17.1 Chimeric Manganese-Super Oxide Dismutase-2 (Mn-SOD-2) Secreted from a Human Liposarcoma with Specific and Selective Cytotoxic Activity on Tumor Cells Expressing Estrogen Receptors (RE)

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A human liposarcoma derived cell line (LSA), growing in a chemical defined medium, releases in this conditioned medium (LSA-CM), proteins for an outocrine growth. From 40 liters of LSA-CM we have isolated, purified and sequenced a mutated form of Manganese Super Oxide Dismutase-2 displaying a specific and selective cytotoxic activity on tumor cells expressing Estrogen Receptors (RE). The recombinant Mn-SOD-2, expressed in E. coli, presents the same characteristics of pure extractive molecule. The Mn-SOD-2 can be secreted, as from LSA cells, and can be internalized by tumor cells expressing ER. TEM observations by using immunocytochemistry, confirm an intense secretion of this molecule that occurs in Golgi apparatus and in the large amount of secretion vesicles surrounding the plasmatic membrane. Mass Spectrometric analysis of rec-Mn-SOD-2 shows a M.W. of 26666±0.9, about 1800 Da higher than the wild type protein, suggesting the occurrence of an additional 15–20 aminoaacids peptide. Particularly interesting was the discovery that the rec-Mn-SOD-2 contains a moiety of the Estrogen Receptors (16349 fmol/mg protein), and confirmed by Immunoblotting, by using two different antibodies raised against the α and β estrogen receptors.

The in vitro Pharmacology analysis of rec-Mn-SOD-2 confirm the tumor suppressive activity on many tumor cell lines expressing ER and low levels of Catalase enzyme. Moreover, when rec-Mn-SOD-2 (1.5 μM) is combined with low amount of Cisplatin (25 μM), a synergic effect on the cytotoxic activity was observed, producing the necrosis of tumor cells in a few hrs. We retain that per se the rec-Mn-SOD-2 is toxic for tumor cells, but when it is combined with the Cisplatin, this drug turning off completely the gene for Catalase, amplifies the necrotic effect of rec-Mn-SOD-2. On these basis a mechanism for the cytotoxicity effect of rec-Mn-SOD-2 might be suggested: rec-Mn-SOD-2 can be internalised into tumour cells due to the presence of the RE moiety and transforms all the free radicals into hydrogen peroxide. This reaction is dose-dependent, and only a normal levels of Catalase might be effective to neutralize the cytotoxicity of hydrogen peroxide. Since many tumors express low levels of Catalase (20 to 40 fold lower than the normal cells), the necrosis produced by rec-Mn-SOD-2 might be ascribed to the excess of hydrogen peroxide. This hypothesis is also confirmed by the observation that the addition of low amount (25 μM) of Cisplatin, which is able to turn off completely the gene for Catalase, dramatically increases the citotoxic effect of rec-SOD-2. L.M. and TEM immunocytochemistry on tumor cells, after rec-Mn-SOD-2 adding to culture medium, displays internalisation of rec-Mn-SOD-2 in cytoplasm, mitochondria and nucleus and a clear necrotic feature of these cells. These results were confirmed by in vivo experiments. Rec-Mn-SOD-2 was daily injected (2 μg/s.c.) into balb-C-IR-ili affected by mammary adenocarcinoma, inducing the necrosis of the tumor mass, as monitored by NMR and histological examination. Moreover, the treated animals did not develop lung metastasis. At time Mn-SOD-2 is under R&D from FIDIA-Pharmaceutical Co. to explore possible therapeutic of this antitumoral agent.

17.2 Investigation on the Mechanism of Growth Inhibition and Differentiation of Tanshinone IIA on Cervical Cancer Cell Line by Functional Proteomics

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Salvia miltiorrhiza Bunge has been commonly used in traditional Oriental herbal medicine to treat either inflammatory disease such as edema, arthritis and cardiovascular disease or neoplasm. However, as a part of a search for new antitumor agents from Chinese medicinal plants, the roots of Salvia miltiorrhiza Bung (Labiatae), collected in China, were investigated. More than 40 compounds have been isolated from this plant so far. Diterpene tanshinone IIA exhibited cytotoxic activities against HeLa human cervical cancer with IC50 values of 2.5 μM. Recent studies showed that tanshinone IIA possesses cytotoxic activity against many kinds of human carcinoma cell lines; induce differentiation and apoptosis and inhibiting invasion and metastasis of cancer cells. Its mechanism is not yet clear. To discriminate the cytotoxic effect of diterpene on human cervical cell lines, two-dimensional electrophoresis and matrix-assisted laser desorption/ionization time-of-flight were performed choose to evaluate their biological function of cytotoxic activity.
Targeted Proteomics for the Detection of Doping Agents

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Peptide hormones are a class of doping agents prohibited by the International Olympic Committee. However, for some of them, such as recombinant human Growth Hormone, rhGH, there is currently no approved method of detection. Measurement of serum GH itself is of limited use for the following reasons: i) recombinant and endogenous GH have identical amino acid sequences with the exception of some allelic differences, ii) the secretion of the endogenous hormone is a pulsative event and iii) the half-life of GH is too short (about 10 minutes) to allow an efficient control. Therefore biomarkers of GH action are being investigated as potential tests for GH abuse. Insulin-like Growth Factor 1 (IGF-1) and Insulin-like Growth Factor Binding Protein-3 (IGFBP-3) appears to be suitable markers as they are under GH control and present a longer half-life in blood owing to bound circulating forms.

Our objective is to develop a test for the identification and quantification of GH administration based on these two markers using the tools of proteomic. This approach could also be extended to identify and quantify treatments with other peptide hormones simultaneously.

Plasma proteins were separated by one dimensional gel electrophoresis and the bands supposedly containing the IGF-1 and IGFBP-3 proteins were excised and trypsinised. The protein content of these bands was identified by MS/MS ion search (Mascot algorithm, Matrix Science) from data collected by nanoLC-MS/MS on an ion trap spectrometer (Esquire HCT, Bruker Daltonics). In another experimental approach, IGF-1 in a complex mix was treated and analysed according to the cICAT strategy (Applied Biosystems).

Our poster presents the results of the optimization for the identification and quantification of these biomarkers.
17.5 Proteomic Analysis of the Therapeutic Effects of Fufangdanshenfang on Atherosclerosis

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Chinese Traditional Medicine can affect signal molecular groups through chemical molecular groups to restore balance of signal molecular networks to cure diseases. The technical system composed by proteomics, gene chip, RT-PCR and molecular blotting was established, and this system could combine signal molecular groups to chemical molecular groups. The effects of Fufangdanshenfang, effective ingredients and combined ingredients to cure atherosclerosis were investigated. The goal of this research was to discover the ingredients and pathways of Fufangdanshenfang to cure atherosclerosis.

Proteomic protocol involved of 2-DE, image analysis and spectrometry detection was set up. Compared with coomassie blue and silver staining, DIGE has high detection sensitivity, high accuracy and good reduplicate. Silver staining was fit to ascertain protein spot in 2-DE gels, coomassie blue was fit to detect high content protein, DIGE was fit to establish difference protein expression map. The increased production of NO, PAI-1, and the decreased production of growth factor such as VEGF, bFGF, EGF, TGF-β1, and adhesion molecules such as ICAM1, VCAM1, E-selectin might be the mechanism of ginsenoside Rg1 to protect HUVECs from TNF-α induced injury. The expression of G-protein coupled receptor kinase, PKC and CaMKK were decreased by Fufangdanshenfang, which farther decreased matrix metalloproteinase production, and oxidation and inflammation related protein were also decreased, while cell cycle related protein p21, p53 were increased by Fufangdanshenfang in TNFα treated VSMC. The levels of fibronogen and Granzyme C were elevated and the time of coagulate blood was prolonged in Fufangdanshenfang treated plasma. The changes of protein expression map, gene expression, and the time of coagulate blood were similar between Fufangdanshenfang and its combined ingredients, and the changes of protein expression map was different between Fufangdanshenfang, Ginsenoside Rg1 and TanshinonelA, thus it could be seen that the effects of combined ingredients were similar to the effects of Fufangdanshenfang.

The main factors which play important roles in the mechanism in the pathogenesis and development of atherosclerosis could be summarized as the following: dysfunction of vein endothelial cells, proliferation and migration of VSMC, weaken of fibrinolysis, strengthen of oxidation and inflammation. The effects of Fufangdanshenfang on atherosclerosis included that: protection of vein endothelial cells, inhibition of proliferation and migration of VSMC, regulation of fibrinolysis and coagulate, weaken of oxidation and inflammation.
17.8
Rapid Finding of Active Proteins from Chinese Crops and Traditional Medicines with High Throughput Screening and Multidimensional LC and MS Technology


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Previous investigations of biologically active natural products have focused on finding small molecule compounds. With the development of protein purification technology, especially proteomics technology such as high throughput screening and multidimensional LC and bio-MS, it becomes possible to find more active proteins. In ethnopharmacological and pharmaecochemical investigations, we found that the unheated aqueous extracts of some Chinese crops and traditional medicine plants could be used as herbal drugs and pesticides. Supported with NSFC and national basic research priorities programme (2001CCA01100) of China, we established several high-throughput screening methods to determine active proteins, and rapidly found some antimicrobial proteins and thrombin inhibitor and proteins with fibrinolysis activity from crops, insects, microbial organisms and traditional medicine plants by using multidimensional LC and MS technology. In some populations, we identified active proteomes composed with proteins which having the same functions but different molecular weights and different amino acid sequences. We hope the knowledge of distribution of active proteins in Chinese crops and traditional medicines and the knowledge of sequence structure of active function proteomes being helpful in protein drugs and protein pesticides development and in understanding the relationship of structure and function in the active proteomes.

17.9
Proteomics Applications in the Drug Discovery Process

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At Novartis the proteomics activities are divided in two areas: one is the identification of proteins correlating with disease states or drug efficacy. The other encompasses identification and characterization of proteins to gain insight into biological mechanisms. For the first area the approach based on separation of proteins followed by formation of peptides by enzymatic digestion is preferred as it conserves a link between protein and resulting peptides. Examples of in-depth, high through-put identifications applied to global proteome- and membrane proteome mapping of the bacterial pathogens H. Influenza and S. Aureus will be presented. Identification however is often not sufficient to understand a proteome, as there are important mechanisms for the regulation of a biochemical pathway that can be investigated only by characterizing the proteins involved. These include mechanisms such as post-translational proteolytic processing and post-translational modifications, and investigations into the composition of molecular complexes. The second part of the presentation will report on the use of mass spectrometry, affinity purifications, enzymatic digestions, and HPLC separations, for the characterization of post-translational modifications of a specific group of proteins, the histones, and the analysis of protein complexes containing histone deacetylases.

17.10
Protein Digest Analysis on an AP-MALDI/TOF System

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Introduction: Compared with vacuum MALDI/TOF, AP-MALDI/TOF advantages include the decoupling of the ionization conditions (e.g., laser power) and parameters (plate flatness) from mass analysis, leading to better mass accuracy. However, early AP-MALDI sources exhibited relatively low sensitivity and were prone to matrix adducts formation. This work demonstrates the enhanced performance of an AP-MALDI/TOF system for protein digest analysis with high sensitivity and mass measurement accuracy.

Methods: Protein digests and peptide standards were analyzed using an AP-MALDI source coupled with an orthogonal acceleration TOF system. Alpha-CHCA was used as the matrix and a nitrogen laser at 337 nm (10 Hz) was used for the ionization. Mass peaks from matrix-related ions, either alone or in combination with a mass peak from a single peptide internal standard, were used for correction of mass assignments.

Results: Absolute detection limits of 100–200 attomoles of peptides from protein digests was achieved using a normal sample spot size of ~2.0–3.0 mm. The sub-femtomole sensitivity is attributed to the used of heated drying gas in AP-MALDI as a means of reducing background and enhancing ionization. For sample amounts of one femtomole or greater, low ppm (<3–5 ppm) accuracy was achieved by using either real-time mass correction or by recalibration of the mass assignment during data processing. Spectra of sample amounts lower than one femtomole were subject to baseline noise interferences, leading to lesser but still acceptable mass accuracy <15 ppm. Mixtures of two protein digests at amount ratios of 20:1 were correctly identified by database searching.
Study on Serum Proteome of Rat Endotoxemia Treated by Figwort Root

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1.11

Xuan Shen (the traditional Chinese medicines of name, figwort root, FR) used in this study are roots of Scrophularia ningpoensis Hemsl, which contain a diverse group of bioactive natural products and are commonly used in treating infection, fever, constipation, Buerger disease, hyperplasia of mammary glands, pharyngitis, neuritis, and laryngitis. Objective: To study the serum proteome of rat endotoxemia treated by figwort root (FR).

Methods: The differences of serum proteome among rats treated with lipopolysaccharide (LPS), FR, LPS + FR and saline respectively were analyzed by two-dimensional electrophoresis (2DE) assay. Results: The volumes of sixteen serum proteins in LPS induced-endotoxemia group were greatly changed compared with that of the control group. Among which, the volume of α1-acid glycoprotein, Ba1 was significantly decreased, the volumes of α-galactosidase, α-lactalbumin, α2-macroglobulin, α1-antitrypsin, α1-antichymotrypsin, Apolipoprotein E precursor, albumin, preprohaptoglobin. Our study demonstrated that we can use proteomic techniques to study the molecular mechanisms of diseases treated by functional Chinese herbs and the combination of different herbs is necessary for the treatment of endotoxemia, as FR could not regulated all the changed proteins induced by LPS.

High-throughput Screening System for Evaluation of the Interactions Between Small Molecule Drugs and Proteins Expressed from cDNA Gateway Clones Using Size-exclusion Chromatography Coupled with Mass Spectrometry


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Full-length cDNA clones from cDNA projects, such as the Full-length long Japan (FLJ) collection of sequenced human cDNAs, have been available on the Gateway universal cloning platform (Invitrogen). To discover novel pharmacological targets with these cDNA collections, a high-throughput screening system using size-exclusion chromatography (SEC) coupled with mass spectrometry (MS) was established to analyze the interactions between small molecule drugs and proteins expressed from human full-length cDNA Gateway clones. The SEC-MS screening system is composed of the following three steps; (1) the protein purification step in which N-terminal His-tagged proteins were expressed in E. coli from full-length cDNA clones on the Gateway destination vector pDEST17 and purified with Ni-NTA spin-columns by BioRobot 8000 (Qiagen), (2) the SEC separation step in which protein fractions were separated from the mixtures of 8- or 16-multiplexed drugs and a protein through SEC using 384-well UniFilter spin-columns (Whatman) packed with Bio-Gel P-6 (Bio-Rad) or a TSK super SW2000 micro LC column (Toso), and (3) the MS analysis step in which drugs eluted with the protein fractions from SEC columns were separated from proteins and identified by mass spectrometry using an ion-trap mass spectrometer Finnigan LCQ deca XP (Thermo Electron) equipped with an ESI probe. The SEC-MS screening system enabled us to evaluate the interactions between one protein (about 17 nmoles) and 800 compounds per two hours. MS Raw files were batch-processed by in-house software to calculate the ion abundance of each drug compound in each protein fraction. This software could typically extract 4,836 ion abundance data from 312 MS raw files for the analysis of 11 proteins and 1 reference versus 26 multiplex (about 400 drugs) in one plate of 384-well spin-columns per 10 hours. For positive control pairs (cyclophilin A-cyclosporin A, FKBP12-FK506) and for other novel hit pairs, the ion abundances of the corresponding drug compounds in the protein fractions specifically increased in a concentration-dependent manner. This SEC-MS screening system without labeling or immobilization of either proteins or drugs is suitable for the target discovery using cDNA collections in the post-genomic era.
Screening and Identification of Potential Targets for Anti-HBV Drugs in HepG2.2.15 Cells

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Target discovery in virology has been limited to the few open-reading frames encoded by viral genomes. However, several recent examples show that inhibiting host-cell proteins can prevent viral infection. A systematic approach to find these potential cellular antiviral targets is host gene expression analysis using DNA microarrays. Hepatitis B virus (HBV) infection is a major health concern around the world. The aim of this study is to screen and identify new potential cellular anti-HBV targets.

By using our in-house virus-infection-related gene oligonucleotide microarrays, we screened and identified the potential cellular anti-HBV targets in HepG2.2.15 cells. In conclusion, by using our in-house DNA microarray, we screened the expression of HBV DNA, HBsAg, HBeAg in HepG2.2.15 cell culture. The results suggest that both fibronectin ASODN and ASGPR ASODN inhibited the expression of potential targets would inhibit the replication and expression of HBV.

The results of microarrays showed that Fibronectin, ASGPR, Aromatise, SPR1, p58IPK, RPN1/EVIL, and endoglin were up-regulated by HBV but down-regulated by anti-HBV drugs, and that ICSBP was down-regulated by HBV but up-regulated by anti-HBV drugs. The expressions of 5 of these 8 genes were confirmed by semi-quantitative RT-PCR, including Fibronectin, ASGPR, Aromatise, SPR1, ICSBP. Fibronectin protein was confirmed to be upregulated by HBV in HepG2.2.15 cells. And also, it was confirmed to be down-regulated by anti-HBV drugs in HepG2.2.15 cells. The antisense oligodeoxynucleotides targeted fibronectin and ASGPR were designed and synthesized and then transfected into HepG2.2.15 cells mediated by lipofectin reagent. The antisense oligodeoxynucleotides which could inhibit the expression of fibronectin or ASGPR were identified. They were transfected into HepG2.2.15 cells and then HBV DNA, HBsAg, HBeAg were examined. The results suggest that both fibronectin ASODN and ASGPR ASODN inhibited the expression of HBV DNA, HBsAg, HBeAg in HepG2.2.15 cell cultures. In conclusion, by using our in-house DNA microarray, we screened and identified the potential cellular anti-HBV targets in HepG2.2.15 cells.

The results shed new light on the pathogenesis of HBV. ASGPR and fibronectin might be the potential cellular anti-HBV targets.
Effect of Calcium Channel Blocker in Experimentally Induced Diabetic Nephropathy in Rats

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Introduction & Aim: Diabetic nephropathy (DNP) is considered a CRD (Chronic Renal Disease); it is a major cause of illness and premature death in people with DM. Furthermore, it is considered the single most important cause of end stage renal disease in the western world and accounts for more than a quarter of all end stage renal diseases. The present study was designed to illustrate the role of CCBs (amlodipine and diltiazem) in prevention and treatment of DNP in rats.

Materials & Methods: Eighty male albino rats weighing (130–180 gm) were used in this study. These animals were subdivided into five equal groups. Insulinopenic diabetes was induced by STZ, two weeks later, 30 minutes of complete ischaemia was induced in the left kidney to induce diabetic nephropathy then treatment was started for 12 weeks. At the end of experiment urine samples and blood samples were taken for biochemical analysis and kidneys were taken after scarification for histopathological evaluation.

Results: Combination of renal ischaemia with DM produced a significant increase in rat weight, rat kidney weight, BUN (Blood Urea Nitrogen) level, K/B (Kidney/Body weight) ratio, random blood glucose, 24 hrs urine proteins, and 24 hrs urine volumes and creatinine clearance. Treatment with diltiazem or amlodipine significantly lowered elevated SBP and elevated 24 hrs urine volumes. Furthermore, treatment with captopril produced a highly significant lowering of elevated SBP and elevated serum creatinine; and a significant reduction in elevated K/B ratio and proteinuria. Light microscopic examination of diabetic kidneys revealed glomerulopathy characterized by thickening of the glomerular basement membrane, mesangial matrix expansion, arteriolar hyalinosis and large proteinaceous deposits occluding some capillary loops and hyaline droplets within the glomeruli. Moreover, examination of kidneys of ischaemic animals by light microscope revealed focal tubular necrosis at multiple points along the nephron, interstitial edema and accumulation of leucocytes within dilated vasa recta.

Conclusion: It can be concluded that, renal ischaemia hasten the progression of DNP, diltiazem and amlodipine have a tendency to reverse of changed parameters toward normal values except biochemical parameters, generally speaking, diltiazem is better than amlodipine in reversing biochemical and histopathological changes produced by DNP, and captopril reversed most of changed parameters except histopathological changes.

Recommendations: Based on the obtained results from the present study, one can recommend that; 1) Diltiazem and amlodipine have a nephroprotective effect in DNP, therefore, they should be used in diabetic patients to protect and/or slow progression of DNP. 2) Captopril might be considered the first therapy for DNP. Moreover, combination of captopril with CCBs could be a more effective tool got protection and/or slow progression of DNP.