A novel "proteomics-based" approach to identifying new anti-cancer agents using a new 2D liquid protein separation & mapping method: Analysis of the total protein expression of untreated and Peninsularinone-treated HCT-116 human colon adenocarcinoma cells.

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Introduction: Knowledge of how standard anticancer drugs exert their effects has aided in the development of new classes of drugs demonstrating improved tolerance and efficacy. This knowledge has also provided a scientific basis for combination chemotherapy and multi-modal therapy. However, a number of the more promising agents identified by this "rational-based", target-oriented drug screening approach are found to have no *in vivo* activity. To improve on this, a more promising approach may be to search active drug candidates for "protein target-oriented" therapy by starting with a drug with proven *in vivo* activity (i.e. therapeutic efficacy) rather than the other way around. Here we can look at the molecular mechanism of a known active drug on a proliferating tumor cell line and investigate the changes in the protein expression, and thus evaluate the importance (and relevance) of these changes to specific cellular pathways using a proteomics-based approach.

In this poster we demonstrate this proteomics-based approach by comparing the protein expression of a drug-treated HCT-116 human colon adenocarcinoma cancer cell line versus this same untreated cell line using a newly developed 2D all-liquid phase protein mapping technique; ProteoSep®. The drug under study is Peninsularone, a novel anti-cancer drug obtained from the Drug Discovery Program of Henry Ford Health System. The ProteoSep method used involves 2D fractionation and mapping of the total protein expression based on protein pI and hydrophobicity for the whole cell lysates obtained. The ProteoSep method has the distinct advantage over all other 2D gel or LC techniques in that MS analysis can be performed on the intact expressed proteins for highly accurate mass determination for use in evaluating post-translational modification. In addition, accurate ID's can be obtained and compared to the intact protein masses using high resolution peptide mapping MS/MS techniques.

The important advantages of the ProteoSep technique:

- **Isolation and purification of intact proteins.**
  - **Liquid (gel-free) pI fractions**
  - **Direct information on post-translational modifications**
  - **Protein fractions can be isolated & analyzed by Western blot, ELISA, Edman sequencing, etc., as well as MALDI peptide mapping analysis, SELDI, or LC/MS/MS.**
- **Works with most biological matrices (sera, tissue, cell lysates, etc.).**
- **Highly reproducible protein maps for easy comparison of samples.**
- **Direct interface with MS possible.**
- **MW values accurate to 150 ppm using ESI-TOF MS.**

Figure 1. 2D ProteoVue™ "lane and band" map detailing the total protein expression for an untreated HCT-116 whole cell lysate.

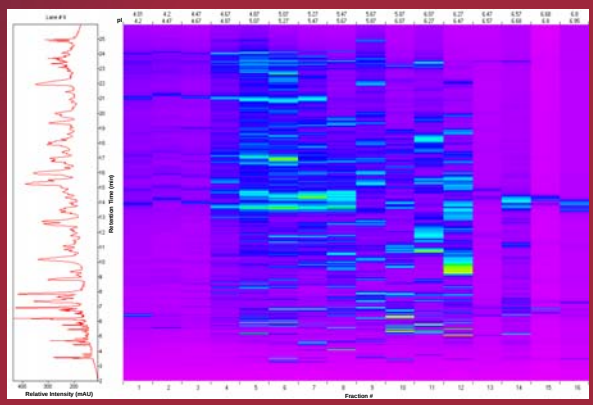
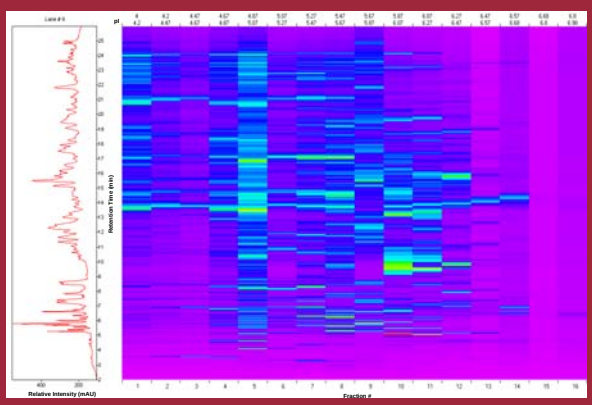


Figure 2. 2D ProteoVue "lane and band" map detailing the total protein expression for a Peninsularinone-treated HCT-116 whole cell lysate. The HCT-116 cell sample, in log-phase growth, was treated with a dose of 0.1ug/ml and exposed for 24 hours (responsible for a clonogenic cell survival of 0.01). Large differences in the protein expression are expected at the high dose of Peninsularinone used.



Results: Two pI lanes [pH 5.9-5.7 and pH 6.5-6.7 of the drug-treated and untreated HCT-116 cells] were analyzed and protein intact MW values measured by ESI-TOF MS. Several of the more abundant proteins were also identified by peptide mapping and database searching using MALDI-TOF MS. **A number of important cancer-related proteins were found in these fractions, namely; Hsp-27, Nucleoside diphosphate kinase, Triosephophate isomerase, Alpha enolase and Annexin II.** The use of ESI-TOF to determine the intact MW is particularly important in identification of these proteins since the peptide mapping and database searching may result in several possible matches; however, the MW value excludes several of the other possible matches. In the case of other protein bands, the peptide mapping, the MW value, and limited MS/MS data did not provide a reasonable match against the database. These proteins may not be in the database or further detailed sequencing work is required to identify these proteins.

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Other protein bands have been identified that appear to be strongly down-regulated [or up-regulated] following drug treatment. More specifically, in the pH 5.9-5.7 fraction, the proteins Annexin I, heterogeneous nuclear ribonucleoprotein H and cold shock domain protein A are strongly up-regulated in the untreated colon cancer sample versus the drug-treated sample. In the pH 6.87-6.67 fraction, histone and RIG protein are strongly up-regulated in the untreated sample versus the drug-treated sample. Further work is presently underway to use this method to study these samples as a function of drug concentration and time to discern more about Peninsularinone's mechanism of action at the molecular level.

Conclusion: A multidimensional chromatographic 2D liquid-phase method for separation of proteins from whole cell lysates has been developed. The method provides a 2D map of the protein content of each cell line based upon pI versus hydrophobicity. The high reproducibility of the 2D maps allow for inter-lysate comparisons using differential mapping analysis of the protein bands displayed with a two-color quantitative representation. Using this differential display method, a comparison between drug-treated and untreated colon cancer cells showed distinct changes in protein expression as a function of drug treatment. This method provides a means of collecting relatively isolated and purified proteins in the liquid phase for mass spectrometric analysis. The liquid phase proteins can be used to obtain the MW value by ESI-TOF MS while peptide mapping can be performed via MALDI-TOF MS. The use of an accurate intact MW, together with the peptide map, can provide identification of the proteins using database searching methods.

Figure 3a-b details the 2D differential display maps produced using DeltaVue™ software comparing two specific pI lanes for the **untreated control (red bands)** and **Penisularinone-treated (green bands)** HCT-116 cells. The relative intensities of the shades of green or red are quantitatively related to the amount of protein detected by UV absorption. The center lane displays the difference in protein expression between proteins. A band with an increased level of expression relative to the Peninsularinone-treated sample shows up in shades of green in the center map while proteins expressed at a increased level in the untreated cell sample relative to the Peninsularone-treated sample will show up in shades of red. In the case where there is no change of expression, the differential map shows up in white.

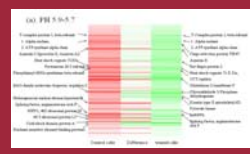

Figure 3

Table 1: MS data comparing drug-treated vs. untreated HCT-116 cell lines

Protein Name pI Range 5.9-5.7	Accession #	Protein MW (Da)	Micromass LCT ESI-TOF Intact Mass (Da) untreated	drug-treated	% change in peak area
Splicing factor, arginine/serine-rich 9	Q13242	25542	25605	25603	-160
Pyruvate kinase, ML isozyme (Pyruvate kinase muscle isozyme) (THBP1)	P14618	57937	57977		+
Heterogeneous nuclear ribonucleoprotein H (hnRNP H)	P31943	49230	49093	49263	+33
Keratin, type I cytoskeletal 6D (Cytokeratin 6D)	P48667	42468.7	42410		+
Glyceraldehyde 3-phosphate dehydrogenase, liver	P04406	36054	35933		+
Deoxyuridine 5'-triphosphate nucleotidohydrolase, mitochondrial precursor (dUTPase) (dUTP pyrophosphatase)	P33316	26707	*	26752	U
Glutathione S-transferase P (GST class-Pi)	P09211	23356	23259		+
T-complex protein 1, epsilon subunit (TCP-1-epsilon) (CCT-epsilon)	P48643	59672	59453		+75
Ret finger protein 2	O60858	47002	47076		+
Annexin II (Lipocortin II)	P07355	38604.4	38532	38649	-24
Cargo selection protein TIP47	O60664	47033	47067		+
Alpha enolase	P06733	47169	47096	47084	+35
ATP synthase alpha chain, mitochondrial precursor	P25705	59751	59595	59582	U
T-complex protein 1, beta subunit (CCT-beta)	P78371	57488.7	57421	57520	-50
Nuclease sensitive element binding protein 1	P16991	35924	35924		-
DNA- binding protein A (Cold shock domain protein A)	P16989	40060	40271		-64
60S ribosomal protein L3 (Hs'-1 TAR RNA- binding protein B)	P39023	46109	46110		-633
40S ribosomal protein S8	P06058	24205	24078		-2800
Nuclear inhibitor of protein phosphatase-1 (NIPP-1)	Q12972	38479	38562		-
Phosphoglycerate mutase 1 (phosphoglycerate mutase isozyme B) (PGAM-B) (BPG-dependent PGAM 1)	P18669	28804	*	28712	U
BAG-family molecular chaperone regulator-3	O95817	61595	61762		-15
Heat shock cognate 71 kDa protein	P11142	70869	70869		-40
phenylalanyl tRNA synthetase beta subunit	7804453	67548	67474		-
Proteasome 26S subunit, ATPase	4506209 M	48634	48518		-224
annexin A2	18645167	38576	38532		U
Prohibitin	P35232	29804	29718	29726	-66

Notes:
1. "(-)": proteins could be detected in the control but not in the treated cells (i.e. drug-modulated down-regulation)
2. "(+)": proteins could be detected in the treated but not in the control cells (i.e. drug-modulated up-regulation)
3. "U": proteins co-eluted and thus could not calculate the peak area
4. Minus value: fold change in peak area of proteins down-regulated in treated as compared to control cells
5. Plus value: fold change in peak area of proteins up-regulated in treated as compared to control cells

Table 2: MS data comparing drug-treated vs. untreated HCT-116 cell lines

Protein Name pI Range 6.87-6.67	Accession #	Protein MW (Da)	Micromass LCT ESI-TOF Intact Mass (Da) untreated	drug-treated	% change in peak area
GST class-Mu 5	P46439	25693	25761		+
Heterogeneous nuclear ribonucleoproteins A2/B1 (hnRNP A2 / hnRNP B1)	P22626	37430	37442	37446	-198
Heterogeneous nuclear ribonucleoprotein A3 (hnRNP A3) (D10S102)	P51991	39686	39561		+
Protein kinase C inhibitor 1(PKCI-1)	P49773	13802	*	13805	0
Alpha-2-macroglobulin receptor-associated protein precursor (RAP)	P30533	41466	41541		-40
Heat shock 27 kDa protein (HSP 27)	P04792	22783	22701	22696	-75
Hypothetical protein CGI-110 (Protein HSPC175)	Q9Y3B4	14585	14498		+
Coproporphyrinogen III oxidase, mitochondrial precursor (CDX)	P36551	40303	40174	40172	+24
54 kDa nuclear RNA- and DNA-binding protein (NMT55)	Q15233	54101	*		-50
Nucleoside diphosphate kinase B (NDK B)	P22392	17298	17211		+
Triosephosphate isomerase (TIM)	P60174	26670	26565	26538	-48
Pyruvate kinase, M2 isozyme	P14786	57914	57849	57804	0
Pyruvate kinase, ML isozyme (THBP1)	P14618	57937	57852	57830	0
Glutamate dehydrogenase 1, mitochondrial precursor (GDH)	P00367	61398	*		-18
Nuclease sensitive element binding protein 1 (DBPB)	P16991	35924	35873		-
60S ribosomal protein L27	P61526	15798	15884		-
60S ribosomal protein L35a	P18077	12538	12653		-
Putative RNA-binding protein 3 (RNPL)	P98179	17171	17221		-
60S ribosomal protein L7 (L23)	P18621	21397	21268		-
60S ribosomal protein L10 (QM protein)	P27635	24577	24481		-
40S ribosomal protein S15 (RIG protein)	P51174	17040	16952		-
Heterogeneous nuclear ribonucleoprotein H3 (hnRNP H3) (hnRNP 2H9)	P31942	36927	37060		-
40S ribosomal protein S18	P17008	16445	16380		-
40S ribosomal protein S18 (KE-3)	P25232	17719	13760		-
Histone H2B.j	Q93079	13892	13812		-
Fibrillarin	P22087	33764	34047		-
Elongation factor 1-alpha 1	P04720	50141	50629		-
Platelet-activating factor acetylhydrolase IB gamma subunit (PAFAH gamma subunit)	Q15102	25734	25645		-
Activin receptor type II precursor (ACTR-II)	P27037	57948	57890		-
40S ribosomal protein S2 (LLREP3)	P15880	31325	31390		-
5'-methylthioadenosine phosphorylase (MTAPase)	Q13126	31250	31149		-
Phosphoglycerate mutase 1 (PGAM-B)	P18669	28804	28714		-
PLAP-endonuclease-1 (MF1)	P97748	42593	41258		-
26S protease regulatory subunit S10B (CADp44)	O95524	44173	44124		-
L-lactate dehydrogenase A chain (LDH-A)	P00338	36689	36597		-

Notes:
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2. "(+)": proteins could be detected in the treated but not in the control cells (i.e. drug-modulated up-regulation)
3. "**": in "LCT mass": proteins identified in w o fractions by MALDI MS, but none matched LCT data

The 2D liquid maps shown in Figures 1 & 2 display protein bands based upon hydrophobicity, which does not correspond directly to MW. The ESI-TOF MS provides a MW value associated with each of the intact proteins accurate to 150 ppm. In addition, the protein bands can be collected and digested producing a peptide map by MALDI-TOF for identification of the proteins present using web-based database searching. These results are tabulated in **Tables 1 & 2.**

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