present ESI-MS result suggests that metal oxygen bond length is responsible for the stabilization of quadruplex in the case of alkaline earth metal ions.

IR-MALDI MASS SPECTROMETRY FOR THE ANALYSIS OF (SMALL) MOLECULES DIRECTLY FROM BIOLOGICAL TISSUE AND FROZEN AQUEOUS SOLUTIONS

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MALDI, infrared laser, metabolites, water ice matrix, plant defense

Material and Methods

Two infrared lasers were employed for infrared matrix-assisted laser desorption ionization mass spectrometry (IR-MALDI-MS). The first was a Er:YAG laser that emits light pulses of ~150 ns duration at 2.94 µm. The second laser is an optical parametric oscillator (OPO) laser that is tunable between 2.71-3.14 µm and has a pulse duration of 6 ns. Employing these lasers allows water to be used as a MALDI matrix and, therefore, facilitates the analysis of biomolecules directly from aqueous solutions and water-containing biological tissue. The MS analysis is aided by the employment of an orthogonal time-of-flight (o-TOF) mass spectrometer utilizing an oMALDI 2 ion source (AB SCIEX) that is operated at a buffer gas pressure of ~1 mbar. Implementation of a custom-made cool stage allowed the sample temperature to be adjusted between -110 °C and room temperature (RT), thereby enabling the analysis of frozen aqueous samples.

Results

In a first set of experiments, we analyzed a variety of biological tissues at RT (e.g, brain slices, seeds, petal leaves) and assessed the detection of lipids (triglycerides, phospholipids, glycolipids), oligosaccharides, and flavonoids. These data showed that IR-MALDI-o-TOF mass spectrometry allows detection of a wide range of metabolites and lipids directly from small pieces of tissue. Notably, it is not necessary to coat the tissue with a matrix, as in standard ultraviolet (UV-)MALDI-MS and UV-MALDI imaging (IMS). This simplifies sample preparation and facilitates data interpretation since matrix-derived background ions no longer overlap with the signals of small molecules.

In a second set of experiments, we refined the methodology for the analysis of secondary metabolites and messenger molecules that were screened from small pieces of healthy and pathogen-infected leaves of Nicotiana tabacum plants [1]. The defense response of the plant against the oomycete Phytophthora nicotianae was profiled between 1–9 h after infection and data evaluated using principal component analysis (PCA). These results show that IR-MALDI-MS allows the time course of the plant defense response to be followed and provides a rapid characterization of the tissue as healthy, irritated, or infected. Interestingly, the presence of an infection in neighboring tissue areas or a mere mechanical wounding could also be differentiated.

In a third set of experiments, we amended the methodology by implementation of a cool stage. Use of the cool stage allowed 1) improved preservation of the tissue water (thus aiding the analysis of less rigid tissue) and 2) the analysis of biomolecules from frozen aqueous solutions. The analysis of peptides/proteins and oligosaccharides from a water ice matrix, and that of a food sample (a Pilsner beer) are shown as examples.

Conclusions

IR-MALDI-o-TOF mass spectrometry constiutes an interesting alternative to common UV-MALDI-MS because it allows the utilization of “endogenous” water as a matrix. Therefore, biological tissue can be analyzed with little sample preparation. The method also induces little fragmentation of analyte ions. Future developments will address using the method for full MS imaging with high lateral resolution and seek to improve the analytical sensitivity.

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QUANTITATIVE ANALYSIS OF SMALL BIOMOLECULES USING LASER DESORPTION IONIZATION MASS SPECTROMETRY

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LDI MS, MALDI MS, Small molecules, Metabolites, Quantitation

Material and Methods

Among the strategies employed for small molecule laser desorption / ionization mass spectrometry (LDI MS), inorganic materials such as silicon and titania have been most widely used. However, small molecule and metabolite analysis using LDI MS for high throughput analysis, tandem MS and quantitation have not been widely investigated. In our group, inorganic particle assisted LDI MS has been explored for small molecules of toxicological importance, bacterial signaling molecules and amino acids, with a focus on their identification and quantitation. LDI MS was performed using ABI Voyager MALDI-TOF system and Waters Synapt HDMS (Manchester, UK) operated in V-positive MALDI-TOF mode. Data analysis following LDI MS was performed using 'MQ', a data processing algorithm developed at NCL for targeted analysis.

Results

Quantitation using inorganic-particle based LDI MS resulted in excellent linearity along with reasonable to good precision and accuarcy for trace analyte detection. The results were found to be comparable to conventional LC-ESI-MS with the added advantage of high throughput and faster analysis. Although matrix interference persists when using organic or conventional matrices, appropriate selection of matrices can lead to a robust method for small molecule analysis.

Conclusions

Tedious sample preparation, derivatization and time consuming chromatographic or method development steps can potentially be eliminated in the MALDI MS mode making it attractive to a large number of users for high throughput analysis of low molecular weight analytes.

DISCOVERY OF NOVEL DROSOPHILA LIPID PHEROMONES USING MASS SPECTROMETRY

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laser desorption/ionization, triglycerides, hydrocarbons, lipids, pheromones

Material and Methods

Cuticular lipids expressed by Drosophila mojavensis and Drosophila arizonae fruit flies were profiled using (i) direct UV-laser desorption/ ionization (UV-LDI) MS (Ref. 1) and (ii) direct analysis in real time (DART™) MS (JEOL). UV-LDI MS was performed with a QSTAR pulsar I™ (AB SCIEX) equipped with a N2 laser (lambda = 337 nm; tau = 3 ns) and a modified oMALDI 2™ ion source (AB SCIEX) that allowed elevated buffer gas pressures in the source of a few mbar. Cuticular lipids were analyzed directly from intact flies with a spatial resolution of 200 µm. DART-MS was performed with a AccuTOF™ MS. Single flies or aliquots from cuticular extracts were placed directly in the DART source. Structural analysis was performed using gas chromatography (GC) MS (Shimadzu) and HPLC-electrospray ionization (ESI) MS/MS with an Acquity™ UPLC system coupled to a Synapt G2 HDMS™ (Waters). Thin-layer chromatography was used for chemical purification of cuticular lipids.

Results

Both UV-LDI and DART MS analyses of D. mojavensis and D. arizonae cuticles revealed the expression of long-chain unsaturated hydrocarbons (C33 – C39) bearing between 1-4 double bonds (alkanes are not detected by the two methods). The cuticular lipid profiles expressed by the two fly species were qualitatively similar. Additionally, males and females within each species showed similar chemical profiles on most of the body parts (e.g., head, abdomen, dorsal surface, and legs). Interestingly, several previously unidentified triglycerides (TGs) and oxygen-containing hydrocarbons were found only in the anogenital regions of males. Moreover, males of both species expressed significantly different amounts of these compounds. UV-LDI MS analysis of recently mated female flies also revealed that these male-specific lipids are transferred to females during copulation. Preliminary structural characterization by HPLC-ESI-MS/MS and GC-MS indicates that the TGs are composed of a longer polyunsaturated fatty acyl chain and two significantly shorter fatty acid moieties. Behavioral experiments measuring courtship activity showed that male-specific TGs are likely to serve as anti-aphrodisiacs. Methodological aspects of the UV-LDI analysis of insect cuticles will also be discussed in the presentation (Ref. 2).

1. Yew, JY, K Dreisewerd, CC de Oliveira and WJ Etges. 2011. Male-Specific Transfer and Fine Scale Spatial Differences of Newly Identified Cuticular Hydrocarbons and Triacylglycerides in a Drosophila Species Pair. PLoS ONE 6: e16898

2. Yew, JY, J Soltwisch, A Pirkl and K Dreisewerd. 2011. Direct Laser Desorption Ionization of Endogenous and Exogenous Compounds from Insect Cuticles: Practical and Methodologic Aspects. Journal of the American Society for Mass Spectrometry 22: 1273-1284.

Conclusions

UV-LDI MS and DART MS analysis identified a putative novel class of sex-specific triglycerides and long-chain hydrocarbons expressed on the cuticular surface of Drosophila. Tandem MS analysis and chemical derivatization indicate that the TGs contain unusually short fatty acyl chains. Behavioral experiments show that these lipid cues are likely to play a role as pheromones that influence the mating choice of Drosophila.

MULTI-OMICS WORKFLOW FOR STUDYING DRUG TREATMENT ON HUMAN CELL LINE

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Proteomics, Metabolomics, Pathways, Mass spectrometry

Material and Methods

Rapamycin is an immunosuppressant drug that inhibits mTOR activity and cellular hyper-proliferation in many cells types. In this study, we aim to understand the effect of rapamycin treatment on various biological pathways using genomics, proteomics and metabolomics analysis of rapamycin treated human embryonic kidney cell line (HEK293). Gene expression, miRNA profiling were performed on respective microarray platforms and differential proteins, metabolites analysis were performed on LC-MS/GC-MS platforms. Proteins extracted from rapamycin treated and control samples were digested and analysed on a QTOF mass spectrometer coupled to HPLC chip MS system. Statistically significant differential features between the rapamycin treated and control samples were identified by targeted MS/MS analysis followed by data base search. Metabolites were extracted using Methanol: water: chloroform mixture. Organic portion was analysed on GC-MS and aqueous portion was analyzed on LC-MS (QTOF). Gene expression, miRNA data, proteomics and metabolomics data were analysed using Genespring software.

Results

Approximately 10000 genes 1000 proteins and 300 metabolites were found differentially expressed between the treated and control samples. Biological processes occur in a sequential manner and are best visualized as pathways. Therefore, pathway analysis is a critical component of multi-omics study. Proteomic analysis suggests that biological processes such as translation, transcription, DNA damage response, cell growth, protein folding, stress response are some of the biological processes affected by rapamycin treatment. These results are consistent with the observation from our gene expression analysis and literature reports. The differential metabolites include several sugars, amino acids and fatty acids. Metabolome analysis using both LC-MS (positive and negative mode) and GC-MS methods increased the total number of metabolites identified. Results from LC-MS and GC-MS analysis are mutually exclusive in nature allowing different classes of compounds to be identified.

Conclusions

This study demonstrates a profile based proteomics/metabolomics approach to find differentially expressed proteins/metabolites and pathways affected by rapamycin treatment in HEK293 cell line.

Translation, transcription, DNA damage response, cell growth, protein folding, stress response are some of the biological processes affected by rapamycin treatment.

Multi-omics approach to understand the effect of a drug treatment will be discussed in the presentation.

LIQUID EXTRACTION SURFACE ANALYSIS (LESA) COMBINED WITH NANOESI-MS FOR DIRECT SAMPLING OF SURFACES

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Material and Methods

Liquid Extraction Surface Analysis combined with nano-electrospray mass-spectrometry (LESA-nESI-MS) is a complementary analytical approach to other surface-oriented MS methods such as DESI, LDI, DART or MALDI imaging MS, where a liquid micro junction surface sampling probe is used to extract the analyte directly from a surface.
A small solvent droplet is placed on the tissue area of interest for analyte extraction and then aspirated into a conductive pipette tip for automated chip based nano electrospray infusion using the Advion TriVersa Nanomate.
Here we applied LESA to different surfaces like whole body animal slices in early stage toxicology studies for determination of small drug molecules such as Propanolol and Diclofenac and a variety of fruits to determine pesticide residues. Both triple quadrupole and high resolution mass spectrometers such as the Exactive were used.

Results

Propanolol tissue analysis; Excellent S:N was obtained for the parent drug (SFN / Propranolol),and the phase II metabolites (SFN-GSH,SFN-NAC / Hydroxypropranolol-glucuronide) when screened in MRM mode in different thin tissue sections (lung, liver, kidney, brain) from the dose and control mouse from thin mouse tissue slices pre dosed with 10mg/kg propranolol.
Diclofenac tissue analysis; Spatial resolution was determined by the contact angle of the extraction solvent on the surface and the overall volume of solvent dispensed. Using 80/20 Methanol/Water as the extraction solvent we determined an initial spatial resolution of about 1.0 mm by optical analysis of the micro liquid junction formed on target Drug and metabolites were clearly detected over control in both positive and negative ion. Diclofenac was analyzed with ca. 10-fold higher sensitivity in negative ion mode MS rather than positive ion mode MS following an exchange of the solvent modifier from 0.1 vol% formic acid to 0.1 vol% ammonium hydroxide
Pesticide study; Carbofuran, Miazinon, Malathion Diazinon were detected at 20-fold below the tolerance level of 400 ng/g when pump spayed directly onto purchased grape, apple and spinach using a high resolution Mass Spectrometer

Conclusions

LESA is capable of detecting small molecules such as drugs, fragile metabolites and pesticides from tissue and plant surfaces in combination with accurate mass high resolution MS and SRM MS with excellent S:N in both positive and negative ion.

NOVEL APPROACHES BASED ON HIGH RESOLUTION AND ION MOBILITY MASS SPECTROMETRY FOR THE QUAL/QUAN ANALYSIS OF PHARMACEUTICALS AND THEIR METABOLITES

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Material and Methods

For microsomes incubations of talinolol and quantitative analysis of paracetamol in human plasma mass spectrometric analysis was performed on a TripleTOF 5600 instrument (AB Sciex). The instrument was operated at a resolving power of 30'000 or higher with duty cycles of 50-100 msec in full scan mode and 25-50 msec in MS/MS mode. Analyses were performed in gradient mode using an ultra high pressure liquid chromatographic system (RSLC, Dionex).
Differential ion Mobility Spectrometry (DMS) Mass Spectrometry analysis samples were performed on a Q TRAP 5500 mass spectrometer (AB Sciex) equipped with a prototype DMS cell in front of the orifice plate. ACN, MeOH, EtOH, toluene, isopropanol and acetone were evaluated as DMS modifiers. Information dependent acquisition (IDA), based on a survey scan of the compensation voltage in SRM mode, and dependent experiments performed in MS/MS and MS3 modes enables simultaneously the screening and the relative quantitation of the analytes.

Results

For paracetamol quantitation and metabolites screening in plasma was achieved in a single LC-MS analysis by combining several targeted and non targeted MS or MS/MS experiments. Using HR-SRM similar limit of quantitation and dynamic range could be achieved compared to a triple quadrupole approach.
Talinolol is a highly efficient and selective β1-adrenoceptor blocking agent. Oxidation at the cyclohexyl moiety has been described as the major metabolic pathway generating several hydroxyl metabolites at various stereochemical positions. In rat liver microsomes six hydroxylated metabolites could be identified using various qual/quan approaches including data dependent acquisition, FragAll or MSeverything and a novel prototype mode of operation Sequential Windows of All THeoretical fragment ion spectra (SWATH) or Global Precursor ions Scan (GPS). SWATH was found to be particular efficient to collect all relevant data in a single run for metabolite fishing, structural elucidation as well as post-acquisition quantitation.
Assignment of the location of the hydroxylation is of importance and various approaches can be considered such as i) comparison with parent drug MS/MS spectrum ii) using fragmentation rules or iii) MSn approaches. We used a strategy based on accurate mass and the structural constrains of the parent drug to locate the hydroxylation site. The PeakView software has the capability for a given structure to generate a list of potential fragments taking into account cation and radical cation formation and to overlay them with the recorded spectra.
The resolving power of the differential mobility device can be significantly improved by the addition of modifiers in particular for isomer separation prior MS detection without chromatographic step. This is nicely illustrated for the analysis of the isobaric benzoylecgonine and norcocaine.

Conclusions

High resolution accurate mass with fast acquisition is essential for qualitative and quantitative analysis in drug metabolism or metabolomics studies. Quantitation can be performed either in HR-SIM or in HR-SRM mode with almost no tuning. For structural elucidation bottom-up approaches should be preferred to complex fragmentation rules. Sequential Windows of All THeoretical fragment ion spectra (SWATH) was successfully explored as an an alternative to classical IDA scheme for the qual/quan analysis of low molecular mass compounds. Finally, differential mobility mass spectrometry with modifiers opens new possibility to separate isobaric compounds or for selectivity enhancement in QUAL/QUAN analysis.

Poster Abstracts

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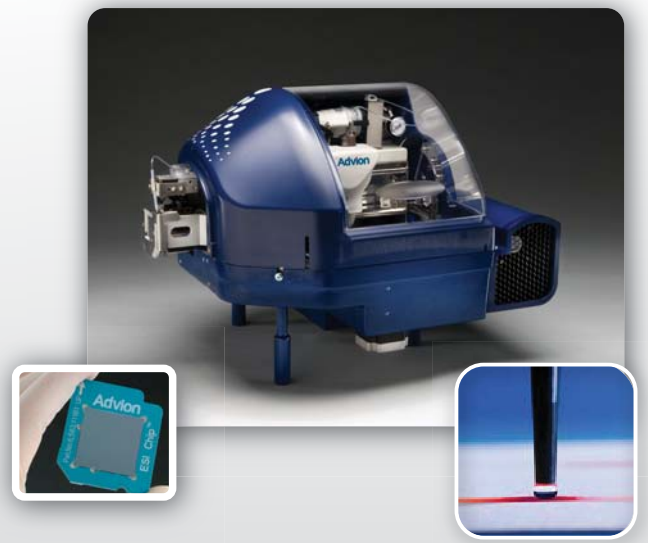

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Poster 1

NEUROLIPIDOMICS-ANALYSES INDICATE ABERRANT LEVELS OF CERAMIDE SPECIES BEFORE DISEASE ONSET IN A DROSOPHILA MODEL FOR LIPID STORAGE DISORDERS

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LC-MS, Ceramide, neurodegeneration, lipidomics, Drosophila

Material and Methods

Drosophila melanogaster were raised under standard laboratory conditions. Fly lines used are spin10403, spink09905, spinEP (Sweeney and Davis, 2002), Oregon R and w1118 (genetic controls).
We have designed a novel approach for the investigation of single dissected Drosophila brains with microscopy followed by an in-depth lipidomics analysis. Brains were dissected in Phosphate-Buffered solution (pH7.4), imaged for lipofuscin using FluoviewFV1000 confocal microscope (Olympus Corporation, Japan; Argon laser, 20x objective: NA=0.7). Post-imaging, brains were homogenized and lipids extracted using an optimized MTBE-based extraction protocol (Schwudke et al, 2008). Mass Spectrometry was performed on a LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Germany) equipped with a robotic nanoflow ion source TriVersa (Advion BioSciences Ltd, USA). Liquid Chromatography was performed on a 1200 micro-LC-system (Agilent Technologies, Germany) with a split flow setup enabling nano-ESI-MS. MS data interpretation was performed with Xcalibur (Thermo Fisher Scientific, Germany).

Results

To investigate the role of perturbed lipid metabolism in maintaining neuronal integrity, we used Drosophila mutants in the spinster (spin) gene. These mutants phenocopy disease pathology of the human Lysosomal Storage Disease cluster, i.e. they exhibit neurodegeneration, short life-span, expanded endo-lysosomal compartments and lipofuscin accumulation (Dermaut et al, 2005, Sweeney and Davis, 2002 and Nakano et al, 2001). Despite evidence that spin mutant-brain extracts accumulate lipofuscin pigment (an indicator for lysosomal malfunction and degeneration) and therefore store lipids, it is unclear as to which lipid species accumulate and how this accumulation relates to the course of disease onset and progression.
To address this, we have combined lipofuscin imaging and lipidomics-based analyses of single, dissected brains in spin mutants. In parallel, we monitored disease onset and progression in spinster mutants with lifespan analyses, behavioral studies and morphological evaluation of the nervous system.
We observed signs of neurodegeneration i.e. a progressive loss of behavior as well as loss of motor neuronal synapses, in these mutants by the first week of adult-life. During this time, massive accumulation of lipofuscin pigment was observed (by measuring autofluorescence through imaging) in these brains. Lipidomics based analyses of these brains revealed increased abundance of membrane lipids, including Ceramide Phosphotidylethanolamine (CerPE), Phosphotidylethanolamine (PE), Phosphotidylcholine (PC), concomitant with the occurrence of lipofuscin. Accumulation of membrane lipids in the subsequent stages of disease is corroborated by electron microscopy which showed evidence of multilamellar whorls and lipofuscin-like structures. Interestingly, prior to disease onset, we observe dramatic changes in one specific lipid class (ceramides: Cer 34:1, Cer 36:1, Cer34:2, Cer 36:2).
Thus storage of membrane lipids in brains and neurodegeneration in a fly LSD model is preceded by increased ceramide levels.

Poster 1

Conclusions

In conjunction with behavioral and morphological evaluations, we used a unique approach to image single brains followed by an in-depth lipidomics-based analyses. Thus, we have (1) made direct correlations between aberrant morphological features and deviations in lipid profiles in a tissue-specific manner, and (2) worked with single brains (100,000 cells) and thus have the advantage of analyzing closer to the dynamic range of the neural lipidome unlike existing methods that utilize enormous amounts of tissue material.

Our current experiments are geared towards using genetic tools for specific manipulation of lipid-metabolic steps in neurons and analyzing the impact on neuronal integrity/degeneration.

Poster 2

A DEEP-LC-MS APPROACH TO CHARACTERIZE THE ACTION OF HEPARAN SULFATE C5-EPIMERASE

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Heparan sulfate . Heparin . C5-epimerase . LC-MS . Hydrogen/deuterium exchange . Proteoglycan biosynthesis

Material and Methods

The CDSNS heparin was purchased from Seikagaku, USA. N-Sulfo heparosan was prepared by chemical N-deacetylation-N-sulfation of N-acetyl heparosan following a reported procedure. Baculovirus system that expresses HS C5-epimerase was a gift from Prof. Rosenberg (MIT, Cambridge), and the expression and purification of HS C5-epimerase was done as reported. Epimerase reactions were performed in MES buffer (pH 7.0). Hydrogen/deuterium exchange experiments with C5-epimerase were performed in D2O. All separations were performed on a Vydac C18 column (4.6×150 mm) using Waters Aliance 6250 HPLC system with a gradient elution at a flow rate of 1.0 mL/min for 30 min, using a binary solvent system composed of water and 70% aqueous acetonitrile, both containing 8 mM acetic acid and 5 mM dibutylamine as an ion-pairing agent. All mass spectrometric data were acquired using LC coupled to LCT Premier ESI-TOF MS (Waters Corporation, USA). All the MS spectra were analyzed using MassLynx V4.1 software.

Results

Currently, radioactive 3H-labeling coupled to paper chromatography is the only method used for determining the epimer content of HS chains. Here, we have developed a non-radioactive, online-LC-MS based method for the separation and quantification of epimeric disaccharides (GlcA-GlcNS and IdoA-GlcNS) generated from K5NS and CDSNS polysaccharides using low pH nitrous acid. The deaminative disaccharide products of K5NS and CDSNS-heparin were subjected to IPRP-liquid chromatographic separation followed by online mass spectrometric detection. The extracted ion chromatogram (for m/z 339.10 [M-H]-1) of K5NS sample yielded a single peak corresponding to GlcA-AManR, whereas the CDSNS-heparin yielded two peaks corresponding to GlcA-AManR and IdoA-AManR. This is the first time that two disaccharides, GlcA-AManR and IdoA-AManR, have been resolved using any HPLC method.

The above discovery was utilized to measure the activity of one of the important enzymes involved in HS and heparin biosynthesis, glucuronyl C5-epimerase. The HS C5-epimerase follows a reversible kinetics in cell-free systems, i.e., some of the newly generated iduronic acid residues are converted back to glucuronic acid residues. Therefore, the integration of peak areas of extracted ion chromatograms for GlcA-AManR and IdoA-AManR does not reflect the total activity and does not provide the extent of reverse reaction catalyzed by the C5-epimerase. To overcome this limitation, we have developed a Deuterium Exchange upon Epimerization Protocol coupled to LC-MS (DEEP-LC-MS) technique, where in C5-epimerase reaction was carried out in D2O. Using DEEP-LC-MS we distinguished and quantified both forward reaction product (DIdoA-AManR m/z 340.1) as well as back-reaction product DGlcA-AManR (m/z 340.1), by measuring relative intensity of mono isotopic ions at m/z 339.1 and 340.1 of the first peak, corresponding to HGlcA-AManR and DGlcA-AManR disaccharides, respectively. As an application, DEEP-LC-MS was used to develop a rapid and non-radioactive assay to measure the total activity of HS C5-epimerase.

Poster 2

Conclusions

We have developed an online-LC-MS technique for rapid determination of epimer content, which is being done for last three decades uisng a tedious and time consuming radioacitve labelling followed by paper chromatography method. Using DEEP-LC-MS technique we have also developed a in vitro, non-radioactive assay for heparan sulfate C5-epimerase enzyme. These two techniques either alone or in combination will become a powerful tool for a broad range of applications in the structural analysis of HS and heparin.

Poster 3

MASS SPECTROMETRIC SEQUENCING OF PEPTIDES: THE DISULFIDES & THE ISOBARIC AMINO ACIDS

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Disulfide Connectivity, Isobaric amino acids, ETD, Conotoxin, Waspotoxin

Material and Methods

Peptides under investigation are isolated from various marine cone snails collected off the coast of India and from the venom of social wasp. The synthetic peptides are synthesized using typical solid phase peptide synthesis protocols, using Fmoc chemistry. All the mass spectrometric experiments are done on a Bruker HCT Ultra ETD-II ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany). The samples are directly injected into the mass spectrometer at flow rate of 120µl h-1 and then subsequently fragmented under ETD and CID conditions. Additionally, the probable structures of the fragment ions generated through fragmentation of the intact disulfide bonded molecules are analyzed for the obtained m/z values through a developed automation protocol.

Results

This report demonstrates the use of ETD and CID methodologies to resolve two existing challenges in mass spectrometric de novo sequencing; distinction between isobaric amino acids and the determination of disulfide connectivity. The presence and corresponding CID activation of the ETD formed z ions are shown to be clinical in distinguishing isobaric amino acids leucine, isoleucine and hydroxyproline (a post translationaly modified amino acid with same nominal mass as 113 Da), through characteristic side chain fragmentation. The CID fragmentation of multiple disulfide bond containing protonated peptides preferentially have been shown cleave along the peptide backbone, with occasional disulfide fragmentation either by Cβ-S bond cleavage through Hα abstraction to yield dehydroalanine and cysteinepersulfide, or by S-S bond cleavage through Hβ abstraction to yield thioaldehyde and cysteine. The structures of these observed fragment ions are then calculated for all the probable disulfide bonded foldmers of the given molecule. In cases where a unique fragment, that exclusively arise from a particular connectivity is not observed, next generation fragment ions are obtained through CID MSn experiments. Subsequently, these MSn fragment ions are mapped against the different probable structures of the parent fragment ions coming from different connectivity patterns. To facilitate the process of structure determination of these complex fragment ions, we have also developed a computer program that generates the probable structures of these fragment ions for a given m/z value, considering all possible fragmentation pathways for molecules with intact disulfide bonds.

Conclusions

Direct mass spectrometric methodologies for distinction between isobaric amino acids and disulfide connectivity determination from the intact molecule have been developed. Further, a program that enables the structure determination of the fragment ions generated through CID fragmentation of the disulfide intact molecules is developed.

Reference:
Gupta K, Kumar M, Balaram P Anal. Chem. 2010, 82, 8313.
Gupta K, Kumar M, Chandrasekhara K, Krishnan KS, Balaram P J. Proteome Res. (Under Review)

Poster 4

TANDEM MASS SPECTROMETRY OF FENGYCIN: IDENTIFICATION OF TWO NEW CYCLIC LIPODEPSIPEPTIDES, FENGYCIN A2 AND FENGYCIN B2 FROM THE BANYAN ENDOPHYTE BACILLUS SUBTILIS K1

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lipodepsipeptide, tandem mass spectrometry, fengycin

Material and Methods

The antifungal compounds produced by the Banyan endophyte, Bacillus subtilis K1 were extracted from cell free culture supernatant by acid precipitation with 6N HCl, followed by solubilization of acid precipitates in methanol. The antifungal compounds were separated by reverse-phase high performance liquid chromatography using a C-18 semi-preparative HPLC column (150 x 4.6 mm; 4□m particle size) using MeOH:H2O:TFA (0.1%) gradient (80 to 95% MeOH for 50min, 95% MeOH for 5 min) pumped at a flow rate of 1mL/min. The molecular masses of compounds eluted in distinct HPLC peaks were determined by using a MALDI-TOF mass spectrometer. The fengycin containing HPLC fractions were subjected to mild alkali hydrolysis to open the depsipeptide ring. To determine the fengycin sequences, tandem mass spectrometry of cyclic and linearised fengycins was carried out using the LIFT option of Ultraflex II TOF/TOF by selecting the appropriate precursor ion (± 3Da window). The data were processed using Flexanalysis software (Bruker Daltonics, version 3.2).

Results

The spectra obtained using MALDI mass spectrometry of crude antifungal extract from Banyan endophyte, B. subtilis K1 showed three clusters of molecular ion peaks: m/z 1030-1073, m/z 1028.0-1095.0 and m/z 1421.8-1521.0, which were putatively assigned to the surfactin, iturin and fengycin groups of cyclic lipopeptides, respectively. Among these cyclic lipopeptides, fengycins precursor ions ranging from m/z 1421.8-1521 were selected for further sequence determination using tandem mass spectrometry. The tandem mass spectrometry of HPLC fractions containing fengycin precursor ions showed four characteristics pairs of daughter ions (m/z 1066, 952; m/z 1080, 966; m/z 1094, 980 and m/z 1108, 994), permitting grouping into four classes. The two pairs of product ions at m/z 1080, 966 and m/z 1108, 994 were found to be identical to those observed in known fengycin classes leading to identification as the lipopeptides, fengycin A and fengycin B, respectively. The product ion pairs at m/z 1066, 952 and m/z 1094, 980 must belong to the new groups of fengycin. The mild alkaline hydrolysis of the intact fengycin opens the lactone linkage leading to linear sequences. The tandem mass spectrometry of the linearised peptides showed a complete series of b and y fragment ions, from which lipopeptide sequences of fengycin homologues could be derived. The general peptide sequence deduced from the tandem mass spectrometry of linear fengycin hydrolysis product was: ß-hydroxy fatty acid-Glu1-Orn2-Cyclo[Tyr3-Thr4-Glu5-Ala/Val6-Pro7-Gln9-Tyr9-Ile/Val10].The de novo sequencing of the fengycin group of cyclodepsipeptides showed the micro-heterogeneity of amino acids at positions 6 and 10. Heterogeneity was also observed at the ß-hydroxy fatty acid in fengycins isomers. On the basis of variation in the amino acid positions, the new classes of fengycins were identified as fengycin A2 (Ala6 and Val10) and fengycin B2 (Val6 and Val10).

Poster 4

Conclusions

In this study, tandem mass spectrometry permitted characterization of two new classes of fengycins. The characteristic product ions of intact fengycin precursor ions should prove useful for quickly identifying the fengycin classes in extract of B. subtilis.

Poster 5

SMILIGN: A NEW METHOD FOR MEASURING STRUCTURAL SIMILARITY OF LIPIDS

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Cheminformatics, Lipidomics, Molecular Similarity

Material and Methods

As basis of our study, we chose a dataset of 1185 lipid molecules of the Drosophila melanogaster sphingolipid metabolism. This dataset is generated based on experimental confirmed structural features of Drosophila sphingolipids, such as Fatty acid chain length, Sphingoid base chain length, number of double-bonds and number of hydroxylations.

Molecules in this dataset are programmatically generated using SMILES chemical notation (Weininger, 1988).

"SMILIGN" method is tested against three other similarity scoring methods, "FINGERPRINT" (http://www.daylight.com/dayhtml/doc/theory/theory.finger.html), "LINGO" (Vidal et. al. 2006) and "BIOISOSTERIC SIMILARITY" (Krier et. al. 2009).

Results

The aim of this study is to establish a method to compare lipidomes consisting of thousands of molecules.

We show that "BIOISOSTERIC SIMILARITY" and "SMILIGN" methods were able to resolve all the structural differences among 1185 molecules, where as other similarity scoring methods such as "FINGERPRINT" and "LINGO" performed poorly and failed to discriminate repeating units and positional isomers.

The similarity score obtained from "SMILIGN" method is representing the structural similarity that exists between a given pair of molecules in relation to overall structural space of the dataset. This feature of "SMILIGN" method is obtained by incorporating multiple sequence alignment algorithms used for protein sequence analysis . The "SMILIGN" method enables to compute scalable chemical spaces for the comparison of lipidomes.

Conclusions

We propose a new method, "SMILIGN" to measure structural similarity of lipids. This method is conceptually different from existing similarity scoring methods and also computationally faster. "SMILIGN" method enables to represent the structural diversity of a lipidome. Out results suggest that "SMILIGN" has the potential to calculate the structural difference between lipidomes consisting thousands of individual molecules.

Poster 6

LIPIDOMICS OF DROSOPHILA INOSITOL 1, 4, 5 TRIPHOSPHATE RECEPTOR MUTANTS REVEALS ALTERED TRIGLYCERIDE HOMEOSTASIS

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InsP3R, Dilp2, Triglyceride (TAGs), Fat body, Metabolism

Material and Methods

Adult *Drosophila* abdomens or heads were homogenized using buffer containing 10mM Tris, 1mM EDTA and 0.1% Triton-X 100 and centrifuged at 4000 rpm for 15 min at 4°C. The supernatant was used for the TAGs measurement, protein measurement and TLC assay. The TLC assay was done in duplicates with the supernatant amount used for each assay normalized to protein content. A mixture of the 70:30:1 (v: v: v) of hexane, di-ethyl ether and acetic acid was used as mobile phase. Lipid bands on the TLC plate were visualized by spraying the plates with 15% H2SO4 in ethanol then charred at 200°C followed by quantification using Image J software. The other TLC plate was sprayed with H20 and TAG bands were marked and scraped from the plate. Lipids were then extracted from silica gel and analyzed by mass spectrometry (Schwudke et al., 2007, 2008).

Results

The Inositol 1, 4, 5-triphosphate receptor (InsP3R) is an InsP3 gated intracellular Ca^{2+} release channel. To understand the importance of InsP3 mediated Ca^{2+} release in drosophila, mutants have been generated in the *itpr* gene coding for InsP3 (Joshi et al., 2004). Recent unpublished work from our group has shown that *itpr* mutant flies have large fat deposits in the fat body and are resistant to starvation. When wild type InsP3R cDNA was expressed in the *Drosophila* insulin-like peptide producing cells in the brain of *itpr* mutant flies using *Dilp2*Gal4, several phenotypes including starvation resistance were rescued (Agrawal et al., 2009 and Manivannan. S et al, manuscript in preparation).

Our data show that TAG levels are higher both in abdomen and in heads of *itpr* mutant flies as compared with the wild type (CS) under fed condition. Under starvation lipid levels reduce in CS flies, but remain elevated for much longer in *itpr* mutant flies. The TAG phenotype can be rescued by expressing InsP3R cDNA in *itpr* mutant flies using *Dilp2*Gal4, whereas partial rescue occurs when InsP3R cDNA is expressed in these mutant flies using *c729*Gal4. Knocking down of *itpr* gene with RNAi in neurons shows alteration in the TAG level but similar knock down in fat bodies does not show any such phenotype. When insulin-signaling components were over-expressed in Dilp cells, the TAG phenotype was only partially rescued when compared to the wild type (CS). Mass spectrometric measurements revealed that the TAG profiles of *itpr* mutant flies are altered and that the mobilization of short chain fatty acids like 12:0 and 14:0 are compromised in the *itpr* mutant flies.

Conclusions

InsP3R in *Drosophila* is a key regulator of metabolic homeostasis. Our result shows that fat store levels are communicated to the brain centers that regulate metabolism (Bader Al-Anzi et al., 2009). Our mass spectrometric result indicates that TAG molecules are differentially mobilized under starvation condition in the wild type and *itpr* mutants are defective in that regard (Eugenio Gutierrez et al., 2007).

Poster 7

SOFTWARE BASED AUTOMATED QUANTITATION OF AMINO ACIDS BY MALDI ANALYSIS

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Amino acids, MALDI, quantitation by software

Material and Methods

Essential amino acids were analysed by MALDI MS to design a high throughput quantitation method for biological matrices. A synthetic amino acid was selected as an internal standard and serial dilutions of amino acids mix was prepared. Analysis was performed on Synapt HDMS and Voyager DE-STR with organic and inorganic matrices, namely CHCA, DHB and Silica. An in-house developed software was used for rapid data analysis and to generate the calibration curves. Manual data analysis was also performed to validate the software processing.

Results

Intense signals with good signal to noise ratio were obtained on all three matrices under study. CHCA signals were found to be most intense at as low as sub nM levels, while Silica provided clean spectra with very low noise levels. Results from software processing were also shown to be comparable to the results from manual processing.

Conclusions

MALDI MS is shown to be an adept analytical technique for analysis of a large analyte set. reduced sample preparation and removal of separation component also lends it an edge over the conventional and generic methods for quantification of amino acids in biological matrices.

Poster 8

A LOGICAL AETIOLOGY FOR HYPOTHYROIDISM IN THE PLAINS

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Iodine deficiency disorder, perchlorate, thyroid hormones, Urinary iodine

Material and Methods

Thyroid deficiency causes goitre. Goitre is very common in areas where iodine is deficient e.g. hills.However, in several areas with iodine sufficiency, goiter is still prevalent in considerable numbers. The definitive reason for this is as yet unknown. One postulate is that the available iodine could have been inhibited from incorporating into the thyroid gland. There are several candidates for this ignominy. Perchlorates are present in considerable quantities around industries manufacturing explosives, fireworks, paints and pharmaceuticals. And, perchlorates have many times more affinity to throxine precursors than iodine and competitively inhibit iodine incorporation. Before accusing perchlorate as the culprit for the persistence of goiter in iodine sufficient areas, we must establish the following: (i) perchlorates are present in considerable quantities in that area. (ii) iodine is available in sufficient levels in that area (iii) clinically establish that goitre is indeed prevalent in considerable numbers in that area.
This study addresses the first two of the above three issues. Three industrial areas (fireworks, explosives, pharma and paints) and three areas without such industries were chosen. From each of these six areas, a minimum of eight samples of drinking water were collected. Groundwater samples were collected from public wells, bore wells, ponds, lakes and rivers. Additionally, several brands of bottled drinking water were purchased from grocery stores (Their source was also ascertained). Urine samples were collected from available humans and cattle. In all these samples, perchlorate levels were estimated by spectrophotometric technique based on Ion-Pair formation with thionine method. And, iodine levels were estimated by Sandell-Kolthoff reaction.

Results

On comparison with the analytical standards, the detection limit for perchlorate in clear drinking water and in clear urine was found to be reliable down to a level of 30 ng/ ml. Iodine concentration of more than 20 µg/ dl in urine indicated iodine sufficiency. High perchlorate levels in the drinking water and surface water sample indicated significant contamination of environment. High presence of perchlorate in the urine indicated significant human exposure to the contaminant.

Poster 8

Conclusions

In the United States, the regulatory authority, namely, the Environmental Protection Agency (US EPA) is actively looking into this aspect. However, so far, no maximal concentration limit (MCL) has been prescribed. However, the US EPA (2005) guidelines for unregulated compounds indicate the human reference level (Rfd) of 0.0007 mg/ kg/ day of perchlorate. Rfd is a scientific estimate of a daily exposure level that is not expected to cause adverse health effects in humans. This level of detection can be achieved by both thionine method and by mass spectrophotometry. In our study, only clear drinking water and clear urine samples were analysed. And, thionine method However, in natural environments, several samples will have impurities and will have additional interfering anions. Hence, ideally speaking, mass spectrophotometry must be used.

Our study was done as a scout in order to identify geographic areas suitable for a clinical study of prevalence of goiter. After identifying these specific areas, the clinical study of goiter prevalence will follow. Further continuation of the full study will be done with the more reliable mass spectrometry. This study has a great community relevance to our country, where salt iodization is a focus area of community development. The ultimate results of this study could impact the very relevance of the salt iodization programme.

Poster 9

FORMATION OF NON-COVALENT COMPLEXES BETWEEN INSULIN PEPTIDE AND DRUGS UNDER ELECTROSPRAY IONIZATION MASS SPECTROMETRY CONDITIONS

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Key words: Insulin, drugs, ESI-MS, non-covalent interactions, gas phase basicity

Material and Methods

Bovine insulin was purchased from Sigma aldrich (Steinhem, Germany). the drugs (lacosamide, pregabalin, and rufinamide were obtained from a local pharma company. Insulin solution (100 μM) was prepared in HCl buffer, pH 2. The lacosamide and pregabalin solutions (100 μM) were made in methanol, and that of rufinamide in dimethylsulfoxide. The 1:5 (v/v) mixture of the insulin and drug solution was vortexed for a minute, and the solution was directly infused into source of mass spectrometer at a flow rate of 5 μL/min using a syringe pump. All the experiments were performed using QSTAR XL (Applied Biosystems, USA) instrument equipped with ESI source in the positive ion mode.

Results

Epileptic seizures have long been recognised as a complication of the clinical syndrome of Alzheimer's disease. Lacosamide is a known drug for antiepileptic activity against epileptic seizures. In another study, we have found that lacosamide acts as an inhibitor towards amyloid β 1-40 aggregation, and it formed non-covalent adducts with amyloid β under ESI conditions. Hence we selected lacosamide and other two related drugs (pregabalin and rufinamide) to study their efficiency to form non-covalent complexes with insulin, which is also an amyloidogenic protein, under ESI conditions.

The positive ion ESI-MS of insulin alone showed the peaks at m/z 1434, 1148 and 956 corresponding to [Insu+4H]4+, [Insu+5H]5+ and [Insu+6H]6+, respectively. The spectrum of insulin in the presence of drug showed the complex ions with charge state 5 and/or 4. For example, the spectrum of lacosamide (mol. wt. 250) showed the [Insu+Laco+5H]5+ (m/z 1198) and [Insu+Laco+4H]4+ (m/z 1497) ions. We have observed that the abundances of the adduct ions were increased when the time of interaction (incubation) increased from zero to 5 hours. We have calculated the binding affinity of the complexes. The collision induced dissociation (CID) spectra of [Insu+Laco+5H]5+ showed [Laco+H]+ as the major product ion in addition to [Insu+5H]5+ and [Insu+4H]4+ ions. Similar results were obtained for the complexes from rufinamide and pregabalin, but there were a little difference in the abundance of [Insu+5H]5+ and [Insu+4H]4+ ions. As in the Kinetic method, this CID data indicates that gas phase basicity (GB) of the drugs are closer to that of insulin (4+ charge state), and GB of the drug is always relatively higher than insulin ion. The formation of non-covalent complex ions under ESI may be controlled by the GB of the drug and the protein charge state.

Conclusions

The selected drugs formed abundant non-covalent complexes with insulin under ESI conditions. The intensity and the type of complex ion formed is found to be selective to the drug used. The binding affinity of the complex increased with the increase in the time of interaction at room temperature. The gas phase basicity of the drug and protein may be playing a role in the formation of the non-covalent complex ions between them in the gas phase. Work is under progress to establish the site of binding of these drugs to insulin by using MS.

Poster 10

ELECTROSPRAY IONIZATION MASS SPECTROMETRIC STUDY OF THE NON-COVALENT DIMERS OF GASEOUS PROTONATED PEPTIDES CONTAINING LYSINE RESIDUES

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peptides, non-covalent dimer, ESI-MS

Material and Methods

A series of position scrambled peptides (C-terminal amidated) containing six alanine and two lysine residues were synthesized and then their various functional groups were chemically modified. Those modified and unmodified peptides were electrosprayed in vacuum from different solvents. The energy resolved mass spectrometric study and molecular mechanics calculation were also undertaken on these peptides.

Results

ESI-MS investigation of those synthetic octapeptides containing six alanine and two lysine residues differing only by their positions showed the formation of non-covalent dimers, which were preserved in the gas phase. Unlike the monomers, the dimers were found to show only singly protonated state. The decrease in the solvent polarity from water to alcohol showed enhanced propensity of formation of the dimer indicating that the electrostatic interaction plays a crucial role to stabilize the dimer. Selective functionalization studies showed that ε-NH2 of lysine and C-terminal amide (–CONH2) facilitate the dimerization through intermolecular hydrogen bonding network. The molecular mechanics calculation supported that the formation of non-covalent intermolecular dimers containing those hydrogen bonds are energitically favorable. The dissociation profile (breakdown curves) of the dimers showed that the strength of noncovalent interactions vary with the change in position of lysine residues in the peptides.

Conclusions

The electrospray ionization was shown to representatively transfer the non-covalent dimers of lysine containing basic octapeptides from solution to gas-phase. The present approach for unraveling the intermolecular non-covalent interaction by ESI-MS may also be applicable in the study of supramolecular assemblies of biologically important molecules.

SYNTHESES, CHARACTERIZATION AND EVALUATION OF LEUCINE ZIPPER ANTIMICROBIAL PEPTIDE SEQUENCE AND ITS ANALOGS

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Keywords: Antimicrobial peptides, LZP, SPPS, ESI-MS

Material and Methods

Antimicrobial peptides are an integral part of host's defense barrier against pathogenic invasions and provide a chemical immune response to organisms and act as the first line of defense against various infections. However, toxicity of these antimicrobial peptides is a major barrier for converting them into drugs. Leucine zipper peptide (LZP) contains heptad repeats of (abcdefg) of amino acids, where at 'a' and 'd' positions leucine residues are present. Studies indicate that minor alteration in the structure of LZP motif results in significant changes in the toxicity and antibacterial activity of this peptide. We have synthesized and evaluated a 21-residue peptide on the basis of classical LZP motif sequence and two of its analogs by substitution of leucine at 'a' and 'd' positions by alanine, utilizing the standard solid phase peptide synthesis (SPPS). In order to examine the progress of the synthesis the molecular mass of the peptides were checked by electron spray ionization mass spectroscopy (ESI-MS).

Results

The amino acid sequence, calculated and observed mass of LZP and its analogs are shown below;
1.LZP, X-NH-LKALKKALKWLKKALKALKKA-CONH2, Calculate mass 2390.151 Da, Observed mass 2391.
2.LZP(L4A), X-NH-LKAAKKALKWLKKALKALKKA-CONH2, Calculate mass 2348.069 Da, Observed mass 2349.
3.LZP(L4A,L8A),X-NH-LKAAKKAAKWLKKALKALKKA-CONH2, Calculated mass 2305.987 Da, Observed mass 2307.
The mass spectroscopic data confirm that the experimental mass of the LZP peptide and its analogs exactly matches with the theoretical mass.
Circular Dichroism studies were carried out to determine the secondary structures of these peptides. Hemolytic assay and antibacterial assay were performed in human red blood cells and bacterial cells to determine their toxicity levels. Flow cytometry experiments were performed in order to evaluate the effect of the peptides on the membrane integrity in bacterial cells. SDS-PAGE gel electrophoresis was performed in order to assess the assembly or nature of oligomerization of the peptides towards the toxicity.
Circular Dichroism studies show that LZP and its analogs form similar helical structure. Studies on hemolytic assay reveal a lower level of toxicity in single alanine substituted analog, which diminish further in double alanine substituted analog as compared to LZP whose activity was the highest. Studies of antibacterial assay reveal that LZP exhibit appreciable antibacterial activity but its analogs show almost similar antibacterial activity with a minimal inhibitory concentration (MIC) of ~ 7.5 μM. Flow cytometry studies reveal that LZP and its analogs induce similar membrane damages to bacterial cells. SDS-PAGE gel electrophoresis studies reveal that LZP formed significantly higher oligomeric structure as compared to alanine-substituted analogs.

Conclusions

As hypothesized the toxicity of antimicrobial peptides seems to depend on the assembly of the sequence. The assembled LZP and its analogs exhibit appreciable hemolytic activity against human red blood cells but little or no effect on antimicrobial activity. The results obtained provide a frame work for leucine zipper based antimicrobial peptides in designing therapeutically relevant molecules.

Poster 12

MALDI MATRIX OPTIMIZATION FOR QUANTITATIVE ANALYSIS OF LIPIDS

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Lipidomics, MALDI, Matrix, Quantitative Analysis, MRM

Material and Methods

Chemicals and materials: Lipid standards (phosphatidylcholine(PC), phosphatidylethanolamine(PE), sphingomyelin(SM), lysophosphatidylcholine(LPC), phosphatidylinositol(PI), phosphatidylserine(PS) and phosphatidic acid(PA)) were purchased from Avanti Polar Lipids. All the MALDI matrices used, 4-Chloro-α-Cyanocinnamic acid (ClCCA), 2,5-dihydroxybenzoic acid (2,5-DHB), 9-aminoacridine (9-AA), 1,8-bis(dimethylamino)naphthalene (proton sponge), were purchased from Sigma Aldrich. 4-Chloro-α-Cyanocinnamic acid (ClCCA) was re-crystallized with ethanol before use.
Lipid extraction from Human Plasma: lipids were extracted from human plasma using the Bligh and Dyer method.
MALDI-MS/MS analysis: all experiments were performed on a FlashQuantTM MALDI sourced 4000 QTRAP system (AB Sciex). The laser was a high repetition rate (1000 Hz). The MRM dwell time was 15 ms per transition. The transitions monitored were previously optimized and parameters adapted to the use of the MALDI source.

Results

Matrix-assisted laser desorption (MALDI) mass spectrometry is increasingly used for lipid analysis, due to the operational simplicity and the robustness against impurities potentially present in crude tissue or body fluid extracts.
The quantitative information achievable from MALDI mass spectra is often assumed to be limited but the use of instruments like MALDI 4000 Q TRAP can give the possibility to perform spectra acquisition in MRM mode combined with very short analysis time. Clearly the quality of the mass spectra is highly dependent on the choice of the matrix. In this work we tested the most used matrices for lipid analysis and we also tried new matrices combinations to define which can work best when the quantitative analysis is as important as the qualitative characterization of the sample.
Two matrices for analysis in positive ion mode (DHB, ClCCA) and two for negative ion mode (9-AA, proton sponge) were tested separately and were then mixed in different ratios.
As, in principle, every single matrix can give better results for specific lipid classes, we decided to combine two different matrices to take advantage of the wider range of detectable molecules in one single experiment. Another advantage came from sample homogeneity when using matrix mixtures. After mixing ClCCA with DHB or 9-AA with Proton sponge, the spot morphology was more uniform and a significant increase of intensity in ionization of the parent ion and MRM transitions was measured all over the spot. The above mentioned combinations of matrices were evaluated for the quantitative analysis of complex biological samples like human plasma.

Conclusions

In this study, the use of different MALDI matrices for quantitative phospholipids analysis using MALDI-MS/MS operated in MRM mode was evaluated. Analysis improvements after using combinations of matrices were obtained in both positive and negative ion mode. Development of appropriate sample preparation protocols and automation of MALDI plate spotting will allow for high throughput quantitative analysis of phospholipids in biological samples.

EFFICIENT PICOMOLAR PROTEIN GEL ELECTROELUTION: APPLICATION TO MASS SPECTROMETRY

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electrophoresis, electroelution, mass spectrometry, protocol, fabrication

Material and Methods

We have designed and fabricated an equipment to perform electroelution on cut gel bands from samples that could be loaded in picomolar range. Our primary aim was to build an equipment that can cater to low volume samples for electroelution. At the same time, we wanted it to be inexpensive and easily sourced locally. Two small (~1cm x 1cm) hollow glass tubes were sealed on a metal filter and made into an inverted bell shape with open ends for electrode insertion. Two platinum electrodes were fixed on a rubber cork on to the open ends and connected to a regulated power outlet. During experiment, cut gel bands are kept on one side of the filter separated compartment and elution buffer was put in both compartment. The polarity of the electrodes are decided based on the isoelectric potential of the protein. The electroeluted samples are mixed with matrix and directly spotted on MALDI plates and spectra are acquired.

Results

We demonstrate by taking picomolar samples of five proteins, bovine serum albumin (BSA), lysozyme (LZM), goat anti-mouse ALP conjugated immunoglogulin (IgG), Uracil-DNA Glycosylase from Mycobacterium smegmatis (UDG) and human tetrameric hemoglogin (HHB) in native and cross-linked dimer of tetramer form. While BSA has an acidic pI, LZM is basic and HHB has a near neutral pI of 6.8. The stability of the proteins also varies, with HHB being the most unstable and readily dissociating into monomers in few hours under the room temperature. Among the five proteins, HHB represents the hardest case, because of its instability and near neutral pI. Our method of electroelution, which was optimized for best recovery of eluted proteins and best signal from MALDI spectra, show that a recovery of as high as 92% of intact tetramer of HHB can be obtained. The optimization and recovery of the other monomeric proteins could be attained in a much shorter and simpler manner due to their better stability and pI values being far away from the neutral. The pH of the buffer was adjusted according to the pI of the protein. For BSA with a pI of 3.7, the buffer pH was 6 and placed in the anode compartment. For IgG, UDG and HEL with a pI of 7.3, 9.5 and 11, respectively, the buffer pH was 8 and placed in the cathode compartment. We propose these standard buffer conditions be used depending on the acidic or basic pI of the protein. If the pI is unknown, the experiment needs to be repeated in both pH conditions, of which electroelution will be successful in one. The results suggest that our simple and cheap inhouse equipment and the protocol for picomolar samples can be adopted by any molecular biology laboratory to significantly shorten the path to final identification of the protein molecule through a combination of gel electrophoresis, electroelution and top-down mass spectrometry.

Conclusions

The main advantage of our equipment is its simplicity and ease of handling. The input voltage and the amperage required are very low, so that even regular alkaline batteries available from the market shelf would be sufficient to perform the experiment. This reduction in cost means that user will be able to save a minimum of 75% of the expenditure compared to any other equipment in the market. Aside from the cost advantage, the soft experimental conditions this equipment provides for electroelution, implies that most fragile of the proteins (even denatured) and complexes can be electroeluted.

Poster 14

INVESTIGATION OF THE INTERACTIONS BETWEEN ANTIEPILEPTIC DRUGS AND AMYLOID B (1-40) PEPTIDE BY ESI-MS

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Amyloid β 1-40 peptide, alzheimer's disease, aggregation, antiepileptic drugs

Material and Methods

Amyloid β 1-40 peptide (M.W. 4329.8) was purchased from sigma aldrich (Steinhem, Germany); lacosamide (M.W 250), pregabalin (M.W. 159), and rufinamide (M.W. 238) were gift from a local pharma company. Solution of amyloid β 1-40 peptide (100 μM) was prepared in ammonium acetate buffer, pH 7.4. Solutions of lacosamide and pregabalin (100 μM) were made in methanol and that of rufinamide in DMSO. Amyloid β 1-40 and ligand solutions were mixed (1:1, v/v) by vortexing for a minute, and the resulting solution was directly infused into ESI source of mass spectrometer at a flow rate of 5μL/min using a Harvard syringe pump. All the experiments were performed using QSTAR XL (Applied Biosystems/MDS Sciex, Foster city, USA) instrument equipped with ESI source in the positive ion mode.

Results

Aggregation of amyloid β peptide (Aβ) finally leads to the formation and deposition of amyloid plaques, the major hallmark in alzheimer's disease (AD). Lacosamide, pregabalin and rufinamide are new antiepileptic drugs and among them, lacosamide is known to have effect on epileptic seizures caused by AD. In the light of the suggested link between antiepileptic drugs and AD, it is proposed that these drugs may offer a therapeutic system for protection against the risk of this disease. For this reason, the formation of non-covalent complexes between amyloid β peptide (Aβ) with these new antiepileptic drugs was studied under mass spectrometry conditions. The stability of the non-covalent complex was examined under several experimental conditions such as pH, presence of organic modifier, concentration and time. Digestion combined with mass spectrometry analysis of the resulting peptide fragments were employed in order to locate the binding site of these drugs in Aβ.

The ESI mass spectrum showed non-covalent complex with lacosamide [Laco] at m/z 1146 [Aβ1-40+Laco+4H]4+ and at m/z 916 [Aβ1-40+Laco+5H]5+, and with rufinamide [Rufi] at m/z 1143 [Aβ1-40+Rufi+4H]4+ and at m/z 914 [Aβ1-40+Rufi+5H]5+. The binding efficiency of the complex increased upon incubation at room temperature for about 3 hours. Digestion with trypsin showed that both the drugs lacosamide and rufinamide bind to the peptide fragment at 17-28, which is the hydrophobic segment of Aβ which was considered as the major aggregating site (KLVFF). Hence we propose that they may act as inhibitors towards amyloid β 1-40 peptide invitro aggregation.

Conclusions

Antiepileptic drugs lacosamide and rufinamide formed non-covalent adducts with Aβ. In the presence of these drugs, Aβ monomeric ions were seen after incubating the samples for 24 hours. However the control sample aggregated completely after 24 hrs incubation, i.e. monomer ions disappeared completely. Hence, we are of the opinion that, these drugs act as inhibitors towards amyloid β 1-40 peptide invitro aggregation. Further work to quantify the inhibitory efficiency of these drugs against amyloid aggregation using mass spectrometry based assay and thioflavin assay is under progress.

Poster 15

DO ORTHOLOGOUS PROTEINS ARE TOPOLOGICALLY INDISPENSABLE? A TOPOLOGICAL ANALYSIS OF PROTEIN-PROTEIN INTERACTION NETWORK

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Protein Interaction Network, degree, betweenness centrality, orthologous proteins

Material and Methods

The PPI dataset was retrieved from IntAct database. Redundant, isolated and self interactions were removed to optimize the dataset. The Yeast protein interaction network composed of 49544 interactions for 5663 proteins, and for Human, 15160 interactions for 3380 proteins. Each binary interaction in the dataset has mapped programmatically to protein-protein interaction(PPI) network. Next, we identified the 537 orthologous proteins between Yeast and Human genome identified. Betweenness centrality of every protein nodes, including orthologous proteins, were identified. Conducted a correlation study between degrees of all nodes, including orthologous proteins, and betweenness centralities values.

Results

Found a positive correlation between degree of the protein nodes with its betweeness centrality. Both degree and betweeness centrality of orthologous follows a power-law distribution.

Conclusions

Orthologous proteins are topologialy more important than other protein nodes in the PPI network. Since orthologous proteins are evolutionarily important, our result postulate, the central role of orthologous proteins may constrain the evolution of PPI netwok. The result also indicates topological organization of protein interaction network can be demonstrated in terms of centrality of orthologous proteins, which evolved over gene duplicating process.

Poster 16

IDENTIFICATION AND CHARACTERIZATION OF NOVEL PROTEINS IN DIABETIC AND DIABETIC TREATED [CYNODON DACTYLON (L.) PERS.] ALBINO RATS

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Alloxan diabetes; 2D Electrophoresis; MALDI-TOF-MS/MS; Cynodon dactylon

Material and Methods

Eight week old male albino rats of same age group and body weight 130–150 g were selected for all the experiments. The study was approved by IAEC No.: 743/03/abc/CPCSEA dt 3.3.03. The albino rats (Four groups; six animals each group) were made diabetic by single intraperitoneal (i.p.) alloxan injection (150 mg/kg/bw) and the treatment with aqueous and ethanolic extract of C. dactylon leaves (450 mg/kg/bw/day) for 2 weeks following diabetes onset (>200 mg/dl were considered to be diabetic). After 15 days of treatment, the animals were euthanized and collected all tissues and subjected to 2D analysis based on O'Farrell method with some modifications (IEF: 1st dimension and SDS-PAGE: 2nd dimension). The completed 2-D gels were stained with colloidal blue. The differential expressed proteins were analyzed with a MALDI-TOF-MS (Bruker-Daltonics, Germany). The results were considered statistically significant if the P values were 0.05 or less.

Results

2DE patterns of rat's tissues proteome were attained by using four rod gels (pH 3–10), Group I: control; Group II: alloxan diabetic; Group III: diabetic treated with aqueous extract; and Group IV: diabetic treated with ethanolic extract. The protein patterns observed for each sample were similar and reproducible from animal to animal and gel to gel. In order to identify the altered proteins, the differentially expressed proteins spot were excised from the 2-DE gels and analyzed by two methods; MALDI-TOF-MS and MS/MS analysis. By using in-gel digest and MS with consecutive database search, we were identified differentially expressed proteins.

The proteomic approach has offered many opportunities and challenges in identifying new marker proteins and therapeutic targets and in understanding disease pathogenesis i.e. by 2D-PAGE and MALDI-TOF-MS. This study shows the up-regulated proteins were found in diabetic rats i.e. Pancreas: adrenodoxin, Mediator of RNA polymerase II transcription subunit 4 and GTPase IMAP family member 4; Brain: PPP1R14D and RAB18; Kidney; RhoA and Ras-related protein–RAB4A and RAB43; Heart: neurotrophin-4; Adipose tissues: SRFBP1 and MAPKK7; Plasma: heat shock proteins B8, tropomyosin alpha 3 chain and preprohaptoglobin. The above mentioned proteins were up-regulated in diabetic rats due to diabetes and associated complication in different organs. These proteins are mainly involved in the prevention of diabetes and associated complication.

This study shows the up-regulated proteins were found in diabetic treated rats; Liver: nucleophosmin, l-xylulose reductase and carbonic anhydrase III; Spleen: Ca2+-binding protein p22, aldose reductase, 78 kDa glucoseregulated protein and coactosin-like protein; Muscle: fructose-bis-phosphate aldolase and glyceraldehyde-3-phosphate dehydrogenase; plasma: apolipoprotein A-IV. The above mentioned proteins were up-regulated in diabetic treated rats due to the effect of C. dactylon extract. These proteins are mainly involved in the prevention of diabetes, associated complication and reduce the severity of diabetes mellitus.

Conclusions

The present investigation has opened avenues for further research especially with reference to the development of potent phytomedicine for diabetes mellitus from C. dactylon leaves. This study provides a preliminary proteome reference map of rat organs that will form a basis for comparative studies on normal and pathology of diabetes. This proteome analysis serve as tool for development of biomarkers for monitoring, predicting and determining treatment efficacy for early prognosis and establishment of categorical stages of disease progression. By using combined phytomedicine and proteome analysis draw the line for discovery of novel possibilities of preventive and effective treatment for diabetes.

Poster 17

A PROTEOGENOMIC APPROACH TO MAP THE PROTEOME OF AN UNSEQUENCED PATHOGEN- LEISHMANIA DONOVANI

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Kinetoplastid, comparative proteogenomics, homology, orthologs

Material and Methods

Kala Azar or visceral leishmaniasis is the most severe form of leishmaniasis and is caused by the protozoan parasite Leishmania donovani. The genome of L. donovani has not yet been sequenced although the genome sequences of three related species that cause a spectrum of less severe diseases, Leishmania major, Leishmania infantum and Leishmania braziliensis, are already available. The goal of this study was to profile the proteome of L. donovani on global scale using a proteogenomic approach. The following approach was used for sample preparation and proteomics. Cell lysates from promastigote (insect) and amastigote (human) stages in the life cycle of L. donovani were prepared fractionated either by SDS-PAGE (proteins) or by SCX chromatography (tryptic digests). The gel and SCX fractions were analyzed by a total of 120 LC-MS/MS runs. The resulting mass spectrometry data were searched using Sequest and Mascot search algorithms. We have used protein and six frame translated genome data from three related species of L. donovani for our analysis.

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Results

This comparative proteogenomic analysis of digenic parasite Leishmania donovani resulted in identification various proteins expressed in both the lifestages of this parasite. From our analysis of the promastigote (insect) and amastigote (human) stages, we identified a total of 22,322 unique peptides from a homology-based search against proteins from L. infantum, L. major and L. braziliensis. These peptides were assigned to 3,711 proteins in L. infantum, 3,287 proteins in L. major and 2,433 proteins in L. braziliensis. Of the 3,711 proteins that were identified based on the annotated proteins in L. infantum which constitutes ~46% of total predicted genes in L. Infantum. Of these 3,711 proteins the expression of 1,387 proteins was detectable in both promastigote and amastigote stages of the parasite while 901 proteins were identified only in promastigotes and 1,423 proteins only in amastigotes. Additionally, we carried out six-frame translated genome database search, which led to identification of 19 novel peptides. Based on these novel genome search specific peptides (GSSPs), we were able to refine gene models in the 3 related Leishmania species. Using peptide evidence and alternative gene prediction tools, we could correct 19 gene models in the 3 Leishmania species (16 N-terminal extensions and 3 C-terminal extension).

Conclusions

We report a comparative proteogenomic approach to map the proteome of an unsequenced pathogen Leishmania donovani.

Poster 18

LC-MS/MS DEVELOPMENT STRATEGIES FOR HIGH-THROUGHPUT QUALITATIVE BIOANALYSIS

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2D electrophoresis, mass spectrometry, LC-MS/MS.

Material and Methods

The use of Liquid Chromatography coupled with tandem mass spectrometry (LC-MS/MS) has become a method of choice in the field of proteomics. 2D electrophoresis followed by nano-LC-MS/MS improved the specificity and accuracy of identification and post-translational modifications of samples. Based on this our aim is to standardize LC and MS parameters for qualitative and quantitative analysis of samples. In this study, standard samples such as Cyt c, Bovine Serum Albumin, 18 kDa, Beta casein was used. Samples were separated using Two Dimensional Polyacrylamide Gel Electrophoresis (2D PAGE) to examine the protein profile and get the data about pI, molecular weight and modifications if any. In-gel and in-solution tryptic digested samples were further taken for analysis by nano LC MS/MS in which different methodology were followed in terms of mobile phase, column dimension, gradient, mode of injection, collision energy, run time etc., to achieve accurate protein identification and modifications.

Results

For sample separation in nano LC µl pick up method was found to be appropriate. The volume and the concentration used for injection were also adjusted according to the protein spot intensity measured using the gel analysis software or pmol/fmol concentration in case of in-solution digested samples. The shallow and longer gradients even upto 60 minutes are required to achieve different chromatographic separation of High molecular weight samples. Column dimension in which pore size, plays a major role was also optimized to get better resolution. Direct injection to MS was also optimized for pure protein and to find modifications in the proteins.

Conclusions

LC-MS methods and sample preparation must be carefully designed for each type of sample to achieve good detection. These high throughput approaches will further enhances analysis speed and sensitivity to quantitative bio-analysis by LC-MS/MS.

Poster 19

DECIPHERING THE CROSS TALK BETWEEN CHANNELRHODOPSINS OF CHLAMYDOMONAS REINHARDTII

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Microbial rhodopsin, channelrhodopsin, eyespot, proteomics, MS

Material and Methods

The C-terminus region of channelrhodopsin1 (ChR1) and c hannelrhodopsin2 (ChR2) were cloned into pET-Sumo expression vector. Protein was expressed in E.coli and purified by immobilized metal affinity chromatography. Polyclonal antibodies were raised against the recombinant C-terminal region of ChR1 and 2. Peptide antibodies were also raised against the peptide sequence present in the extracellular loop region of rhodopsin domains of ChR1 and ChR2 respectively. Peptide sequences were synthesized and KLH (Keyhole limpet hemocyanin) was conjugated at C-terminus, before injecting in animal. Specificity of antibody was checked by immunoblotting. Cellular localization of ChR1 and ChR2 was performed using C-terminus and pept ide antibodies as primary antibody and fluorescence tagged IgG as secondary antibody. Immunoprecipitation, immunoblotting and Nano-LC-MS/MS techniques were used to decipher the direct interaction between ChR1 and ChR2.

Results

Photobehavioural responses (phototaxis and photophobic) in Chlamydomonas reinhardtii are mediated by rhodopsin based photoreceptor i.e, channelrhodopsin 1 (ChR1) and channelrhodopsin 2 (ChR2). RNAi experiment suggested that the expression of ChR1 in organism depends on the relative abundance of ChR2 and vice-versa. It clearly suggests interaction between ChR1 and ChR2 at genetic level. However, there is no evidence of direct interaction between these channelrhodopsins. Using immunoblotting technique with the antibody raised against the C-terminus region, we detected ChR1 (78kDa) and ChR2 (80kDa) respectively in Chlamydomonas total cell protein. Immunoprecipitation followed by nano LCMS/MS confirmed the antigen identity. Both ChR1 and 2 were found to be localized in to the eyespot region of the cell. Due to high homology between the amino acid sequences of ChR1 and ChR2, antibody raised against C-terminus region of both resulted in cross-reactivity. Antibodies raised against the peptide sequence with least homology between ChR1 and ChR2 resulted in generation of specific antibodies. Immunolocalization with specific antibodies confirmed that both ChR1 and ChR2 are present onto the eyespot region. ChR1 and ChR2 were pulled down together in the form of protein complex using immunoprecipitation. Channelrhodopsin complex was detected by imunoblotting with specific antibodies.

Conclusions

In this report we present the preliminary data indicating the interaction between ChR1 and ChR2. Our study indicates that both ChR1 and ChR2 are localized in the eyespot. Co-immunoprecipitation followed by nano LCMS/MS studies will be performed further to establish interaction between ChR1 and ChR2 and/or to identify novel interacting partners of light-gated ion channel proteins.

Poster 20

USING MASS SPECTROMETRY TO DETECT ESTRUS-SPECIFIC PROTEINS IN VAGINAL MUCOUS OF SYNCHRONIZED MURRAH BUFFALOES (BUBALUS BUBALIS)

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Mass-spectrometry, Vaginal mucous, Binding protein, Estrus-specific, Buffaloes

Material and Methods

For estrus synchronization, six Murrah buffaloes were selected and treated with the progesterone impregnated Controlled Internal Drug Releasing (CIDR) kit. After 48 to72 hours, the animals came to estrus (the period of ovulation). Vaginal mucous were collected by rectal palpation during proestrus and estrus stage. The samples were processed and concentrated then used for 12% SDS-PAGE. Protein bands from SDS-PAGE gel were excised and t he gel plug was destained and undergone for tryptic digestion. Further, the mass spectrometric analysis were performed using an LTQ- Orbtrap(Discovery) hybrid mass spectrometer with a nanoelectrospray ionization source(ThermoElectron, Scan Jose, CA, USA) coupled to a nano-flow high-performance liquid chromatography system(Agilent Technologies 1200 series, Germany). The mass spectrometry dataset was analyzed using Xcalibur software (Version 2.0 SR1). Product ion scans obtained from the MS/MS experiments were investigated using the database search software SEQUEST (TURBO).

Results

The protein profiles of estrus stage vaginal mucous were compared to proestrus, there were 12 different bands exhibited in coomassie brilliant blue stained gel and their molecular mass ranged between 15 and 133 kDa. Densitometry analysis showed the staining intensity of high molecular weight polypeptides were found to be similar in proestrus and estrus. In the low molecular ranges, there were three polypeptides showed in the estrus and postestrus 27, 20, 15 kDa respectively. By comparing, the staining intensity of polypeptide 20 and 15 kDa was higher in the estrus (band area 147.05 and 200) to proestrus (53.83 and 97.1). Interestingly, 27 kDa low molecular weight protein present only in estrus stage. Then the tryptic digested band were analyzed in LC/MS (Liquid chromatography/mass spectrometry), there were 267 proteins exhibited in proestrus. In case of estrus nearly 332 proteins were identified. Among these proteins, two of the individuals such as 27 kDa Heat Shock Protein1 and 20 kDa Enolase proteins were specifically expressed during the estrus stage.

Poster 20

Conclusions

Mass spectrometry results showed 20% of proteins in estrus stage have binding functions. It may confirm that the pheromonal communication of female buffalo with male through these proteins during the estrus stage. Interestingly 27 kDa Heat Shock Protein1 and 20 kDa Enolase protein were specifically expressed during the estrus stage. These results suggest that these differentially expressed proteins may be used as biomarker, in view of artificial insemination. It would help to enhance the buffalo population for future need. Further studies are mandatory to pinpoint the role of differentially expressed proteins in estrus-specific stage.

Poster 21

2D DIGE ANALYSIS OF MITOCHONDRIAL PROTEOME ALTERATION DURING IN VITRO HEMATOPOIETIC CELL DIFFERENTIATION

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Differentiation, Mitochondria, DIGE and MALDI

Material and Methods

Erythroleukemic cell line K562, stably transfected with Notch 1 Intracellular domain and K562 cells treated with Hemin was cultured to ~5×107 cells. From these cells mitochondria were isolated using differential centrifugation and sucrose gradient for further enrichment. Mitochondrial protein extracted using 2D DIGE Buffer were labeled with Cy2, Cy3, and Cy5 following the manufacturer's protocols. 2D-PAGE was run (5-8 pI/ 7-10 pI, 10% PAGE) and scanned by Typhoon scanner. Image analysis was done using Decyder. Spots with differential expression were excised from preparative gel for MALDI based identification. Protein identification was processed and analyzed by searching the MSDB protein database for Homo sapiens using the MASCOT search engine integrated in the Global Protein Server Workstation. The mass tolerance was limited to 50 ppm and MS and MS/MS spectra were accepted when the GPS score confidence was higher than 95%. Validation was done by western blot.

Results

K562 stably transected with Notch 1 Intracellular domain (KNICD) ie. with activated Notch pathway, shows down regulation of CD235a (a erythroid marker); while K562 treated with hemin (KHem), shows an upregulation of hemoglobin synthesis. These two systems were used as a model for hematopoietic cell differentiation. Mitochondria isolated from these cells were compared to control cells to understand the alteration of mitochondrial proteome during erythroid differentiation. Mitochondria enrichment was judged by immunoblot of various markers and by Citrate Synthase Activity. DIGE images were analyzed using Decyder software in BVA mode where the normalized and matched gels were compared. Comparison of K562 and KNICD showed alteration of some of the proteins. From the analysis of 5-8pI range out of 330 matched spots a total of 65 spot shows significant (confidence > 95%) down regulation and 48 spots showed significant up-regulation. Proteins belonging to protein import in mitochondria were getting altered like GRP75_HUMAN, LONM_HUMAN, and LPPRC_HUMAN etc. For the comparison of K562 and KHem it was expected to show an opposite variation of protein expression which was true for some like QCR1_HUMAN, IMMT_HUMAN etc. but many other proteins remained unaltered and new proteins showed alteration. Among the altered proteins PDIA3_HUMAN is interesting as it is increased in KNICD but remains unaltered in KHem in the mitochondrial fraction. All the proteins were identified using MALDI based mass-spectrometry followed by database matching. Some of the alterations were validated using western blot too.

Conclusions

K562 can be differentiated to either erythroid or megakaryocytes. In this study we observe that an activation of the Notch pathway makes it becomes less erythroid like and this causes an alteration of the mitochondrial proteome. The proteome content is distinct and is significantly different from that of mitochondria collected from cells undergoing erythroid differentiation. This clearly indicates that mitochondrial proteome is altered depending on cellular lineage commitment. Further study is being done to understand the role of these mitochondrial proteins in cell fate determination.

Poster 22

ARSENATE(V) INDUCED DIFFERENTIAL EXPRESSION OF PROTEINS IN BACTERIA ISOLATED FROM INDUSTRIAL WASTE WATER.

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Bioremediation, Arsenate(V), Proteins, Redox enzymes

Material and Methods

The sample was collected from Mula River at Chichawad, Pune, from the site where industrial effluent is dumped, isolate having higher minimum inhibitory concentration (MIC) against arsenate(V) was picked and identified using ribotyping, Bacterial cells treated with different concentrations of arsenate(V) (100,200,300mM), were harvested in logarithmic growth and lysed in 50mM phosphate buffer pH 7.0 for catalase and superoxide dismutase enzyme activity. However, for revealing differential protein expression under 100mM stress, cells were lysed in lysis buffer. Proteins of interest were excised from 2-Dimensional Gel Electrophoresis (2-DE) gels, in-gel trypsin digestion at 37oc using sequencing grade trypsin. The mass of each extracted peptide was determined with Voyager STR MALDI-TOF-MS, were recorded in the reflector mode using delayed ion extraction. Peptide mass fingerprinting was used for protein identification from tryptic fragment sizes using the MASCOT search engine based on NCBInr protein database.

Results

Over the past decade, the consumption of metals and chemicals in the process industries has increased dramatically. So, the control of environmental pollution has become the major and one of the most difficult tasks.

The present work is a little step in solving the above problem wherein, water sample collected from the site, river Mula, showed the presence of few promising heavy metal resistant bacteria. Initial isolation showed one culture to be highly resistant to arsenate (V), the latter has shown sensitivity to almost all antibiotics. The production of reactive oxygen species is known as one of the major mechanisms of the toxicities exerted by heavy metals; increase in specific activity of catalase enzyme in the presence of arsenate can be attributed to this stress. Interestingly, the specific activity of superoxide dismutase was repressed.

Protein profile alterations following exposure to 100mM arsenate(V) were examined on 2-DE and proteins were analyzed through MALDI-TOF-MS. Arsenate resistance revealed a down regulation of enzymes involved in glycolysis, TCA cycle (enolase, malate dehydrogenase, fructose 1,6 bisphosphate aldolase). This suggests the lowering of energy production and lowered cell growth. This could explain the repression of α & β subunit of ATP synthase. There was inhibition of Elongation factors Tu,Ts,G, however, ribosome recycling factor, ribosomal proteins S1, S2 were high in abundance. Arsenate(V) appeared to upregulate the expression of glycine hydroxymethyl transferase enchancing the accumulation of serine, the study shows high in adundance of two enzymes pyridoxine biosynthesis protein and pyridoxal phosphate dependent acyl transferase for its synthesis. The study also showed high abundance of protein involved in motility under arsenate(V) stress. Urea cycle enzyme ornithine transcarbomylase involved in arginine biosynthesis was in high abundance under arsenate(V) stress. Arginine is a precursor in polyamines, polyamines metabolism is stimulated by a variety of environmental stresses such as heat shock. An upregulation of cysteine synthetase was observed. Further, cysteine represents one of the limiting factors for glutathione (GSH) biosynthesis (Noctor, 1996), GSH contributes actively to detoxification of plants, probably acting as intracellular heavy metal chelating agent (Bruns, 2001).

Conclusions

Functional assessment of proteins with altered expression levels revealed mechanisms which could be involved in the survival of the organism in the presence of arsenate(V), this information will contribute towards developing bioremediation processes for arsenate detoxification and may help to gain better information and deeper understanding into the toxic mechanism of this metal.

Poster 23

RICE PROTEOME ANALYSIS REVEALS THAT SALICYLIC ACID ALLEVIATES CADMIUM TOXICITY BY RESTORING ELECTRON TRANSPORT CHAIN ACTIVITY AND PREVENTING OXIDATIVE STRESS

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Proteome, Cadmium toxicity, Oxidative stress, Salicylic acid, Rice

Material and Methods

Seedlings of rice (Oryza sativa L.) cv. Malviya-36 were raised in purified sea sand saturated with Yoshida nutrient medium which served as control, or supplemented with 150 µM Cd(NO3)2 alone or in combination with 30 µM salicylic acid (SA). Seedlings grown 12 days were used for all the experiments conducted in triplicates. Various reactive oxygen species (ROS) and antioxidative enzyme activities were determined using following protocols:

H202 content (Jana and Choudhuri, 1981)
Lipid peroxides (Heath and Packer, 1968)
Thiols content (de Kok and Kuiper, 1986)
Superoxide dismutase activity (Fridovich, 1971)
Catalase activity (Beers and Sizer, 1952)
Guaiacol peroxidase activity (Egley, 1983)

Proteins were isolated following the method of Kim et al., 2001 and separated by 2-D electrophoresis (Kim et al., 2004). PD Quest selected spots from the 2 -D gels of control, Cd-stressed and S A+Cd treated plants were subjected for MALDI-TOF/TOF analysis for further characterization.

Results

When growth of rice seedlings was examined at 12th day in sand culture, it was observed that, Cd toxicity significantly reduced the vigour of rice plants in comparison with control. Treatment with SA significantly alleviated the Cd-toxicity effect in comparison with control. SA treatment caused reduced production of H2O2 in rice seedling grown under Cd-toxicity. SA treatment mitigated Cd mediated damage to cell wall integrity and lipid peroxidation. Cd caused significant increase in the level of SOD expression, which was found to be regulated by SA treatment in comparison to control. Catalase activity also decreased with SA treatment in comparison of Cd-stressed rice seedlings, even though it was more than the level of control. Catalase activity was more in shoots than in roots in all the cases. Level of guaiacol peroxidase (APX) was regulated to level of control expression with SA treatment in comparison with Cd-stressed rice seedlings. PD Quest and MALDI-TOF/TOF analysis showed a r emarkable change in the expression pattern of many important proteins. PD Quest analysis of 2D proteomes revealed changes in protein spot patterns in all three gels. SA treatment caused impressive proteomic regulation of Cd toxicity by changing expression level of many proteins, which were further characterized by MALDI-TOF/ TOF analysis.

From SA treated rice leaf proteome some spots selected by PD Quest analysis were subjected to MALDI-TOF/ TOF analysis. Mass characterization of two spots among all these showed that Cd toxicity caused inhibition of electron transport chain (ETC) enzymes. The two analyzed spots were characterized as photosystem II oxygen-evolving complex protein 1 [nominal mass (Mr): 26.603], and s pot of ferredoxin [2Fe-2S] I precursor [nominal mass (Mr): 15.169] which were down regulated under Cd toxicity.

Conclusions

Cadmium adversely affects germination of seeds, plant growth and metabolism. The most common effect of Cd toxicity in plants is stunted growth, leaf chlorosis and alteration in the activity of many key enzymes of various metabolic pathways. SA is a direct scavenger of hydroxyl radical and an iron chelating compound, thereby it inhibits the direct impact of hydroxyl radicals as well as their generation via the Fenton reaction. In addition to the negative effects of Cd on the photosynthetic carboxylation reactions, PSII electron transport and especially oxygen-evolving complex are found to be very sensitive to the effect of Cd. Different components of electron transport chain are proposed as primary targets of Cd. All these observations indicate that SA treatment alleviates Cd-stress induced oxidative damage by promotion of plant's metabolism toward normal status.

Poster 24

CYCLIC AMP-REGULATED PROTEIN LYSINE ACETYLATION IN MYCOBACTERIA

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Cyclic AMP (cAMP), Universal Stress Protein (USP)

Material and Methods

Radioligand binding assay: Test proteins were incubated at 37°C for 1 h with [3H]-cAMP and filtered through nitrocellulose membrane. The bound radioligand was measured in a liquid scintillation counter. Specific binding was confirmed using competition with unlabelled cAMP.

GST-pull down assay: Purified GST and GST-MSMEG_5458 were incubated with M. smegmatis lysates for 1 h at 4°C. Interacting proteins were subjected to SDS-PAGE and identified using MALDI-TOF.

In vitro acetylation assay: Acetylation of substrates by MSMEG_5458 was detected using acetyl-lysine antibody using immunoblotting. Rate of acetylation was measured using an enzyme-coupled reaction where the rate of NADH production is measured at 340 nm spectrophotometrically.

Tandem mass spectrometry: In gel tryptic digestion was carried out. 2, 5-dihydroxybenzoic acid was used as matrix and samples were analysed by MALDI-TOF (Ultraflex TOF/TOF, Bruker Daltonics, Germany).

LC-MS/MS: Complex mixtures of proteins were subjected to tryptic digestion and LC- MS/MS analysis was carried at the NCBS MS facility.

Results

Cyclic AMP synthesized by M. tuberculosis plays an important role in pathogenesis. However, the high levels of intracellular cAMP found in both pathogenic and non-pathogenic mycobacteria suggests that additional and important biological processes are regulated by cAMP in these organisms.

We describe here the biochemical characterization of a conserved novel cAMP binding protein from M.smegmatis, MSMEG_5458 (ortholog of Rv0998 from M. tuberculosis H37Rv), that contains a mammalian protein kinase A and G-like cyclic nucleotide binding domain (CNB) fused to a GNAT family acetyl transferase domain.

MSMEG_5458 bound cAMP with a high affinity (IC50 ~100 nM) and showed selectivity for cAMP, showing a 50 fold lower IC50 for cGMP. The protein MSMEG_4207 (USP) was found to be a substrate for MSMEG_5458, and the acetylation of USP by MSMEG_5458 was regulated by cAMP binding to the CNB domain of MSMEG_5458. The site of acetylation on USP (K104) was mapped using MS/MS and confirmed using mutagenesis of K104 to Arg. Using a strain deleted for msmeg_5458 (KO), we showed that USP was indeed an in vivo substrate for MSMEG_5458.

The KO strain showed a reduced viability in the stationary phase of growth which was rescued by complementation with a wild type copy. Reduced viability was not seen in a strain containing the mutation USPK104R, suggesting that the phenotype of the KO strain was not due to the lack of acetylated USP, but due to other substrates of MSMEG_5458.

To identify other substrates of MSMEG_5458 we fractionated KO lysates using gel filtration chromatography and subjected the fractions to in vitro acetylation by MSMEG_5458. These were then analysed by LC-MS/MS. Eight proteins were found to be acetylated only on incubation with MSMEG_5458, and thus may be possible substrates for MSMEG_5458.

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Conclusions

The substrate of MSMEG_5458 was found to be the protein MSMEG_4207 (USP), which is acetylated at the Nε- group of Lys104. To our knowledge, this is the first report of protein acetylation at the Nε- group of lysine in mycobacteria. We also report for the first time a protein acetyl transferase regulated allosterically by cAMP binding.

The domain fusion of an acetyl transferase domain with a cNMP binding domain is unique to mycobacteria and therefore studying its importance in mycobacterial physiology would allow us to understand the need for this cross talk between cAMP and protein post- translational modification.

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Poster 25

PROTEOMIC STUDIES OF AN ARSENATE REDUCING BACTERIUM

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arsenate stress, proteomics, two dimensional gel electrophoresis, MALDI-TOF-TOF

Material and Methods

The arsenate tolerant bacteria were isolated from river Mulla, Pune, Maharashtra, India. The minimum inhibitory concentration (MIC) of arsenate metal was established with macrodilution method by using sodium salt of arsenate in Luria-Bertani broth. Of 12 isolates screened, an isolate, MR4, showing the highest MIC was identified using 16S ribotyping and was thus selected for further study. Growth kinetic of MR4 was studied by using varying concentration of sodium arsenate (0mM, 100mM, 200mM, 250mM, 300mM). The ability of isolate to reduce arsenate [As(V)] to arsenite [As(III)] was tested by spectrophotometric assay (Bachate et al., 2008). Effect of arsenate on the activity of antioxidant enzymes of isolate MR4 checked by spectrophotometric assay and ingel activity staining. Comparative proteomic analysis using two-dimensional gel electrophoresis (2-DGE) and matrix assisted laser desorption ionization-time of flight-time of flight (MALDI-TOF-TOF) was used to monitor the proteins undergoing changes in expression levels under 200 mM As(V) stress.

Results

An arsenate, As (V), tolerating bacterium, MR4, isolated from Mulla river Pune, India, capable of reducing As(V) to As(III) and was identified as K. pneumoniae. The minimal inhibitory concentration (MIC) of sodium arsenate was determined to be more than 400 mM in Luria-Bertani broth. In the presence of As(V), isolate MR4 showed a prolonged lag phase compared to control. Doubling time of K. pneumoniae increases with increase in arsenate concetration. Around 350 protein spots with a pI range from pH 4 to pH 7 and molecular weight from 6.5 to 116.25 kDa were separated on 2-D gels in the absence and presence of 200 mM As(V). From these, 65 most intense protein spots were excised from both gels and 9 differentially expressed protein spots were successfully identified by MALDI-TOF-TOF with a significant mascot score(>70). These identified proteins were classified into the following functional categories (i) outer membrane proteins (OMP), membrane transport and binding proteins; (ii) antioxidant proteins; (iii) stress proteins. Among the outer membrane proteins, two porins viz., OmpK36 precursor (spot no 0301), and OmpA precursor (spot no 4206), were down-regulated by 4.5, 3.5 fold respectively and one ABC transporter protein was down-regulated and identified as ABC periplasmic dipeptide binding protein precursor (DppA; 1.5 fold). Among the antioxidant enzymes, thiol peroxidase, superoxide dismutase (Mn-SOD and Fe-SOD) were up-regulated by 2.8, 2.5 and 2 fold, respectively. Functional analysis of SOD shows that activity of SOD was increased in As(V) stress. In this study three stress proteins were up-regulated under As(V) stress and identified as DnaK type molecular chaperones (2.0 fold), Hsp60 (2.4 fold) and a protein similar to GroES protein (3.3 fold).

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Conclusions

In conclusion, K. pneumoniae able to tolerate As(V) concentration up to 400mM, and capable of reducing As(V) to As(III) was isolated. Survival of K. pneumoniae under stress is enhanced by physiological changes at the biochemical level that enable the organism to overcome arsenate stress. The results obtained in this study suggest that multiple factors, which includes up- regulation of stress and antioxidant proteins has played an important protective role during As(V) stress, while down-regulation of porins and membrane transport and binding proteins reduced the uptake of As(V) in K. pneumoniae.

Poster 26

SECRETOME ANALYSIS OF GASTRIC CANCER

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SILAC, cell lines, secretome, gastric adenocarcinoma

Material and Methods

To identify proteins that are more abundant in secretome from gastric cancer cells as compared to normal cells, we have employed Stable Isotope Labeling by Aminoacids in Cell culture (SILAC). The following gastric cancer cell lines (AGS, SNU-1, SNU-1, SNU-5, SNU-16, NCI-N87, AGS and KATO-III) were procurred from ATCC and were grown in normal medium. The normal gastric epithelial cell line, HFE145 (provided by Dr. Duane T Smoot, Howard University) was grown in a medium containing heavy arginine and h eavy lysine. Equal amount of protein from pooled tumor secretome and f rom normal secretome were mixed. Proteins were subjected to fractionation by SDS-PAGE electrophoresis and strong cation exchange chromatography . The fractionated proteins were subjected to LC-MS/MS analysis using LTQ-Orbitrap Velos mass spectrometer. The raw data was searched and quantitated using Maxquant With Andromeda search engine.

Results

Gastric cancer is one of the highly prevalent cancers in Asia associated with poor prognosis and high mortality. Comparing differentially expressed secreted proteins between normal gastric epithelial cells and malignant gastric epithelial cells could yield potential biomarkers of gastric cancer. SILAC-based quantitative proteomic analysis was performed using gastric cell lines. LC-MS/MS analysis of 25 in-gel digested proteins and 12 S CX fractions resulted in the identification of 1, 700 pr oteins. Among the identified proteins, 481 proteins were found to be pr esent in higher abundance and 143 proteins with lower abundance in tumor cells secretome as compared to normal cells secretome. Some of the proteins that were found with higher abundance in tumor cells secretome includes , Immortalization up-regualted protein (IMUP), Tumor necrosis factor superfamily, member 11, (TNFSF11) and Lec tin galactoside-binding, soluble 4 (LGALS4) with 7.7- fold, 7.5-fold, 7-fold and 6-fold overexpression respectively. Some of the protiens that were below the detection limits in normal cells secretome includes Fibulin-7 (FBLN7), Annexin A-6 (ANXA6) and Insulin-like growth factor binding protein 3 (IGFBP3). A subset of the proteins that were highly abundant in tumor cells will be validated by immunohistochemical analysis using tissue microarrays.

Conclusions

Secreted proteins are important class of molecules that could be detected in body fluids and could be potential biomarkers. However, due to the complexity and dynamic concentration range of proteins, it remains challenging to identify these proteins directly from proteomic analysis of body fluids especially serum. Proteins that are secreted from tumors are often present in low abundance in serum and are masked by highly abundant proteins not secreted by the cells under investigation. Cell culture-based models are suitable tools to identify secreted proteins from tumors which could further be validated for its presence in serum.

Poster 27

SG2NA-DJ-1 INTERACTION: A PLATFORM FOR NEUROPROTECTION

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SG2NA, Interacting proteins, DJ-1, MALDI-TOF/TOF, Parkinson's disease

Material and Methods

The ORFs of SG2NA variants were PCR amplified from mouse brain derived cDNA and cloned into pGEX vectors. To identify the interacting partners, recombinant GST-tagged proteins were subjected to pull-down assay. The eluted samples were resolved on SDS-PAGE; putative candidate bands were excised, and analyzed by MALDI-TOF/TOF. DJ-1 ORF was derived from Neuro 2A cells (mouse neuroblastoma) and its interaction with SG2NA was confirmed by co-immuno- precipitation of flag tagged DJ-1 and myc tagged SG2NAs followed by western analyses. To check its phosphorylation status, DJ-1 was also immunoprecipitated from Neuro2A cells treated with 100 µM H_2O_2 for 6 hours and analysed by LC MS/MS. shRNAs for SG2NA were designed and cloned into pSilencer vector followed by stable transfection into Neuro 2A cells to verify whether downregulation of SG2NA leads to more neuronal cell death upon oxidative stress.

Results

SG2NA is a WD-40 repeat containing protein belonging to the striatin subfamily. It was first discovered as a nuclear auto-antigen in a cancer patient. Our laboratory has shown that SG2NA has multiple variants that are generated by alternative splicing. SG2NA has potential scaffolding functins and it interacts with protein phosphatase 2A (PP2A), caveolin, calmodulin etc. It also plays significant role in estrogen signaling. Drosophila homolog of SG2NA/striatin family is CKA, acts as a signaling mediator of JNK pathway. To further delineate its functions, we looked into its novel interacting partners by means of GST-pulldown assay followed by mass spectrometric analysis. Amongst potential interacting partners of 78 kDa SG2NA were REST Corepressor, DAAX, MAP kinase 4 and DJ-1. DJ-1 has been attributed to familial Parkinsonism and cancer but its physiological function is poorly understood. Co-immunoprecipitation followed by Western analysis showed that all four variants of SG2NA, viz. 35, 52, 78 and 87 kDas interact with DJ-1. Since DJ-1 is an activator of the Akt pathway in neuronal cells and SG2NA is a potential scaffold, we tested whether Akt is also present in the DJ-1-SG2NA complex (immunoprecipitated). The presence of Akt was confirmed only in case of 78 and 87 kDa SG2NAs. shRNA mediated downregulation of SG2NA in Neuro 2A cells results in enhanced cell death under oxidative stress induced by H_2O_2. Cell fractionation assay showed that in response to oxidative stress, SG2NA helps phosphorylted DJ-1 to be accumulated in the membrane.

Conclusions

Loss of dopaminergic neurons is one of the principal causes for neurodegeneration in Parkinson's disease. In response to oxidative stress, DJ-1 protects dopaminergic neurons by activating Akt pathway. It has also been shown that under oxidative stress, DJ-1 helps Akt to accumulate in the membrane activating the survival pathways. Our results thus suggest the mechanistic insight into this process wherein SG2NA may act as a platform for DJ-1-Akt interaction specifically in the plasma membrane.

Poster 28

OCULAR PROTEOMICS: TOWARDS IDENTIFICATION OF DISESAE SPECIFIC BIOMARKERS

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ocular proteomics,aquous fluid,vitreous fluid and serum.

Material and Methods

Human ocular fluids are characterized with very small volume and complex protein constitutes with a very large orders of magnitude. The proteome analysis of these provides a unique dataset (i.e., specific protein markers or protein patterns) that may be correlated to more effective diagnosis, prognosis, and response to therapy. Ocular samples (vitreous humour / aquous humour / tear) was collected from the controls and patients with ocular infections attending Aravind Eye hospital Madurai. Samples were processed and analysed using 2D gel elctrophoresis and nano LC MS/MS.

Results

Several variations starting from optimization of the sample preparation methodologies were found in each ocular samples. Analysis of the proteome of the ocular fluids of Diabetic retinopathy and fungal keratitis patients shows several major variations in proteome map when compared to respective control samples, that will be discussed .

Conclusions

Analysis of the proteome of ocular fluids indicate that the proteome based technologies will be better tool towards understanding the pathogenesis of many ocular infections including Diabetic Retinopathy and Fungal Keratitis. Future studies are ongoing to characterize the mechanism of pathogenesis of these diseases

EXPRESSION PROFILING OF HSP70, NF-KB AND INFLAMMATORY CYTOKINES (IL-1B AND TNF-A) IN CHICKEN PERIPHERAL LEUCOCYTES UNDER THERMAL-STRESS BY REAL-TIME PCR

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Heat shock protein 70, heat-stress, TNF-a, IL-1, Real Time PCR.

Material and Methods

The present study was undertaken to characterize chicken 70 kDa heat shock protein (HSP70) and study its expression level along with NFκB and two of the most potent pro-inflammatory cytokines TNF-α, IL-1β at different temperatures (42º, 44º, 46º and 48º C) in vitro in six divergent strains/breeds of chickens (viz. Rhode Island Red (RIR-B and RIR-C strains), Dahlam Red, Punjab Red and White Leghorn (PL2 and Naked Neck strains). Total RNA was extracted from chicken leukocyte and cDNA was synthesized by reverse transcription. HSP70 gene was amplified from cDNA using specific primers, cloned and sequenced. Further the expression profile using Real Time PCR (ABI 7500) under normal and heat-stressed conditions were performed in vitro (42°C-48°C for 30 min) in different strains/breeds of chickens.

Results

Upon cloning and sequencing of Hsp70 amplicon, Gen Bank Accession No: GU980869 was obtained. Sequence comparison and phylogenetic analysis of Hsp70 revealed high degree of identity suggesting that Hsp70 is conserved among species and is under purifying selection. The expression of Hsp70, NFκB, TNF-α and IL-1β was found to increase from 42°C to 44°C and decrease from thereon, being lowest at 48°C. This may be attributed to the inability of the cells to survive high temperatures beyond 44°C as indicated by trypan blue dye exclusion test. Among the strains/breeds highest and lowest expression of Hsp70, NFκB, TNF-α, and IL-1β was observed in RIR-C and Punjab Layer strain, respectively. The expression of these genes was observed to be higher in Red varieties (RIR-B, RIR-C, DR, PR) than the White varieties (NN, PL2). Among the Red varieties, the expression of Hsp70 in Punjab Red was higher than its parental breeds (RIR-B and Dahlam Red).

Conclusions

The results of the present study indicate that the expression of Hsp70 is correlated with the expression of NFκB and potent pro-inflammatory cytokines TNF-α and IL-1β. Further the expression level of such genes can be studied at their protein level along with different kinases protein interaction with Hsp70 in order to explore its signaling mechanism.

Poster 30

DIFFERENTIAL EXPRESSION OF CELL SURFACE MOLECULES IN ADHESIVE AND LESS ADHESIVE HUMAN ENDOMETRIAL CELL LINES

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Endometrial cells, Cell Surface Proteins, 2DE, Differential expression, Cell adhesion

Material and Methods

Adherent human endometrial cell lines RL95-2 (CRL-1671) and HEC-1-A (HTB-112) were obtained from American Type Culture Collection .Confluent monolayers of RL95-2 and HEC1-A cells grown in 75cm2 tissue culture flasks were labeled with Sulpho NH-SS-Biotin and cell surface proteins were obtained as per instructions in the Pierce Cell Surface Protein isolation kit. The proteins were resolved by two dimensional polyacrylamide gel electrophoresis afterfocussing on 11c m immobiline strips in the linear range of pH 4-7. The gels were analyzed for differential abundance of proteins using 2D Imagemaster Platinum Software. Differentially abundant spots were excised; trypsin digested and subjected to MALDI TOF MS/MS. Validation was carried out using immunoblottingto determine relative levels of the identified antigens in total lysates as well as in cell surface extracts. In situ localization of differentially abundant proteins on cell surface was determined by confocal microscopy. The extent of colocalization was determined by staining the cells with phalloidin, an impermeable dye.

Results

Analysis of the four pairs of cell surface extract revealed that 194 protein spots were present in RL95-2 and 244 protein spots in HEC-1-A out of which 138 spots were paired. 55 spots were found to be differentially expressed by at least two fold, in three of the four pairs of gels; run for comparing the cell surface proteomes of RL95-2 and HEC1A, of which eleven could be identified.These included Tubulin beta 2C and Elongation factor-1-beta which were up regulated in HEC-1-A cell surface protein extract and HSP70, gsp96, Calreticulin, HSP27, ADAMTS3,Tapasin-ERP57 Heterodimer, Human Pyruvate Dehydrogenase and Protein disulfide-isomerasewhich were up regulated in RL95-2 cell surface extract. A search through the Gene Ontology database also indicated the presence of Calreticulin, HSP70, Protein disulfide-isomerase, ADAMTS3, HSP27,Tapasin-ERP57 Heterodimer as cell surface componentsin humans.

Calreticulin and ADAMTS were found more abundant on the cell surface of RL95-2 as compared to that of HEC-1-A. This increased expression on cell surface was probably because of inherently increased synthesis of these proteins in RL95-2, as revealed by immunoblotting of total cell lysates. While other proteins such as HSP70 and tubulin beta, found more abundant on RL95-2 cell surface, did not show any significant difference in their cellular expression levels as compared to that in HEC1A. This may implicate the role of differential trafficking in RL95-2. Confocal microscopy analysis also demonstrated differential abundance of these cell surface molecules in RL95-2 cells as compared to HEC-1-A. An interesting observation was differential abundance of cytoskeletal proteins in RL95-2, a more adhesive cell line.

Conclusions

Our investigations reveal differences in the cell surface protein profiles of two endometrial cell lines which differ in their ability to adhere with embryonic cells. While some of these differences may arise because of the different origins of these two cell lines, some of these may account for their differential abilities to adhere with embryonic cells. Studies are in progress to determine whether differential abundance of some of these proteins is indeed responsible for differential adhesiveness of these cell lines to embryonic cells.

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Poster 31

PROTEOMIC ANALYSIS REVEALS THE PLASTICITY AND DIVERSITY OF PLANT MEMBRANE PROTEINS

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grain legume; membrane proteome; 2-DE; hydropathicity; transmembrane domain

Material and Methods

Chickpea (Cicer arietinum L.) seedlings were grown in environmentally controlled conditions and aerial tissues were harvested from 3-week-old seedlings. The cells were fractionated and membrane fractions were systematically assessed using standard marker enzymes. Isoelectric focusing of the membrane proteins was performed with 100 µg protein on 13 cm variable pH gradient strips (pH 3-10 and 4-7). The strips were subjected to 12.5% SDS-PAGE. The silver-stained gel images were analyzed by PDQuest ver 7.2.0 (Bio-Rad, USA). Three replicates gels were used to generate the composite image conventionally known as the 'standard gel'. Protein spots present in, at least, two of the three gels were considered for further analysis. The 'high quality' protein spots were excised, tryptic-digested, and subjected to MALDI-TOF/TOF and LC-ESI-MS/MS. The identified proteins were functionally classified into various categories. Multiple bioinformatics tools were used to predict putative transmembrane domains (TMDs) and compute hydropathicity of the identified proteins.

Results

The hydrophobicity, low abundance and physicochemical heterogeneity of the membrane proteins are major limiting factors for membrane proteomics. This study was focused on developing the membrane proteome of chickpea, aiming at global identification of intrinsic and extrinsic proteins, and also those interacting with membrane. The enrichment of various membranous structures viz. plasma membrane, mitochondrial, vacuolar, and Golgi membranes was confirmed by higher activities of respective marker enzymes in the isolated membrane fraction. Various combinations of chloroform/methanol used for protein extraction revealed 6:3 ratio as optimal for maximal extraction of membrane proteins. Electrofocusing was performed using different pH gradient strips and sample loading methods of in-gel rehydration or cup loading, the latter yielding maximum representation of spots in pH range 4-7. The experiments were performed with at least two biological and three technical replicates which led to the detection of 280 protein spots with high reproducibility. A total 130 spots were subjected to MS/MS analysis that led to the identification of 100 proteins with significant match. The identified proteins were functionally classified into various categories including bioenergy, metabolism, protein biogenesis and translocation, stress responsive and signal transduction, among others. The proteomic analysis revealed many resident integral membrane proteins as well as membrane-associated proteins including several novel candidates. The proteins with unknown function (accounting for 15%) were subjected to domain analysis which indicated their functional implications. The membrane association of identified proteins was validated by determining the TMDs and Grand Average of Hydropathicity (GRAVY) values. Five different programs were used to predict TMDs. Significantly, >70% of the identified proteins were predicted to have putative TMDs. The GRAVY values indicated positive trends ranging 0.038 to 0.179 (proteins) and 0.014 to 1.287 (peptides). A comparative analysis of membrane proteome datasets revealed overlapping as well as exclusive proteins at organismal level.

Conclusions

A high resolution 2-DE based proteome map of membrane was developed from chickpea, whose genome is yet to be sequenced. The proteomic analysis led to the identification of 100 proteins with high confidence. Membrane-association of the identified proteins was validated by assessing hydropathicity, and the presence of TMDs. The proteins having unknown functions were subjected to domain analysis to determine their putative functions. This study would not only provide an understanding of the diversity and complexity of membrane proteins, and their networks, but also provide a platform for functional genomics studies associated with stress and developmental pathways in plants.

Poster 32

COMPARATIVE NUCLEAR PROTEIN PROFILING OF TWO CHICKPEA GENOTYPES WITH CONTRASTING DEHYDRATION TOLERANCE

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dehydration; nuclear proteome; ROS-catabolism; contrasting tolerance

Material and Methods

Chickpea (Cicer arietinum L. c.v. ICCV-2) seedlings were grown and maintained under environmentally controlled conditions. Gradual dehydration condition was applied on 3-week-old seedlings by withdrawing water, and tissues were harvested for a period upto 144 h. Nuclear proteins were prepared from nuclei-enriched fraction using TriPure Reagent (Roche). Isoelectric focusing was carried out with 150 µg protein on IPG strips (13 cm; pH 4-7) using IPGphor system (GE Healthcare). The strips were then overlayed on 12.5% polyacrylamide gel for SDS-PAGE. Electrophoresed proteins were stained with silver stain plus kit (Bio-Rad, USA). Digitised gel images were obtained using Bio-Rad FluorS equipped with a 12-bit camera. PD Quest version 7.2.0 (Bio-Rad, USA) was used to create the matchsets. Protein spots of interest were excised mechanically and in-gel digested with trypsin (Sigma), and analyzed using LC-ESI-MS/MS (Applied Biosystems, USA). Nuclear localization of the identified proteins was validated by different bioinformatics tools.

Results

This study was directed to develop dehydration-responsive nuclear proteome from a sensitive chickpea cultivar ICCV-2 and compare with that of a tolerant cultivar. Intact nuclei were isolated from unstressed and dehydrated seedlings, and nuclear integrity was assessed by DAPI staining. Nuclear enriched fraction displayed a distinct 1-D protein profile when compared with the crude extract. The enrichment of nuclear proteins was further evaluated by immunoblot analysis using antibodies for proteins specific to the nucleus. The temporal changes in the nuclear proteome were monitored during 0-144 h of dehydration. To reduce gel-to-gel variation, each protein preparation was analyzed on at least four parallel 2-DE gels from two biological replicates.Three representative gels were computationally combined using PDQuest to generate a standard gel, the first-level matchset. To analyze the abundance changes for each spot across the time points, a second-level matchset was created. The differential proteome revealed 189 dehydration-responsive proteins (DRPs), which showed more than 2.5-fold difference in expression values, at least at one time point. Mass spectrometric analysis led to the identification of 118 DRPs. The identified DRPs were grouped into ten functional categories based on their putative functional roles. The proteins for which no known function could be assigned were grouped under the unknown category, representing the most abundant class. Prediction of nuclear localization was carried out using six different programs; proteins that displayed positive output in at least three analyses were considered as nuclear residents. This proteome was then compared with that of c.v. JG-62. Several divergent trends as well as commonalities were identified in the proteomes of the two contrasting varieties. The array of differentially expressed proteins was found to be significantly higher in c.v. JG-62 compared to ICCV-2.

Conclusions

The study summarizes the differential response of two cultivars of chickpea to dehydration in terms of the dynamic changes in the nuclear proteome. The differential physiological state of the varieties at cellular level was reflected in the variance of the nuclear proteome under dehydration. Several of the key enzymes involved in dehydration response, notably the ROS-catabolism were found to be highly regulated specifically in c.v. JG-62. It is thus proposed that the differential protein profile and the superior ability to cope with oxidative stress might render JG-62 better tolerance to dehydration as against ICCV-2.

Poster 33

HIGH THROUGHPUT QUANTITATIVE MALDI MS

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MALDI MS, Small molecules

Material and Methods

To demonstrate quantitative analysis mixture of triazines and melamine was prepared and diluted to generate calibration curve. One of triazines was used as internal standard. All the analysis was performed on Synapt HDMS and Voyager DE-STR mass spectrometer. DHB was selected as matrix for all analytes. To validate MALDI MS result LC-ESI MS analysis was also carried out. Regression coefficient, slope, intercept and determination of unknown were calculated both manually and with MQ algorithm. A highthroughput experiment was also performed with triazines and melamine to demonstrate rapidness of MALDI MS with accurate and reproducible results.

Results

Quantitative data from LC-ESI-MS compares well with the MALDI MS data. Peak-intensity based MALDI MS analysis is comparable to the corresponding values from both peak areas and peak intensity measurements in the LC-ESI or LC-PDA mode. Results from the software processing tool ‘MQ’ are in agreement with those obtained manually.

Conclusions

MALDI MS is capable of performing highthroughput quantitative analysis. Tedious sample preparation, derivatization and time consuming chromatographic or method development steps can potentially be eliminated in the MALDI MS.

Poster 34

DETERMINATION OF ALMOTRIPTAN, IT'S IN VIVO METABOLITES IN RAT PLASMA, URINE AND FECES BY LC-ESI–MS/MS: ITS APPLICATION IN PHARMACOKINETICS

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Almotriptan, sumatriptan, LC-ESI–MS/MS, Metabolites, rats

Material and Methods

A highly sensitive and specific liquid chromatography/tandem mass spectrometric (LC-ESI-MS/MS) method for investigating the metabolites and pharmacokinetics of almotriptan in rats was developed. The almotriptan, IS and its metabolites were extracted from rat plasma, urine, feces using simple solvents acetonitrile and methanol by vortexing and centrifuging at 4500×g for 20min as the sample clean-up procedure. Chromatographic separation was achieved on a Lichrospher RP-18 column (250×4.6 mm, 5 µm), using 20 mM ammonium acetate (pH adjusted to 3.5 with trifluoroacetic acid) and acetonitrile (60:40 v/v) as mobile phase at 25º C. Detection was performed by positive ion electrospray ionization in multiple reaction monitoring (MRM) mode while monitoring the transitions of m/z 336, 296, 281, and m/z 368 (M+H) for almotriptan, IS and its two metabolites, respectively. The validated method was successfully applied to pharmacokinetic studies of almotriptan after an oral administration to rats.

Results

1.0 Liquid chromatography–ESI-tandem spectrometry (LC–ESI-MS/MS)
The present study was aimed at developing a chromatographic system capable of eluting and resolving almotriptan, IS in rat plasma and its two metabolites in rats plasma, urine and feces. Almotriptan, IS, and its two metabolites were subjected to separation by reverse-phase (LC–ESI-MS/MS) on a Li chrospher C18 column using column (250×4.6 mm, 5 µm), using 20 mM ammonium acetate (pH adjusted to 3.5 with trifluoroacetic acid) and acetonitrile (60:40 v/v) as mobile phase at 25º C.

2.0 Metabolism study of almotriptan in rat plasma, urine and feces sample
The full scan mass spectrum of free fraction of rat plasma, urine and feces after administration of almotriptan was compared with those of blank plasma, urine and feces samples and almotriptan solution to find out the possible metabolites in rat plasma urine and feces. Then, these compounds were analyzed by LC-ESI–MS/MS. Their retention times, changes in observed mass and s pectral patterns of product ions were compared with those of almotriptan standard to identify metabolites and elucidate their structures.
Based on the method mentioned above, the main drug and its main metabolites were found in rat plasma, urine and feces after administration of almotriptan at 24h and 72hr. Their molecular ions ([M]+) were at m/z 336, 368, and 281, almotriptan, The λ-aminobutyric acid metabolite formed by oxidation of the pyrrolidine ring , A sulphonamide metabolite resulting from the loss of the pyrrolidine ring moiety in rat plasma, urine and feces respectively. LC–MS/MS spectra of the metabolites of almotriptan were obtained via fragmentation of molecular ions were characterized by the HR-MS for structural identification of metabolites.

3.0 validation
A full validation according to the FDA guidelines (US DHHS et al., 2001) was performed for the assay in rat plasma.

Conclusions

A highly sensitive and specific method for the determination of almotriptan and its two metabolites was developed using LC-ESI–MS/MS. LC-ESI–MS/MS spectra of the metabolites of almotriptan were obtained via fragmentation of molecular ions were characterized by the HR-MS for structural identification of metabolites. It was shown to be sensitive and rapid for the simultaneous determination of almotriptan and its metabolites in rat plasma, urine, feces. The method is rapid and practically applicable to the pharmacokinetic studies of almotriptan in rats.

GAS PHASE BEHAVIOR OF RADICAL CATIONS OF 2,4,6-TRI SUBSTITUTED S-TRIAZINES: UNUSUAL LOSS OF NH3 INVOLVING OPENING OF THE S-TRIAZINE RING

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s-triazines, Electron Ionization, Skeletal rearrangement

Material and Methods

Tris-benzyl-triazines (1 and 2) were obtained on t rimerization reaction of phenyl acetonitriles in the presence of triflic acid in good yields. Various benzyl amines were treated with cyanuric chloride and K2CO3 and 18-Crown-6 as catalyst in dry toluene to yield tris-benzylamino-triazine derivatives (3-7). Third series of compounds, tris-benzyloxy derivatives (8 and 9), were prepared by conducting a reaction between cyanuric chloride and various benzyl alcohols in the presence of base. Electron ionization (70 eV, source temperature 230 oC) mass spectra of compounds 1-9 were recorded using a Shimadzu GC-MS instrument (GCMS-QP2010 Plus). The data acquisition was under the control of GC-MS solution software. The samples were introduced through a direct insertion probe.

Results

The EI mass spectra of all these compounds show the M+. ion. The fragmentation pattern depended significantly on the nature of the substituents at the 2,4,6-positions of the s-triazine ring. S-triazine derivatives 1(tris-benzyl-triazine) and 2 (tris-p-chlorobenzyl-triazine) constitute one series of compounds. The EI mass spectrum of 1 shows an a bundant molecular ion at m/z 351. Interestingly, the spectrum shows the base peak at m/z 334 c orresponding to the loss of NH3. A mechanism involving a s keletal rearrangement followed by elimination of NH3, has been proposed for the formation of this ion. The mechanism may involve a rearrangement of the molecular ion with the successive migration of three of the benzylic hydrogens at C-2 and C-4 positions to the ring nitrogen resulting in expulsion of ammonia. The proximity effect of the pendant benzyl to the amine group may be a driving force for the elimination of ammonia with the formation of a t etra cyclic system. A similar skeletal rearrangement involving loss of NH3 has been observed in 2. Whereas this fragmentation pathway is absent for 3-9.

Conclusions

Three series of 2,4,6-tri substituted s-triazines with antibacterial and antifungal activity have been characterized by electron ionization (EI) mass spectrometry. Compounds1 and 2 show ions corresponding to the loss of ammonia (17u). One attractive rationale of this process invokes a skeletal rearrangement of the molecular ion followed by expulsion of ammonia. This fragmentation pathway is absent for 3-9. The fragmentation of tribenzyl derivatives indicates that benzylic hydrogens play a significant role in the loss of ammonia from the molecular ions of compounds 1 and 2.

CHARACTERIZATION OF AMINO ACID DERIVED BETAINES BY ELECTROSPRAY IONIZATION TANDEM MASS SPECTROMETRY

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betaines, ESI, MS/MS, isomeric differentiation

Material and Methods

All amino acids were obtained from Sigma–Aldrich (St. Louis, MO, U.S.A). Methyl iodide was purchased from spctrochem pvt.ltd. (Mumbai, India). HPLC grade sovents were purchased from Merck. All the betaines were synthesized by the reaction of the corresponding amino acids with methyl iodide using a known procedure. Electrospray ionization (ESI) mass spectrometric analysis of all the compounds were done under the positive ion ESI conditions using a Q-TOF mass spectrometer (Q-Star XL, Applied Biosystems, USA). Collision induced dissociation (CID) spectra were obtained by selecting the m/z value of the ion of interest by the quadrupole, colliding with nitrogen in the collision cell, followed by detecting the product ions by TOF. The typical positive ESI conditions were: capillary voltage, 5.00 kV, declustering potential 60V, focusing potential 220V; resolution10000 (FWHM).

Results

Betaines are small quaternary ammonium compounds, which act as os molytes as well as work as a catabolic source of methyl groups in many biochemical pathways. Characterization of betaines is important to understand the metabolomic picture of a cell or tissue. Theoretically all amino acids may be converted to betaines [Me3N+-CH(R)-COO-], however, only a f ew of them were characterized in the literature. This prompted us for synthesis of all natural amino acid derived betaines and their characterization by ESI-MS and MS/MS methods. The ESI-MS analysis of betaines are expected to produce [M+H]+, [M+Na]+ and [M+K]+ ions, because the cells contain sodium and postassium salts. Therefore, the analysis of betaines has two important problems, i.e., isomeric betaines, and overlapping m/z values for certain species such as the m/z value of [M+Na]+ of serine betaine matches with [M+K]+ of alanine betaine. Here, we successfully used HRMS and MS/MS methods for characterization of all the betaines.

The positive ion ESI mass spectra of the studied betaines showed [M+H]+, and in the presence of sodium and potassium they produced the corresponding [M+Na]+ and [M+K]+ions. In general, cleavage of N-C bond with or without hydrogen migration is the major fragmentation observed in these species. If the charge is retained on quaternary ammonium ion, they produced m/z 58, 59 and 60; and if the charge is on acid part they produce structure indicative fragment ions. The isomeric betaines showed a clear-cut differences in their CID spectra. Similarly, the nature of amino acid (aliphatic, aromatic, acidic and basic) is also very much reflected in the fragmentation of all the species. For example, the CID of [M+Na]+ and [M+K]+ from all aliphatic betaines showed loss of NC3H9 and NC3H9+CO, whereas those from the aromatic betaines showed loss of NC3H9+CO2. Cyclic betaines characteristically showed the ion at m/z 58.

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Conclusions

The ESI-MS/MS analysis of all the amino acid derived betaines showed isomer/structure selective fragmentation that enabled successful characterization of one betaine another. The heterolytic cleavage of C-N bond is the key fragmentation observed in the CID of all protonated and cationized (Na+ and K+) species of the betaines. This cleavage produced the ions m/z 58, 59, 60 and other structure indicative fragment ions depending on the location of the charge. The betaines having a β hydrogen showed the ion at m/z 60. The isomeric betaines and other m/z overlapping species were successfully discriminated.

Poster 37

DIRECT DETECTION OF METABOLIC CHANGES BY LASER ABLATION ELECTROSPRAY IONIZATION: A STUDY ON UNFERTILIZED EGGS OF XENOPUS LAEVIS

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Laser ablation electrospray ionization, Metabolomics, Xenopus Laevis, High resolution mass spectrometry, tandem mass spectrometry

Material and Methods

Unfertilized eggs collected from Xenopus Laevis were immediately stored at -800 C until use. A single egg was directly placed on a microscope slide for LAESI analysis. Laser pulses of 2940 nm wavelength (Nd:YAG laser system with 100-Hz repetition rate) were used to ablate the sample placed at 90□ angle on a microscope slide at a 15 mm distance below the spray axis (average laser spot ~ 300□m diameter). A home built electrospray system was used for post-ionization of ablated neutral species. A 50% methanol solution containing 0.1% acetic acid was infused (300 nl/min) through a metal tapered tip (length 5 cm, tube OD 320 □m and tip ID 50 □m) at 2800 V. The LAESI ion source was mounted on a Q-TOF premier mass spectrometer and full scan mass spectra were recorded over the mass range of m/z 50-2000 using TOF analyzer at a resolution of 8,000 (FWHM).

Results

The laser ablation electrospray ionization (LAESI) technique, developed in our laboratory at GWU, USA, enables direct analysis of live tissue samples under atmospheric pressure conditions without involving any sample preparation steps. We applied the LAESI technique for the identification of metabolites present in the Xenopus laevis' unfertilized egg, a large single cell that has been used as a model system in molecular and developmental biology. Though the broad aim of the study is for investigation of metabolic changes at various stages of development after fertilization, initially we focused on metabolomic profiling of mature unfertilized egg. The high resolution LAESI spectrum obtained from multiple laser ablations showed mostly singly charged ions, but included a few doubly charged ions. Various metabolites appeared in the spectrum below m/z 450, and abundant lipid peaks appeared in the high mass region (450-900). The peak assignments of metabolites were done based on searches in metabolomic databases with accurate mass values and later verified by their isotope distribution patterns; the typical metabolites were further confirmed by tandem mass spectrometry analysis.

The identified peaks refer to protonated/sodiated/potassiated species of approximately 35 metabolites, excluding lipid peaks. The lipid peaks were dominant because a major part of the egg is yolk platelets (composed of proteins and lipids). The most abundant lipid peaks in the spectrum correspond to different glycerophosphocholines (PC), followed by lysophosphatidylcholines (Lyso–PC or LPC). A few weak peaks corresponding to glycerophosphoethanolamines (PE) and glycerophosphoserines (PS) were also seen in the spectra. The metabolic composition of the eggs with and without jelly coat appeared the same. Changes in the metabolite composition of eggs were clearly seen between the eggs stored at -800 C and at room temperature.

Conclusions

The LAESI technique was successfully used for detection of metabolites directly from unfertilized eggs of Xenopus laevis. The identified metabolites include choline, spermine, phosphocholine, GPC, IP3, and many organic mono- and dicarboxylic acids, including those in the citric acid cycle. Most abundant lipid peaks were PCs and Lyso-PCs, in which palmitic acid occupies one end. The fatty acid compositions and lipids found are in agreement with the literature reports, but the LAESI technique enabled direct detection of these important lipids along with other metabolites within seconds without any sample preparation. The use of LAESI in the detection of metabolic changes was also demonstrated.

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ENANTIOSELECTIVE DISCRIMINATION AND MEASUREMENT OF OPTICAL PURITY OF DRUGS BY ELECTROSPRAY IONIZATION MASS SPECTROMETRY

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Chiral drugs, ESI-MS, enantioselective discrimination, optical purity

Material and Methods

The chiral references (3, 5-Diiodo tyrosine, 4-Iodo phenylalanine) and analytes (D-, and L-DOPA, R- and S-Atenolol, R- and S-Isoproterenol, R- and S-Propranolol) were purchased from Aldrich (Steinheim, Germany). The R- and S-Zolmitriptan were a gift from local pharmaceutical company. Methanol (HPLC grade) was obtained from Merck (Mumbai, India) and CuCl2 from Spectrochem, India. All the experiments were performed using a LCQ ion trap mass spectrometer (Thermo Fisher, San Jose, CA, USA), equipped with an ESI source. The data acquisition was under the control of Xcalibur software. A mixture of a chiral reference (50 µM), D or L- analyte (50 µM) and copper chloride (12.5 µM) was made and introduced into ESI source using an infusion pump.

Results

The positive ion ESI-MS spectra obtained for a mixture of a chiral reference (ref) and analyte (A) in the presence of copper chloride include [A+H]+, [ref+H]+, [(ref)2+H]+, and [(ref)2+A+Cu-H]+ ions. The interested trimeric ion, [(ref)2+A+Cu-H]+ was selected for CID experiments at optimized conditions. The secondary interactions (namely, π-π interaction and d-π interaction) play a vital role in the stability of the trimeric adduct ion which inturn reflects in the relative abundances of the product ions formed in the decomposition. In the case of DOPA and Isopreterenol, the CID spectra showed both the dimeric product ions and their relative abundances were considered for the enantioselective discrimination by applying the kinetic method. Rest of the drugs, however, showed only one product ion; and thereby CR method was applied for the enantioselective discrimination and quantification. Both the 3, 5-Diiodo L-tyrosine and 4-Iodo phenylalanine showed more selectivity towards S-isomers rather than its antipode R-isomer of the studied drugs. The 3, 5-Diiodo L-tyrosine showed relatively better discrimination for the drugs DOPA, Zolmitriptan and Propranolol when compared with 4-Iodo phenylalanine; on the other hand, 4-Iodo phenylalanine showed a little better discrimination for Atenolol and Isoproterenol. The suitability of this method in measuring enantiomeric excess (ee %) was also demonstrated using a set of enantiomeric mixtures of DOPA.

Conclusions

Herein we demonstrated the use of 3, 5- Diiodo Tyrosine and 4-Iodo phenylalanine as chiral references for the enantioselective discrimination and optical purity determination of a set of drugs. The Rchiral values obtained for the drugs are more than the reported values, and both the reference molecules showed comparable discrimination. We have also validated this method for the optical purity measurements, which will be much useful in the pharmaceutical area.

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CHARACTERIZING ENZYMATIC CONVERSION OF DRUGS TO METABOLITES LIQUID CHROMATOGRAPHY AND MASS SPECTROMETRY

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Mass Spectrometry,Chromatography,Enzymes,microsomal proteins, Metabolites

Material and Methods

Importance of obtaining pure organic compounds by enzymes and microorganisms is increasing considerably. Catalysis by enzymes and microorganisms occur in water, neutral pH and room temperature which are healthy, safe and ideal for large scale applications in industries. Strains from enzyme, microorganisms metabolize drugs and also helpful in synthesizing metabolites which are not possible chemically

Liquid Chromatography coupled with Mass Spectrometry is presently used in this study to understand the enzyme assisted reactions. Enzymes present in microsomal proteins and serves as a catalyst for organic conversions. Drugs with sulfonylurea urea groups are incubated in microsomal proteins and the structure of the resulting metabolites are characterized by liquid chromatography and mass spectrometry.

Results

Sulfonylurea urea drugs with different types of functional groups are chosen in the present study. Enzymatic conversion of sulfonyl urea drugs such as glyburide, tolbutamide, sulfaphenazole and sulfasalazine to its metabolites are compared using results obtained from liquid chromatography and mass spectrometry. Liquid chromatography is used to separate the mixture of metabolites while fragmentation pattern obtained from mass spectrometry is used to characterize the structure of metabolite.

Each of these sulfonyl urea compounds has different functional groups resulting in different type of metabolites. Glyburide results in the formation of seven different metabolites which is due to oxidation of aliphatic functional group; tolbutamide shows two major metabolic pathways due to oxidation of aliphatic functional group whereas sulfasalazine and sulfaphenazole are very stable in enzymes. Eventhough all the above drugs have common functional group sulfonyl urea their metabolic pathways are very different.

Conclusions

Invitro incubation of drugs containing sulfonyl urea groups with microsomal proteins result in different types of metabolites. Glyburide and Tolbutamide shows mono oxidation as the major metabolic pathway where cyclohexyl group and methyl groups are hydroxylated. Sulfasalazine and Sulfaphenazole are very stable compounds and are not metabolized. Binding of the drugs to the particular site of the enzymatic proteins and cytochrome P450 enzymes present in the microsomal proteins plays a significant role in deciding the metabolic pathway.

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DIFFERENTIATION OF ISOMERIC CATIONIC AMPHIPHILES BY ELECTROSPRAY IONIZATION TANDEM MASS SPECTROMETRY

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Cationic amphiphiles, ESI-MS/MS, Mc Lafferty Rearrangement, isomeric discrimination

Material and Methods

The isomeric lipid pairs [(R1)(R2)N+(-X-CO-C13H27)2] and [(R1)(R2)N+(-CO-X-C13H27)2], where R1=CH3, R2=n-C3H7 or -C2H4OH, and X= O or NH, were synthesized through different synthetic schemes and characterized by NMR and MS. HPLC grade solvents were obtained from Merck (Mumbai, India). All the full scan and MSn (collision-induced dissociation, CID) experiments were performed using a LCQ ion trap mass spectrometer (Thermo Fisher, USA), equipped with an ESI source and Xcalibur software. The HRMS data was obtained from quadruple time-of-flight mass spectrometer (QSTAR XL, Applied Biosystems, USA) with analyst QS software.

Results

The clinical success of gene therapy is remaining critically dependent on the availability of safe and efficacious gene delivery reagents, popularly known as transfection vectors. Cationic amphiphiles consisting hydrophilic (positively charged) and a non-polar region (long chain fatty acid), are being reported as nonviral alternatives. The linker group controls the conformational flexibility. Recently, our laboratory has designed and synthesized structurally isomeric cationic amphiphiles with varied linker (ester and reverse ester) functionality. Despite having such striking structural similarities, only the lipid having –O-C(O)- (normal ester) linker showed efficient in vitro gene delivery, in contrast, its isomer with –C(O)-O- (reverse ester) was found to be incompetent. Here we aimed to characterize the lipid isomers (normal and reverse esters/amide linkers) under ESI-MS/MS conditions.

All the compounds, being preformed cations, exhibited fantabulous sensitivity under positive ion ESI conditions, and the formed ions are denoted as M+ (refers to quaternary ion). The CID of M+ ions were studied to evaluate structural effects in the fragmentation of the isomers. The CID spectra of M+ from all the isomeric pairs showed clear-cut differences that enabled easy characterization of one isomer from another. For example, the normal ester results in prominent ion at m/z 255 and a low abundant ion at m/z 330, whereas its reverse ester shows the ions at m/z 402, 298, 220, and 116. The spectra obtained at difference collision energies revealed that the reverse ester is more stable than normal ester. We extended the study to isomeric cationic amphiphiles with amide functionality instead of ester. Normal amide exhibited same fragmentation as normal ester, where as the reverse amide failed to show a few fragment ions when compare to reverse ester.

Conclusions

The isomeric cationic amphiphiles are successfully characterized and studied by ESI tandem mass spectrometry. The MS-MS spectra were distinctive for normal and reverse esters/amides by which one isomer can be unambiguously discriminated from another isomer. The spectra also show linker specific fragment ions.

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HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHIC-FLUORESCENCE METHOD FOR DETERMINATION OF GEMIFLOXACIN MESYLATE IN DRIED BLOOD SPOTS, APPLICATION TO RAT PHARMACOKINETIC STUDIES

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GFX, Pharmacokinetics, DOE, HILIC, DBS

Material and Methods

A highly selective, liquid chromatographic method has been developed and validated to quantify gemifloxacin in dried blood spots. The gemifloxacin and internal standard (ciprofloxacin) were used. Chromatographic separation was achieved on a reversed phase Zwitterionic hydrophilic interaction liquid chromatographic ZIC®HILIC-C18 (4.6mm×100mm; 5 µm) column with a mobile phase of (80:20, v/v) acetonitrile: 10mM Ammonium acetate at (pH 3.5) using isocratic elution (at flow rate 0.6mL/ min) at room temperature (27ºC). The analytes were monitored by Fluorescence detection, at 269 and 393nm for excitation and emission, respectively. The assay exhibited a linear range of 25–5000ng/mL for GFX in dried blood spots. The recovery of GFX from dried blood spots was greater than 94.0%. Stability of GFX in DBS samples were storage in a freezer at 81°C. The method was validated in terms of accuracy, precision and linearity by Design of Experimental (DOE) as per ICH guidelines.

Results

The analytes were well separated from DBS samples after Vortex and centrifugation under the present chromatographic conditions at retention times of 4.9 min and 6.2 min for IS and gemifloxacin respectively. The total run time was 7 min. The peaks were of good shape, completely resolved one from another at therapeutic concentration of gemifloxacin. Collected sample was sufficient to isolate the gemifloxacin and ciprofloxacin from DBS without any interfering endogenous peaks at 269nm and 393nm for excitation and emission, respectively, and also calculated the in terms of accuracy, precision, system suitability, linearity, selectivity, sensitivity, matrix effect, stability were calculated as per ICH guide lines. The developed HPLC method has been successfully used to quantification of the gemifloxacin concentration in dried blood samples after oral administration of gemifloxacin. DBS samples were analyzed with own calibration curve and QC samples as one batch in a single analytical run. The standard calibration curve, including blank sample and standard zero samples, was used to determine the sample concentrations in the unknown DBS samples. QC samples (at each of low, medium and high concentrations) were analyzed together with the unknown DBS samples and were allocated judiciously taking into consideration the estimated drug level through the batch, in order to detect any analytical drift. The terminal phase of gemifloxacin in the study was well characterized and the analytical assay was able to detect low concentrations at the end of the DBS concentration- time profile.

Conclusions

The developed HPLC method of analysis provided a reliable, reproducible and specific assay for GFX in dried blood samples. The method described here is sensitive enough to detect as low as 10ng/mL. The validation method allows quantification of gemifloxacin in dried blood samples for the purpose of bioequivalence study was linear from 25-5000ng/mL. The present assay method assessed extensive validation parameters as per FDA guideline. The method has shown acceptable precision, accuracy and adequate sensitivity for use in the pharmacokinetic studies and deems to be suitable for use in all laboratories equipped with sophisticated or unsophisticated instruments.

LASER DESORPTION/IONIZATION MASS SPECTROMETRY OF BACTERIAL SIGNALLING MOLECULES

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LDI-MS, quorum sensing, N-acyl homoserine lactones, inorganic particles

Material and Methods

In this work, we report the accurate and sensitive LDI MS detection of homoserine lactones, which are a class of the bacterial quorum sensing molecules. LDI MS has unique advantages such as simplified sample preparation and high throughput analysis with minimal matrix interference thereby allowing accurate and sensitive small molecule pattern analysis.

MALDI MS of N-acyl homoserine lactones using 2, 5-Dihydroxy benzoic acid was used for comparison with inorganic particles that were synthesized and characterized in house. Mass spectrometric studies were carried out on SYNAPT HDMS in MALDI mode. For the LDI-MS the inorganic particles dispersed in methanol were spotted onto the surface of target plates before the analytes were placed on the surface. Pseudomonas aeruginosa biofilm was cultured on polystyrene controlled flow models using synthetic media. The ethyl acetate extracts of the culture supernatants was used for the detection of N-acyl homoserine lactones..

Results

A cleaner background was obtained for the 1-500 m/z region with the LDI-MS using inorganic particles as compared to 2, 5-dihydroxy benzoic acid. The analytes were resolved with 10ppm mass accuracy and sub-picomole sensitivities were obtained with the standards. MSMS of the molecular ion using CID yielded signature fragmentation patterns characteristic to the N-acyl homoserine lactones. N-acyl homoserine lactones relevant to the Pseudomonas aeruginosa were detected using LDI-MS of the ethyl acetate extracts of the culture supernatants of P. aeruginosa

Conclusions

LDI-MS provides a cleaner background and hence allows unambiguous detection of small molecules (m/z <500). LDI-MS can thus be successfully applied for the study of metabolomics.

CHROMATOGRAPHIC ENANTIOSEPARATINS OF DARUNAVIR ON IMMOBILIZED POLYSACCHARIDE-BASED CHIRAL STATIONARY PHASES AND ITS COMPREHENSIVE STUDY WITH COATED POLYSACCHARIDE-BASED CHIRAL STATIONARY PHASE

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Darunavir, Chiral separation, Chiralpak IA, Chiralpak IC, Chiralpak AD-H

Material and Methods

Chiral separation of darunavir was conducted on an immobilized polysaccharide-based chiral stationary phases, Chiralpak IA and Chiralpak IC columns and studied comprehensively with coated polysaccharide based chiral stationary phase, Chiralpak AD-H column. The experiments were performed under normal-phase conditions. The solvents n-hexane, polar modifier (Iso propanol, Ethanol and 1-propanol) and DEA (0.1%) were suitable for LC/APCI/MS/MS and LC/APPI/MS/MS used as mobile phase solvents. Effect of type of modifier (Iso propanol, Ethanol and 1-propanol), percentage of modifier (15% to 60%) by varying column temperature (20ºC to 40ºC) on the retention and enantioseparation was studied. The chiral separations of darunavir with Chiralpak IA and Chiralpak IC columns were done in Iso propanol, Ethanol and 1-propanol modifier systems, where as with Chiralpak AD-H column were done in Iso propanol and Ethanol systems. Better resolution was achieved with all three chiral columns in all polar modifier systems.

Results

Immobilized polysaccharide-based chiral stationary phases, which contains amylose tris (3, 5-dimethylphenylcarbamate), Chiralpak IA; cellulose tris (3, 5-dichloro phenylcarbamate), Chiralpak IC were the first in a series of polysaccharide-derived CSPs from Daicel compatible with all ranges of organic miscible solvents. In this work, several chiral columns Chiralpak IA and Chiralpak IC with different mobile phases were used and studied comprehensively with coated polysaccharide based chiral stationary phase, Chiralpak AD-H column in the enantioseparations of darunavir.

Chiralpak IA column
Effect of type of polar modifier
When ethanol was used, large α value were obtained. The highest α value was 1.353. However, the α value decreased gradually when the alcohol modifier was changed in the order of 1-propanol > IPA.
Effect of the content of polar modifier
At a particular temperature of the column, as the content of polar modifier increases the retention times as well as resolutions of darunavir decreased.
Effect of temperature of the column
At a particular content of polar modifier, as the temperature increases the retention as well as resolutions of darunavir decreased.

Chiralpak IC column
Effect of type of polar modifier
When ethanol was used, large α value were obtained. The highest α value was 1.512. However, the α value decreased gradually when the alcohol modifier was changed in the order of 1-propanol > IPA.
Effect of the content of polar modifier
At a particular temperature of the column, as the content of polar modifier increases the retention times as well as resolutions of darunavir decreased.
Effect of temperature of the column
At a particular content of polar modifier, as the temperature increases the retention as well as resolutions of darunavir decreased.

Chiralpak AD-H column
The reasonable retention times with better resolutions and large enantio separations achieved by using Chiralpak AD-H column.

Conclusions

Effect of type of modifier, percentage of modifier by varying column temperature on the retention and enantioseparation was studied. Better resolution was achieved with all three chiral columns in all polar modifier systems. The conditions which were used in better resolutions in all polar modifier systems are useful in LC/APCI/MS/MS and LC/APPI/MS/MS to apply the method in biological samples of darunavir.

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HIGH THROUGHPUT MALDI MS BASED QUANTITATION OF METABOLITES IMPLICATED IN DISEASES

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MALDI MS, quantitation, metabolites, disease, biofluids

Material and Methods

MALDI MS of metabolites like S-adenosyl methionine, S-adenosyl homocysteine, etc. were studied using organic matrices such as a-cyano hydroxy cinnamic acid and dihydroxy benzoic acid. Mass spectrometric studies on standards (from Sigma) were carried out on a SYNAPT HDMS MALDI-TOF MS instrument which is equipped with a 355nm Nd:YAG laser, 8kDa quad and a sample target plate with 96 wells. The instrument was operated in a V-mode for increased sensitivity. The samples were premixed with internal standard and spotted on the plate. Quantitation was done in automated mode.

Results

With appropriate choice of MALDI matrices and proper sample preparation, the interference from the inherent matrix peaks was overcome. Tandem MS based analysis further confirmed the nature of the analyte which correlates well with available literature. With the use of an internal standard, reproducible quantitation was obtained with good linearity (R value > 0.9).

Conclusions

MALDI MS based detection (MS and tandem MS) and quantitation of amino acids is possible and can be further used as a high throughput method for screening biological samples. The method might prove useful in an application where levels of different metabolites determine the fate of the cell.

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LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY OF PRE-IONIZED GIRARD P DERIVATIVES FOR QUANTIFYING ESTRONE AND ITS METABOLITES IN POSTMENOPAUSAL WOMEN'S SERUM

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LC/SRM/MS, Girard P, Estrones, Qunatification, Stable Isotope Dilution

Material and Methods

An ultrasensitive stable isotope dilution liquid chromatography/selected reaction monitoring/mass spectrometry (LC/SRM/MS) assay has been developed for serum estrone, 16α-hydroxyestrone, 4-methoxyestrone, and 2- methoxyestrone. The enhanced sensitivity was obtained by the use of Girard P (GP) pre-ionized derivatives coupled with microflow LC.

Results

The limit of detection for each estrogen using 0.5 mL of serum was 0.156 pg/mL and linear standard curves were obtained up to 20 pg/mL. Serum samples from 20 postmenopausal women (10 lifetime non-smokers and 10 current smokers) were analyzed using this new assay. Mean serum concentrations of estrone and 2-methoxyestrone were 14.0 pg/mL (±1.56 pg/mL) and 3.30 pg/mL (±1.00 pg/mL), respectively, for the 20 subjects enrolled in the study. The mean estrone concentration determined by our ultrasensitive and highly specific assay was significantly lower than that reported for the control groups in most previous breast cancer studies of postmenopausal women. In addition (and contrary to many reports) serum 16α-hydroxyestrone was not detected in any of the subjects, and 4-methoxyestrone was detected in only one of the subjects. Furthermore, there were no significant differences in the mean serum concentrations of estrone and 2-methoxyestrone or the ratio of serum 2- methoxyestrone to estrone between the non-smoking and smoking groups. Interestingly, the one subject with measurable serum 4-methoxyestrone (2.3 pg/mL) had the lowest estrone and 2 -methoxyestrone concentrations.

Conclusions

Using this assay it will now be possible to obtain definitive information on the levels of serum estrone, 4-methoxyestrone, and 2-methoxyestrone in studies of cancer risk using small serum volumes available from previous epidemiology studies.