"Proteomic Technologies"

TECHNIQUES & STRATEGIES IN MOLECULAR MEDICINE
12th December 2007

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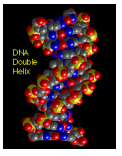
UCD Conway Institute
of Biomolecular and Biomedical Research

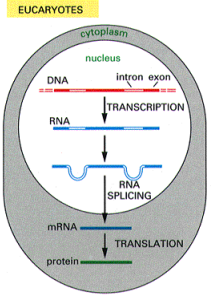

Proteomic Technologies

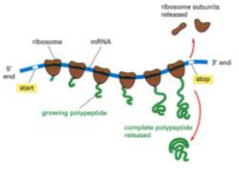
- Background
  - What is a protein?
  - What is proteomics?
  - In general, how is it done?
  - Is it worth doing?
- Biomedical applications of proteomics
  - 1-D and validation - Neurocalcin delta binding proteins
  - 1-D and validation - Topoisomerase binding proteins
  - Sample preparation, validation, new discoveries both clinical and biological - Pancreatic cancer
  - Biomarker discovery: sample preparation, validation - Prostate cancer
  - 2-D; isotope labelling, quantitative proteomics - Drug toxicity in the liver
- Conclusions
- *Conway Institute Proteome Research Centre*

DNA structure



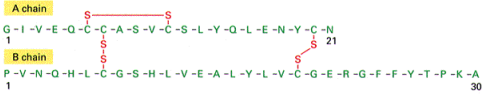
From DNA to protein





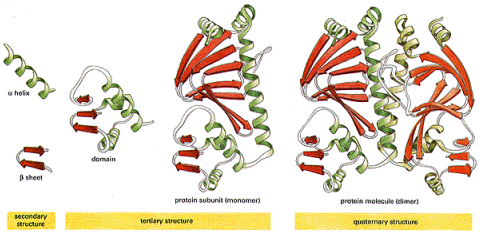
Numbers .....

Protein primary structure



A	= Ala = alanine	G	= Gly = glycine	M	= Met = methionine	S	= Ser = serine
C	= Cys = cysteine	H	= His = histidine	N	= Asn = asparagine	T	= Thr = threonine
D	= Asp = aspartic acid	I	= Ile = isoleucine	P	= Pro = proline	V	= Val = valine
E	= Glu = glutamic acid	K	= Lys = lysine	Q	= Gln = glutamine	W	= Trp = tryptophan
F	= Phe = phenylalanine	L	= Leu = leucine	R	= Arg = arginine	Y	= Tyr = tyrosine

Protein structure



What is a Proteome?

Oxford English Dictionary: [< *prote-* (in PROTEIN *n.*) + *-ome* (in GENOME *n.*).]

The entire complement of proteins that is (or can be) expressed by a cell, tissue, or organism.

1995 V. C. WASINGER et al. in *Electrophoresis* **16** 1090. **2000** *New Scientist* 15 July 67 (*advt.*) We have the unique ability to select disease relevant targets from the proteome.

(1994 – Marc Wilkins – PhD student – Siena)

What is a "Biomarker"?

WebSearch: the webpages from Ireland: Results 1 - 10 of about **591,000** for **Biomarker** [definition]. (0.14 seconds)

"A specific physical trait used to measure or indicate the effects or progress of a disease or condition:

Biomarkers of aging include thinning of the hair and diminished elasticity of the skin."

Oxford English Dictionary: [< BIO- + MARKER *n.*]

A substance used as an indicator of the presence of material of biological origin, of a specific organism, or a physiological condition or process; *spec.* a diagnostic indicator of (predisposition to) a medical condition.

What is Proteomics?

Static - identification of all the proteins produced from a genome

identification
characterisation

Dynamic - analysis of (up to) several thousand proteins at a time

Numbers again - Human proteome: 30,000 genes? – 250,000 proteins?

What is Proteomics?

Measurement of protein expression: *expression proteomics*
(1-D, 2-DE, LC, ICAT, iTRAQ etc..)
Measurement of protein composition of cellular organelles (spliceosome, phagosome, speckles etc.): *cell map proteomics*
Analysis of post-translational modifications; of protein:protein interactions; of protein:drug interactions:

Ultimately these will lead to new diagnostic protein biomarkers, new drug targets

And…. the determination of protein function and a more detailed understanding of biological systems

A general Proteomics workflow

- Sample acquisition and preparation
  - Biological fluids
  - Tissues – biopsies (disease vs normal)
  - Cells
  - Sub-cellular components (membranes/mitochondria/nuclei etc…)
- Protein/peptide separation
- Protein/peptide detection
- Protein/peptide identification
  - *Sounds easy!*

Validation and functional analysis

Proteomics …. is not easy

- Proteins have diverse physico-chemical properties
- There are large numbers of them
- They are very dynamic
- Methods for their analysis are complex and still evolving (rapidly)
- Interested in *protein function ….*

Protein function

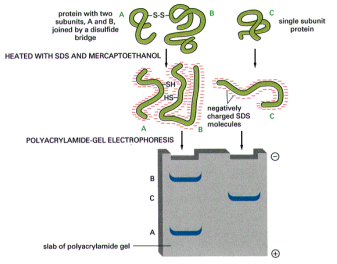
- How is the function (activity) of a protein regulated?
  - Expression
  - Folding
  - Processing
  - Post-translational modifications
  - Interaction with other proteins
  - Sub-cellular localisation (cf. NFkB)
  - Degradation
  - Others (cf. StAR)
- How is the function of a protein determined?

With (great) difficulty ….

So - why do Proteomics?

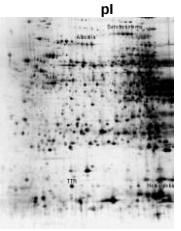
- mRNA expression analysis – (transcript profiling) - does not always reflect the expression level of proteins
- Biological samples such as CSF, serum, urine etc. are often not suitable for mRNA expression analysis
- It focuses on gene products - the active agents in cells/tissues/organisms
- Supports the analysis of the modification of proteins that are not apparent from DNA sequence i.e. post-translational modifications

Protein separation by SDS-PAGE



Protein separation by 2-DE

- Developed in mid/late 1970s
- Separates by pI and Mr – up to 10,000 spots
- Protein detection – several stains including fluorescent dyes
- But, protein identification….



High resolution, sensitivity, and reproducibility make this technique a powerful analytical tool which could potentially find use in a wide range of investigations.

High Resolution Two-Dimensional Electrophoresis of Proteins*

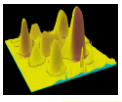
(Received for publication, September 5, 1974)

Patrick H. O'Farrell‡

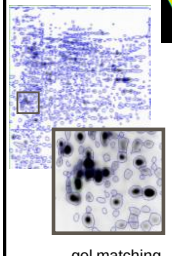
From the Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, Colorado 80302

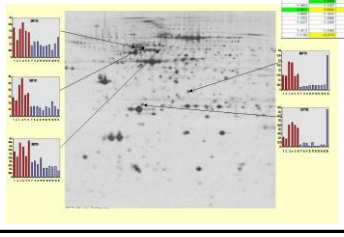
Gel image analysis *(the bottleneck)*

filtering, spot detection



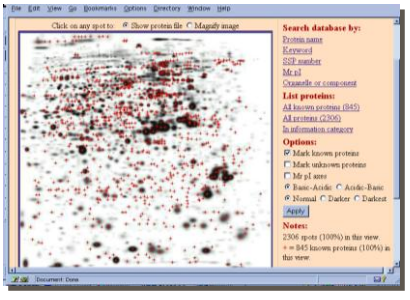
quantitative analysis of detected proteins





gel matching

2-DE maps and databases



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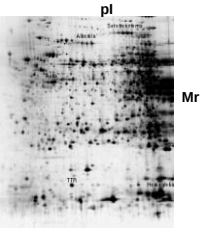

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 250, No. 10, Issue of May 25, pp. 4007-4021, 1975
Printed in U.S.A.

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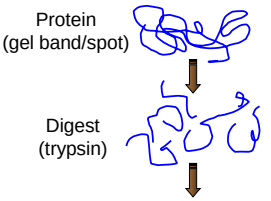
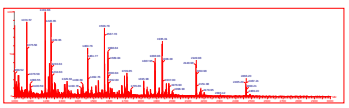
Peptide mass fingerprinting



Why trypsin?

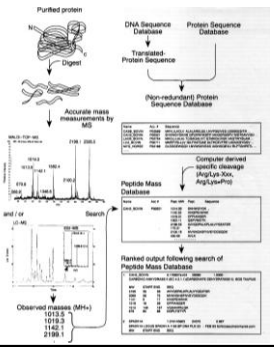
(lys/arg charge + frequency of cleavage)

MALDI
MS spectrum



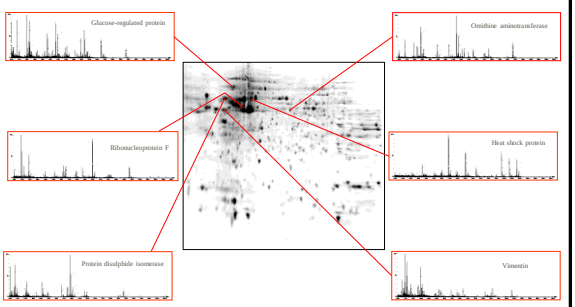
Peptide mass fingerprinting

In lab



In silico

Protein identification



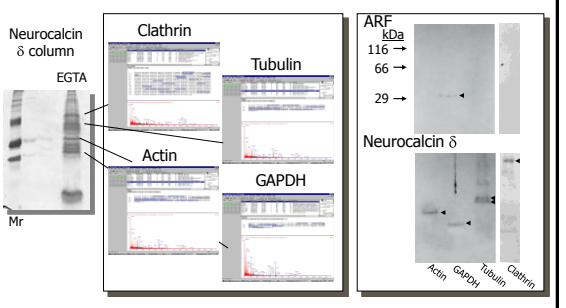
Can proteomics deliver?

- Benchmark Pharma example – Novartis (van Oostrum)
  - Bengamide – inhibitor of tumour growth
  - Unknown mode of action
  - Transcript profiling reveals no transcriptional response
  - 2-DE protein expression profiling (15-20,000 protein features)
  - Novel spot change in 14-3-3 protein
  - Detailed (painstaking and slow) analysis and validation leads to identification of protein modification and target for bengamide
  - Methionine aminopeptidases (24 including novel enzymes)
  - New compounds (1 in clinical trial – others to follow)
- Benchmark biomedical example – many emerging (phagosome proteome)

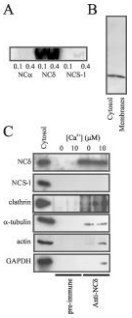
Answer – yes

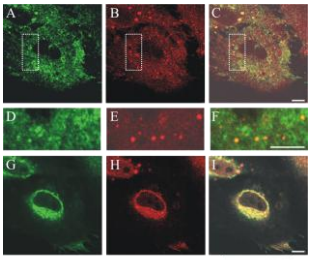
Can you believe everything you read?

Neurocalcin δ binding proteins



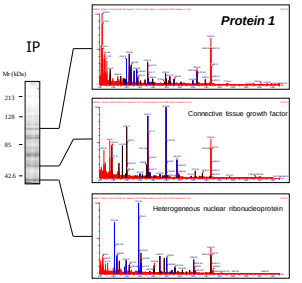
NCδ protein interactions





Ivings et al. Biochem. J. 363, 599-608 (2002)

Topoisomerase binding proteins



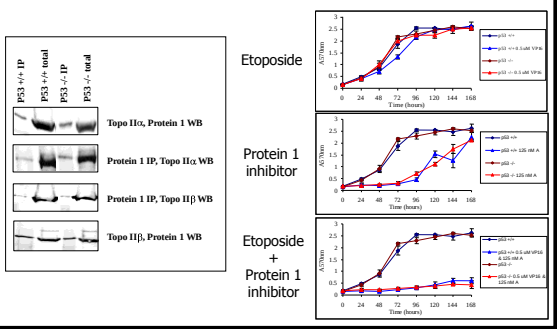
HCT 116 cells were exposed to 50μM VP16 for 24 hrs.

Proteins extracted and immuno-precipitated with antihuman topoisomerase-γ antibody.

Proteins were separated under reducing conditions by SDS-PAGE.

Individual bands were excised from the gel and subjected to tryptic digestion.

Functional interaction



Colon tumour xenografts

Mice treated when tumour volume = 140 mm³

Etoposide administered intraperitoneally (i.p.) at doses of 20 and 30mg/kg weekly.

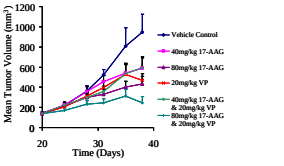
17-AAG administered i.p at doses of 40 and 80 mg/kg 3 times per week.

Experiment ended when control xenografts reached 1000mm³

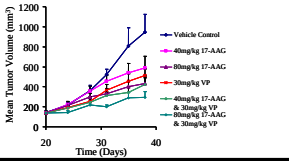
17-AAG
17-allylamino-17-demethoxygeldanamycin

VP
Etoposide

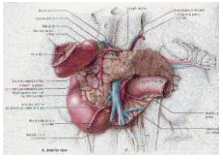
40 or 80mg/kg 17-AAG and 20mg/kg etoposide



40 or 80mg/kg 17-AAG and 30mg/kg etoposide

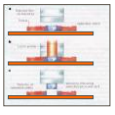


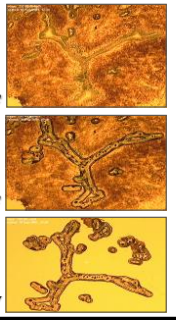
Pancreatic ductal adenocarcinoma



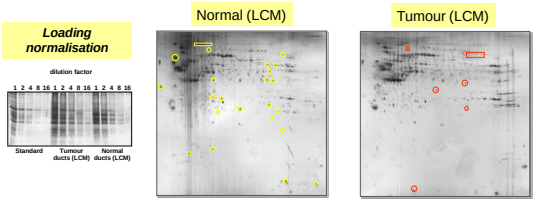
- Accounts for 90% of all pancreatic cancer
- Pancreatic cancer is the 4th most common cause of cancer-related death in the western world
- Responsible for 7000 deaths per year in the UK (40,000 in Europe)
- Majority of patients present with advanced disease
- 10-15% of these are suitable for potential curative surgery.
- 5 year survival range between 10-24%
- Arises from ductal cells of pancreas

Laser capture microdissection



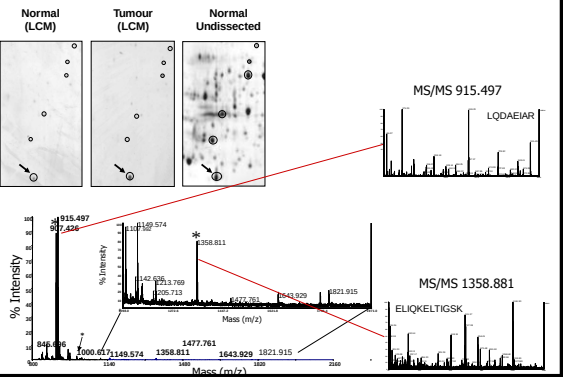


Matched comparisons

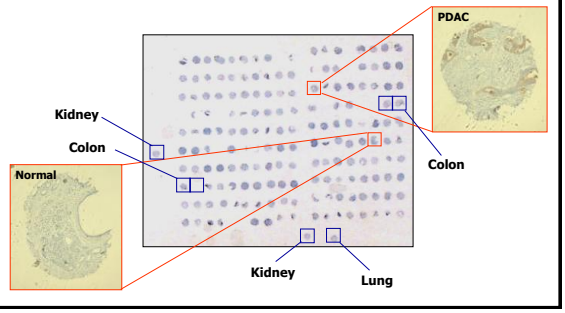


Several of the differentially expressed proteins have been identified – validation against tissue arrays is in progress

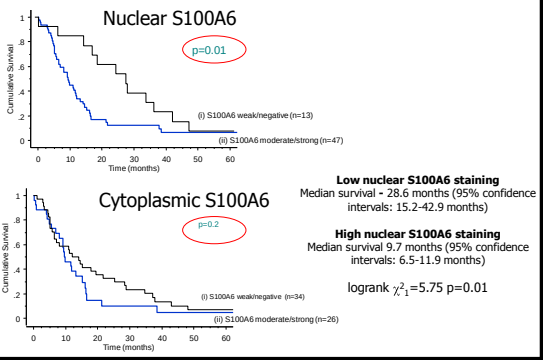
Identification of protein spot 1 – S100a



Validation: pancreatic tissue arrays



Validation - linkage



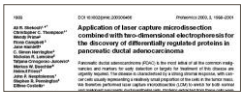
Low nuclear S100A6 staining
Median survival - 28.6 months (95% confidence intervals: 15.2-42.9 months)

High nuclear S100A6 staining
Median survival 9.7 months (95% confidence intervals: 6.5-11.9 months)

logrank χ^2_1=5.75 p=0.01

Pancreatic ductal adenocarcinoma Conclusions

- Discovery

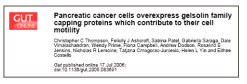


Application of laser capture microdissection combined with two-dimensional electrophoresis for the discovery of differentially regulated proteins in pancreatic ductal adenocarcinoma

- Linkage

Vimalachandran D, Greenhalf W, Thompson C, *et al.* High nuclear S100A6 (Calcyclin) is significantly associated with poor survival in pancreatic cancer patients *Cancer Res* 2005;**65**:3218-25.

- Functional validation



Pancreatic cancer cells overexpress gelsolin family capping proteins which contribute to their cell motility

Prostate Cancer

Serum Biomarker *Discovery*

Proteomic workflow

Sample Aquisition *(SOP)*

Fractionation (Removal of abundant proteins)

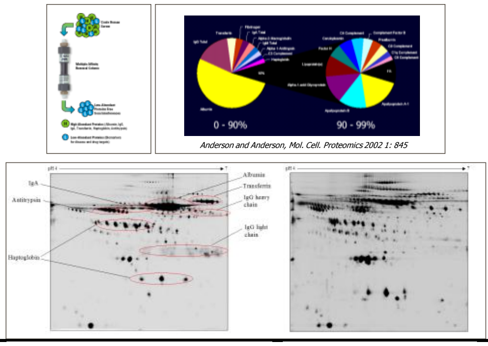
Protein separation by 2-DE (DIGE)

Gel image analysis *Scanning –Typhoon scanner & Analyisis - Progenesis*

Protein identification by MALDI-Tof/Tof analysis - 4700

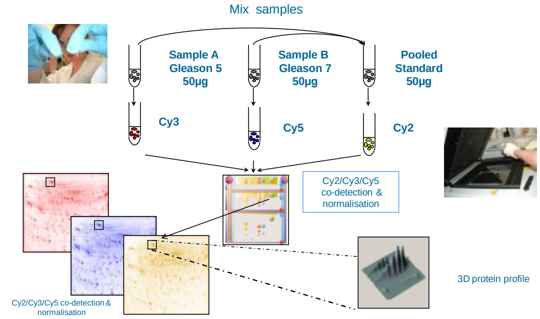
Validation

Prostate Cancer
Serum Biomarker *Discovery*



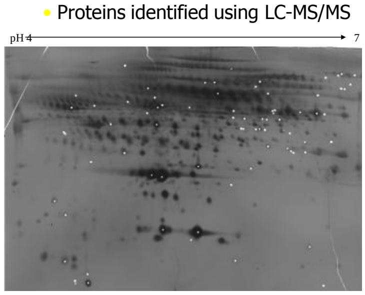
Anderson and Anderson, Mol. Cell. Proteomics 2002 1: 845

DIGE workflow

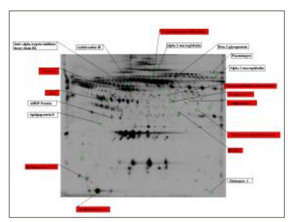


MS Analysis

- 57 spots have been cut from silver stained prep gel
  - Proteins identified using LC-MS/MS



Prostate Cancer
Serum Biomarker *Discovery*



- Zinc alpha 2-glycoprotein 1.4x↑ - association with tumour stage and predictive of biochemical recurrence (IHC) (Hale *et al.* Clin Cancer Res 2001)
- Pigment epithelium-derived factor 1.8x↓ - decrease in metastatic human and rat prostate tumours (Halin S *et al.* Cancer Res 2004)
- Proteasome subunit β type 6 1.6x↑ - unknown

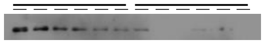
Protein *Identification*

Acc No	Protein Description	Peptides		Expression in Gl 7 vs. Gl.5	Fold Chang
P28072	Proteasome subunit beta type 6	2 (2)		Increased	2.53
P02790	Hemopexin	47 (84)		Decreased	1.17
P02749	Beta-2-glycoprotein 1	1 (2)		Decreased	1.61
P25311	Zinc-alpha-2-glycoprotein	5 (18)		Increased	1.36
P01024	Complement C3	4 (6)			
P02652	Apolipoprotein A-11	6 (17)		Increased	1.51
P01024	Complement C3	2 (2)		Decreased	1.26
P08670	Vimentin	2 (4)		Decreased	1.88
P02749	Beta-2-glycoprotein 1	2 (2)			
P02749	Beta-2-glycoprotein 1	2 (2)		Decreased	1.71
P05160	Coagulation factor XIII B chain	4 (10)		Decreased	1.65
P36955	Pigment epithelium-derived factor	1 (1)		Decreased	1.76
P01028	Complement C4	5 (8)		Decreased	2.1
P02656	Apolipoprotein C-III	2 (2)		Increased	1.42
P10809	60kDa Heat Shock Protein	6	CARRY OVER	Decreased	1.41
O75636	Ficolin 3	3			
P10909	Clusterin	12		Decreased	2.12
P01011	Alpha antichymotrypsin	5			
Q96KN2	Glutamate carboxypeptidase	4			
P04004	Vitronectin	2			
Q14624	Interalpha trypsin inhibitor heavy chain	3		Decreased	1.55
Q14624	Interalpha trypsin inhibitor heavy chain	9		Decreased	1.24
P01028	Complement C4	4		Decreased	2.21
Q03591	Complement factor H related protein	3			
P02749	Beta-2-glycoprotein	3		Decreased	1.22
P81605	Dermicidin	2		Decreased	1.66
Q03591	Complement factor H related protein	1			
P01028	Complement C4	5		Decreased	1.27
P05160	Coagulation factor XIII B chain	5		Decreased	2.05
P01028	Complement C4	4(6)		Decreased	4.74
P05090	Apolipoprotein D	2(2)		Decreased	2.31
P01008	Antithrombin III	1(4)		Increased	1.93
P00450	Ceruloplasmin	2(2)			
P01876	Ig alpha-1 chain C region	1(2)			
Q14624	Inter-alpha-trypsin inhibitor heavy chain	14(24)		Increased	2.44
P01008	Antithrombin III	3(4)			
P02774	Vitamin D binding protein	3(5)			
P02749	Beta-2-glycoprotein	1(1)		Increased	1.27
P00747	Plasminogen	6(9)		Increased	1.24
Q02325	Plasminogen-regulated protein B	1(1)		Increased	1.28
P00747	Plasminogen	11(19)		Increased	1.27

Prostate Cancer
Serum Biomarker *Validation*

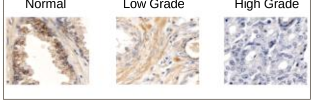
Western blotting

Gleason 5 Gleason 7



Immunohistochemistry

Normal Low Grade High Grade



Validation: antibody-dependent

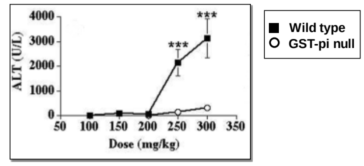
Paracetamol toxicity in GST-pi "knockout" [GST-P1/2$^{(-/-)}$] mice

Increased resistance to acetaminophen hepatotoxicity in mice lacking glutathione *S*-transferase Pi

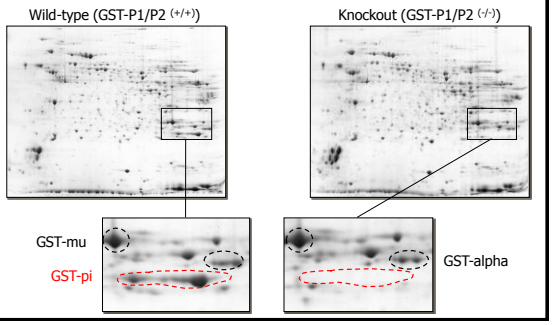
Colin J. Henderson*†‡, C. Roland Wolf*†, Neil Kitteringham§, Helen Powell§, Diana Otto*, and B. Kevin Park§

*Imperial Cancer Research Fund Molecular Pharmacology Unit, Biomedical Research Centre, Level 5, Ninewells Hospital and Medical School, Dundee, DD1 9SY, United Kingdom; and §Department of Pharmacology and Therapeutics, University of Liverpool, New Medical Building, Ashton Street, Liverpool, L69 3GE, United Kingdom

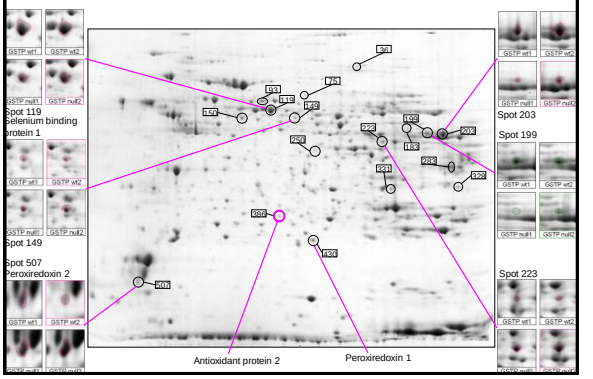
Proc. Natl. Acad. Sci. U.S.A. 97, 12741 (2000)



Role of GST-pi (P1/P2) in paracetamol toxicity



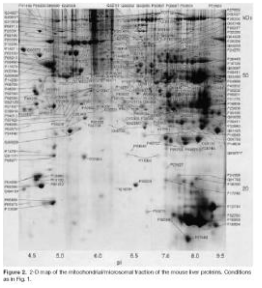
Proteomic analysis of GST-P1/2$^{(-/-)}$ mice



Where are the P450s?

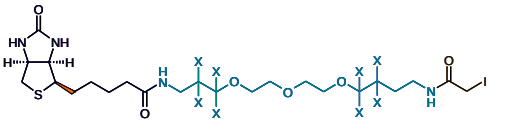
Fountoulakis *et al.*, 2001

- Mouse liver
- Excised 5800 spots
- Identified 2500 proteins
- 328 unique genes
- No P450



Isotope-coded affinity tagging (ICAT)

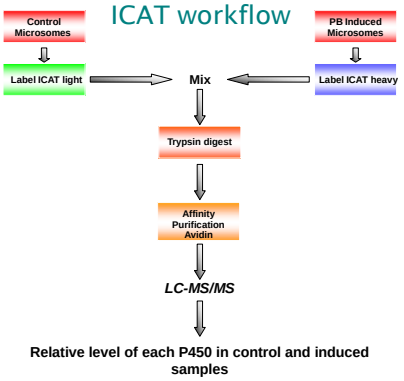
ICAT reagents



Biotin tag | **Linker** | **Thiol specific reactive group**

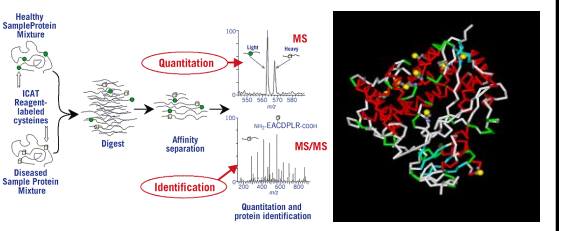
X = H, light linker
X = D, heavy linker

ICAT workflow



Relative level of each P450 in control and induced samples

P450 3D structure: *Cysteine residues*



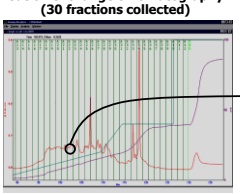
ICAT labelling of liver microsomes

Comprehensive analysis of proteins present in microsomes

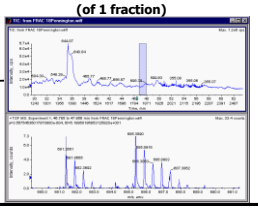
Investigate whether can use the approach to identify P450 and quantify their expression

Label, digest, cation exchange, avidin affinity isolation; nano-LC MS

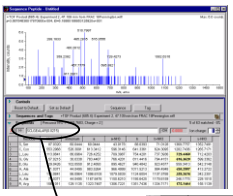
Cation exchange chromatography (30 fractions collected)

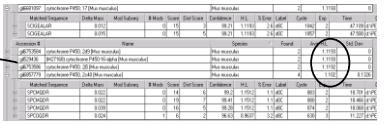


Nano-LC MS (of 1 fraction)



Identification & quantification





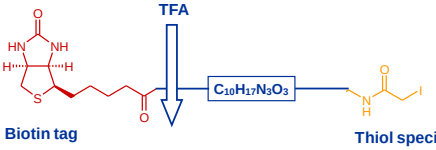
Analysis shows:

Several cytochrome P450 proteins (Cyp 2C, 2E, 3A)

Cytochrome P450 reductase

Microsomal glutathione transferase

Evolving technology: Cleavable ICAT



Biotin tag

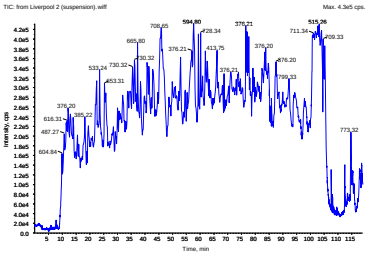
Thiol specific group

9 x ^{12}C, light linker
9 x ^{13}C, heavy linker

- Less complex MS/MS
- More peptides
- Linker modified to improve co-elution

Analysis of mouse microsomal proteins with cleavable ICAT reagents

Unfractionated sample - TIC

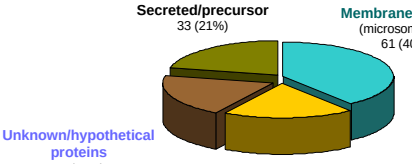


- 2 hour LC-MS run
- 1903 survey scans
- 1685 MS/MS spectra
- 830 peptides matched
- 155 mouse proteins identified

Identification of P450's

Family	Isoform	Peptides found at confidence level		
		75%	90%	99%
Cyp1	**cyp1a2**	2	1	
Cyp2	**cyp2a4**	2	1	1
	cyp2a12	1		
	cyp2c29	4	4	3
	cyp2d9	1	1	
	cyp2d11	1		
	cyp2e1	3	3	
	cyp2f2	2		
	cyp2j9	1		
Cyp3	cyp3a11	1		
Cyp4	cyp4v3	1	1	
Cyp8	cyp8b1	1		
	TOTAL P450s	12	6	2
	TOTAL Proteins	155	82	33

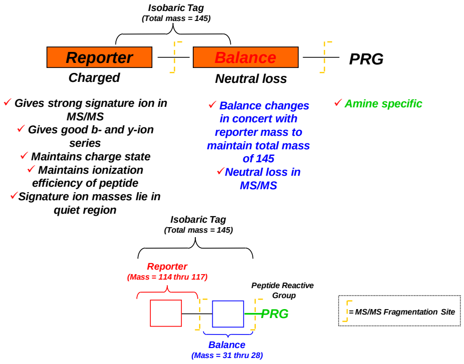
Sub-cellular localisation of ICAT labelled proteins



155 proteins – single LC run

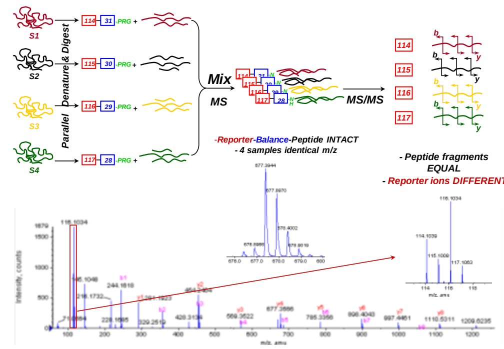
But…. would it be the same 155 next time?
Do we want to see this group of 155?

iTRAQ™ Reagent Design



Isobaric Tag
(Total mass = 145)
Reporter
Balance
PRG
Charged
Neutral loss
✓ Gives strong signature ion in MS/MS
✓ Gives good b- and y-ion series
✓ Maintains charge state
✓ Maintains ionization efficiency of peptide
✓ Signature ion masses lie in quiet region
✓ Balance changes in concert with reporter mass to maintain total mass of 145
✓ Neutral loss in MS/MS
✓ Amine specific
Isobaric Tag
(Total mass = 145)
Reporter
(Mass = 114 thru 117)
Peptide Reactive Group
PRG
Balance
(Mass = 31 thru 28)
= MS/MS Fragmentation Site

Isobaric Tagging - General Method(4-Plex)



Cyp2C's sequences and unique peptides

```
Cyp2C29    MDLVVFLALTLSCLILLSLWRQSSGRGKLPPGPTPLPIIGNFLQIDVKNISQSFTNFSKA 60
Cyp2C40    MDPFVVLVLCLSFLLVLSLWRQRSARGNLPPGPTPLPIIGNYHLIDMKDIGQCLTNFSKT 60
Cyp2C50    MDPILVLVFTLSCLFLLSLWRQSSERGKLPPGPTPLPIIGNILQINVKDICQSFTNLSKV 60
             ** .:.*.: ** *::****** * **:************    *::*:* *.:**:**.
Cyp2C29    YGPVFTLYLGSKPTVILHGYEAVKEALIDRGEEFAGRGSFPMAEKMIKGFGVVFSNGNRW 120
Cyp2C40    YGPVFTLYFGSQPIVVLHGYEAIKEALIDHGEEFSGRGRIPVFDKVSTGKGIGFSHGNVW 120
Cyp2C50    YGPVYTLYLGNKPTVVLHGYEAVKEALVDHGEEFAGRGRLPVFDKATNGMGIIFSKGNVW 120
             ****:***:*.:* *:******:****:*:****:*** :*: :*  .* *: **:** *
Cyp2C29    KEMRRFTLMTLRNLGMGKRNIEDRVQEEAQCLVEELRKTKGSPCDPTFILSCAPCNVICS 180
Cyp2C40    KATRVFTVNTLRNLGMGKRTIENKVQEEAQWLMKELKKTNGSPCDPQFIIGCAPCNVICS 180
Cyp2C50    KNTRRFSLTTLRNLGMGKRSIEDRVQEEARCLVEELRKTNGSPCDPTFILGCAPCNVICS 180
             *  * *:: **********.**::*****: *::**:**:****** **:.*********
Cyp2C29    IIFQNRFDYKDKEFLILMDKINENVKILSSPWLQVCNSFPSLIDYCPGSHHKIVKNFNYL 240
Cyp2C40    IVFQNRFDYKDKDFLSLIGKVNECTEILSSPGCQIFNAVPILIDYCPGSHNKLFKNHTWI 240
Cyp2C50    IIFQDRFDYKDRDFLNLMEKLNEITKIMSTPWLQVCNTFPVLLDYCPGSHNKVFKNYACI 240
             *:**:******::** *: *:** .:*:*:*  *: *:.* *:*******:*:.**.  :
Cyp2C29    KSYLLEKIKEHKESLDVTNPRDFIDYYLIKQKQVNHIEQSEFSLENLASTINDLFGAGTE 300
Cyp2C40    KSYLLGKIKEHEESLDVTNPRDFIDYFLIQRRQKNGIEHMDYTIEHLATLVTDLVFGGTE 300
Cyp2C50    KNFLLEKIKEHEESLDVTIPRDFIDYFLINGGQEDGNYPLKNRLEHLAITVTDLFSAGTE 300
             *.:** *****:****** *******:**:  * :     .  :*:**  :.**. .***
Cyp2C29    TTSTTLRYALLLLLKYPDVTAKVQEEIDRVVGRHRSPCMQDRSHMPYTDAMIHEVQRFID 360
Cyp2C40    TLSSTMRFALLLLMKHTHITAKVQEEIDNVIGRHRSPCMQDRNHMPYTNAMVHEVQRYID 360
Cyp2C50    TTSTTLRYALLLLLKYPHVTAKVQEEIEHVIGKHRSPCMQDRSRMPYTDAMIHEVQRFID 360
             * *:*:*:*****:*:..:********:.*:*:*********.:****:**:*****:**
Cyp2C29    LLPTSLPHAVTCDIKFRKYLIPKGTTVITSLSSVLHDSKEFPNPEMFDPGHFLNGNGNFK 420
Cyp2C40    LGPNGVVHEVTCDTKFRNYFIPKGTQVMTSLTSVLHDSTEFPNPEVFDPGHFLDDNGNFK 420
Cyp2C50    LVPNSLPHEVTCDIKFRNYFIPKGTNVITSLSSVLRDSKEFPNPEKFDPGHFLDENGKFK 420
             * *..: * **** ***:*:***** *:***:***:**.******* *******: **:**
Cyp2C29    KSDYFMPFSTGKRICAGEGLARMELFLILTTILQNFKLKSLVHPKEIDITPVMNGFASLP 480
Cyp2C40    KSDYFMPFSAGKRICVGESLARMELFLFLTTILQNFKLKPLVDPKDIDMTPKHSGFSKIP 480
Cyp2C50    KSDYFMPFSTGKRICAGEGLARMELFLFLTSILQNFNLKPLVHPKDIDVTPMLIGLASVP 480
             *********:*****.**.********:**:*****:**.**.**:**:**   *::.:*
                     Cyp2C29    PPYQLCFIPL- 490
                     Cyp2C40    PNFQMCFIPVE 491
                     Cyp2C50    PAFQLCFIPS- 490
                                  * :*:****
```

cyp2c29	cyp2c40	cyp2c50
MDLVVFLALTLSCLILLSLWR	MDPFVVLVLCLSFLLVLSLWR	MDPILVLVFTLSCLFLLSLWR
GSPCDPTFILSCAPCNVICSIIFQNR	DIGQCLTNFSK*	IMSTPWLQVCNTFPVLLDYCPGSHNK
ILSSPWLQVCNSFPSLIDYCPGSHHK*	TNGSPCDPQFIIGCAPCNVICSIVFQNR	FIDLVPNSLPHEVTCDIK*
FIDLLPTSLPHAVTCDIK*	VNECTEILSSPGCQIFNAVPILIDYCPGSHNK	
	YIDLGPNGVVHEVTCDTK	
	ICVGESLAR	

Quantitative Proteomics

Research Article

Relative and absolute quantitative expression profiling of cytochromes P450 using isotope-coded affinity tags

Rosalind E. Jenkins[1], Neil R. Kitteringham[1], Christie L. Hunter[2], Sally Webb[3], Tony J. Hunt[2], Robert Elsby[3], Rod B. Watson[4], Dominic Williams[1], Stephen R. Pennington[5] and B. Kevin Park[1]

[1] Department of Pharmacology, University of Liverpool, Liverpool, UK
[2] Applied Biosystems, Framingham, MA, USA
[3] AstraZeneca, Loughborough, UK
[4] Applied Biosystems, Warrington, UK
[5] Conway Institute, University College Dublin, Dublin, Ireland

Swiss-Prot acc. no.	Cytochrome P450	Peptide	Level in control (fmol/μg total protein)	95% uncer-tainty	Level in PB in-duced (fmol/μg total protein)	95% uncer-tainty	Average ratio PB:control	95% CIs		p value (t-test)
								Upper	Lower	
P00184	1a1	CIGETIGR	5.38	0.35	5.21	1.14	0.96	1.18	0.78	0.670
P00186	1a2	CIGEIPAK	1.38	0.24	1.25	0.18	0.91	0.95	0.87	0.013
Q64429	1b1	CIGEELSK	14.11	1.01	14.99	0.51	1.06	1.13	1.00	0.031
P15392	2a4	YCFGEGLAR	11.53	1.22	13.02	0.26	1.13	1.28	1.00	0.043
P56593	2a12	FCLGDSLAK	15.07	1.62	14.99	1.32	1.00	1.07	0.93	0.905
P12790	2b9/10/13/20	ICLGESIAR	11.41	1.95	68.97	5.24	6.07	7.24	5.09	<0.0001
Q64458	2c29/37/50	ICAGEGLAR	55.84	4.03	171.18	23.03	3.06	3.53	2.66	0.001
P56655	2c38/39	VCAGEGLAR	7.58	1.09	7.48	0.96	0.99	1.06	0.92	0.844
P56657	2c40	ICVGESLAR	16.15	1.93	15.85	1.97	0.98	1.03	0.93	0.625
P11714	2d9/11	SCLGEALAR	12.42	0.70	7.56	1.39	0.61	0.70	0.52	0.002
P24456	2d10/22/26	SCLGEPLAR	21.68	1.05	19.61	0.42	0.90	0.96	0.86	0.007
Q05421	2e1	VCVGEGLAR	35.13	1.58	30.38	2.78	0.86	0.91	0.82	0.006
P33267	2f2	LCLGEPLAR	21.74	3.34	16.27	1.96	0.75	0.78	0.72	0.0003
O54749	2j5	ACLGEQLAK	9.05	0.94	8.82	0.62	0.98	1.02	0.93	0.008
Q64459	3a11/13/16	NCLGMR	5.48	0.63	19.56	1.16	3.58	3.96	3.23	<0.0001
O88833	4a10/11/12/14	NCIGK	2.71	0.86	4.32	0.93	1.61	1.98	1.31	0.003

a) Levels listed are in fmol/μg total protein. Ratios in bold were subjected to the first tier statistical analysis (comparisons with the mean ratios of all peptides), whereas the rest were subjected to the second tier analysis (comparisons with the mean ratios of all peptides minus the three induced). This is described in greater detail in Section 2.

Proteomics workflows are evolving…

- 2-DE – introduction of fluorescent dyes - DIGE
  - Multiple (3) samples on a single gel
  - Easier image analysis
  - Minimal labelling (less interference, lower sensitivity)
  - Saturation labelling (?)
- iTRAQ reagents
  - Multiple (4) samples in a single LC run – soon 8
  - Improved fragmentation
  - Requires MS/MS for quantification
- Off-line LC-MALDI
- MS-imaging
- MS instrumentation

Conclusions

What is the question/hypothesis?

- Teamwork is essential – proteins work in teams so why not us?
- Discovery requires subsequent 'validation'
- Proteomic biomarker discovery is only as good as the samples and the experimental design
- Proteomics does not 'stand-alone' but need to be integrated with other datasets from other sources and technologies (clinical/metabolomic/genomic etc.)
- Are antibody-based assays the future of validation or routine measurement?
- What considerations are there for taking biomarkers into clinical practice?