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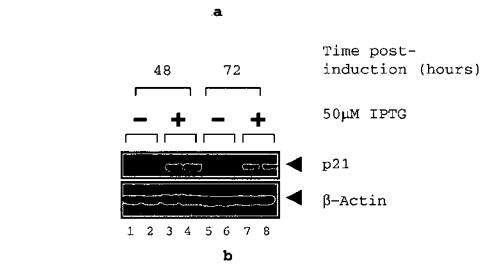
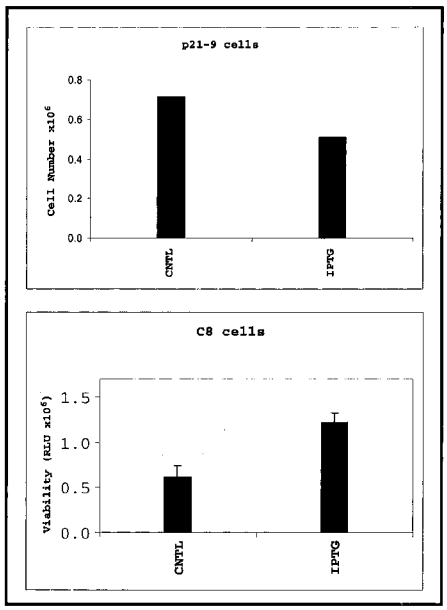
(54) Title: METHODS AND COMPOSITIONS FOR IDENTIFYING AND USING P21-REGULATED PROTEINS





(57) Abstract: This disclosure identifies p21-regulated proteins that may be used to induce anti- apoptotic and/or mitogenic cellular effects, both paracrine and within the p21-expressing cell itself. Three proteins released from HT- 1080 human fibrosarcoma cells displaying inducible p21 expression were identified; β-2-microglobulin, cystatin C, and pro-platelet basic protein. The disclosure also provides therapeutic compositions, methods for using, and methods for identifying p21-regulated proteins.

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METHODS AND COMPOSITIONS FOR IDENTIFYING AND USING p21-REGULATED PROTEINS

BACKGROUND

1. Technical Field

The present disclosure relates to compositions and methods useful for modulating cellular senescence.

2. Description of the Related Art

Cellular senescence is the phenomenon where cells lose the ability to divide. It can be activated spontaneously in stressful cellular environments or induced during therapeutic intervention. The response of tumor cells to this senescent event is complex, poorly understood and can have significant consequences for prognosis and subsequent cancer treatment [1]. Cells displaying a senescent phenotype are not responsive to mitogens, but remain in a dynamic state, serving as pools of secreted factors with tumor-inhibiting and promoting activities [2, 3, 4]. Whether these reservoirs promote a favorable environment for tumor progression is thought to be mediated in part by the expression of cyclin-dependent kinase inhibitors, such as $p21^{Waf1/Cip1/Sdi1}$ (hereon referred to as p21).

As a negative regulator of cell cycle progression in response to genotoxic insult, p21 plays a key role in preserving the integrity of the genome. However, p21 may also mediate oncogenic effects and as such contribute to tumor progression and promote carcinogenesis [3]. Such paradoxical duality in terms of activity is not without precedence among central regulators of the cell cycle. E2F, for example, regulates the expression of proliferation-dependant genes, thereby acting as an oncogene; however, it also displays noted pro-apoptotic activity [5].

p21 classically acts downstream of p53 during the DNA damage response as a broadspecificity inhibitor of cyclin-dependent kinases (CDKs) [6]. Inhibition of CDKs by p21 results in the hypophosphorylation of Rb, which inhibits the activity of E2F transcription factor complexes and leads to cell cycle arrest [7]. p21 can also bind and sequester PCNA,

which is required by DNA polymerase δ, thus blocking DNA replication and affecting growth arrest [8].

p21 can bind directly to c-Myc, STAT3 and E2F1, negatively modulating their activity [9, 10, 11]. Despite a lack of discernable affinity for DNA and no apparent DNA-binding motif, p21 is also found in transcription complexes positively influencing the transcriptional activity of NF-κB and C/EBP [12, 13], both of which are implicated in cell growth and differentiation. p21 also selectively controls the transcriptional activity of Estrogen receptor α in a histone acetyltransferase-dependent manner [14] and stimulates transactivation by p300 and CBP through the inhibition of a potent transcriptional repression domain (CRD1) present in both proteins [15].

p21 has also been implicated in mediating the proliferative effect of IGF-1 and Ets-1 [16, 17] and increased cytosolic p21 has been shown to result in cell cycle progression in vascular smooth muscle cells [18]. Indeed, within the p21-expressing cell itself, subcellular localization is a key determinant of function. Phosphorylation by Akt causes the stabilization and cytoplasmic accumulation of p21 [19, 20], leading to a proposed "gain of function" anti-apoptotic activity when retained in the cytoplasm. p21 was found to be predominantly cytoplasmic in many breast tumors, a feature associated with poor prognosis [21]. Even within the nucleus, p21/cyclin/CDK complexes that are catalytically active have been identified [22] and p21 inhibition with antisense oligonucleotides in vascular smooth muscle cells results in loss of assembly of cyclin D complexes and inhibition of growth factor-stimulated proliferation [23]. Also, the mTor inhibitor, RAD001, has been shown to sensitize tumor cells to cisplatin-mediated cell death through the inhibition of p21, further supporting an anti-apoptotic function [24]. Finally, p21 overexpression is an early event in the genesis of some cancers [25] and also occurs in response to mitogenic stimulation [26].

Ectopic expression of p21 and subsequent examination of global alterations in gene expression by DNA microarray analysis revealed that p21 inhibits the expression of a wide variety of genes associated with mitosis, DNA replication and proliferation, but also induces the expression of genes implicated in a number of age-related disorders, including cancer, Alzheimer's disease and amyloidosis [27]. Several p21-induced genes encode secreted proteins with known paracrine effects on mitosis and apoptosis. In addition, conditioned

medium from cells in which p21 was induced was found to have anti-apoptotic and mitogenic activity [27].

An anti-apoptotic function for p21 is consistent with the observation that it is cleaved by caspase 3 at the onset of apoptosis [28], indeed p21 can form a complex with procaspase 3, an interaction that provides resistance to Fas-mediated cell death [29]. p21-deficient tumors also display increased radiosensitivity [30] and p21-disrupted colon cancer cells have a higher sensitivity to cisplatin and nitrogen mustard [31]. In addition, p21 inhibits pro-apoptotic signalling kinases, such as SAPK and ASK1 [32, 33], as well as its inhibitory effect on transcription factors involved in stimulating programmed cell death, such as E2F and Myc [11, 34].

As previously mentioned, alterations in the levels of some $p21^{Waf1}$-inducible genes has been found to be mediated, in part, at the level of transcription, as the inhibitory and stimulatory effects of $p21^{Waf1}$ can be reproduced through the use of promoter-reporter constructs (Zhu *et al.*, 2002; Poole *et al.*, 2004). The inhibitory effects on gene transcription of $p21^{Waf1}$ expression have shown to be mediated through E2F, c-myc and STAT complexes [34]. Indeed, many of the genes inhibited by $p21^{Waf1}$-induction [27] were found to have E2F sites in their promoters. The lesser known stimulatory effects of $p21^{Waf1}$-induction were examined in a study by Poole *et al.* (2004) where they found that $p21^{Waf1}$ expression stimulated the promoters of six $p21^{Waf1}$-responsive genes, including the anti-apoptotic secreted protein, prosaposin. This effect was found to be mediated through NF-κB, which interacts with the transcriptional co-activator proteins, p300 and CBP, factors that $p21^{Waf1}$ has been shown previously to interact with [13]. They also examined the promoters of a subset of $p21^{Waf1}$-inducible genes *in silico*, and found putative NF-κB binding sites in 19 of 21 promoters tested (Poole *et al.*, 2004). Of the three proteins, both β-2-microblobulin and cystatin C have putative NF-κB binding sites in their promoters, while PPBP has no observable binding sites, as identified using *in silico* analysis. However, the promoters of some $p21^{Waf1}$ inducible genes have no detectable NF-κB sites; indeed induction of prosaposin promoter by $p21^{Waf1}$ was only weakly modulated by SR-I-κB, an NF-κB repressor (Poole *et al.*, 2004).

SUMMARY OF THE INVENTION

This disclosure provides methods and compositions for identifying and using p21-regulated proteins. In one aspect, the invention provides a method for inhibiting apoptosis in a cell (e.g., a human or mammalian cell) by administering to said cell, an effective amount of a p21-regulated protein. Suitable methods for administering a p21-regulated protein to a cell include, for example, contacting the cell with a p21-regulated protein and introducing into the cell, or neighboring cells (and subsequently inducing the transcription thereof) a nucleic acid encoding a p21-regulated protein, wherein the nucleic acid is operably linked to a transcriptional promoter.

In useful embodiments, the cells are at increased risk for undergoing apoptosis. The increased risk for apoptosis may be a result of any cause including, for example, exposure to ionizing radiation, a chemotherapeutic or radiomimetic agent, a toxin, hypoxia, traumatic injury, neurodegenerative disease, or an immunological reaction.

In another aspect, the invention provides a method for inducing apoptosis in a cell, said method comprising reducing the biological activity of a p21-regulated protein in the cell, or neighboring cells. Suitable methods for reducing p21-regulated protein biological activity include, for example, administering, or immunodepletion with, an antibody that specifically binds to the p21-regulated protein, or introducing into the cell, or neighboring cells (and subsequently inducing the transcription thereof) a nucleic acid encoding a sequence complementary to the nucleic acid sequence of a p21-regulated protein (e.g. antisense or RNAi), wherein said nucleic acid is operably linked to a transcriptional promoter. In useful embodiments, the nucleic acid comprises at least 12 nucleotides, although other amounts are contemplated, by way of non-limiting example, greater than 50 is contemplated within the scope of the present disclosure. In other useful embodiments, the cell is a cancer cell including, for example cells of a breast cancer, ovarian cancer, skin cancer, lung cancer, cervical cancer, colon cancer, bladder cancer, prostate cancer and other known cancers affecting the human body.

In another aspect, the invention provides a method for assessing the effectiveness and outcomes of an anti-neoplastic treatment in a patient. The method comprises the steps of: (i)

assessing the level of a p21-regulated protein prior to an anti-neoplastic treatment regimen; (ii) assessing the level of the p21-regulated protein following the anti-neoplastic treatment regimen; and (iii) comparing the results of the assessing step (ii) with the results of the assessing step (i), wherein a decrease in the level of the p21-regulated protein is indicative of efficacy, and an increase in the level of the p21-regulated protein is indicative of residual cellular senescence and the potential for tumor recurrence. Suitable methods for assessing the levels of the p21-regulated protein include, for example, assessing the RNA levels and directly assessing the protein levels.

Particularly useful p21-regulated proteins for any of the foregoing methods include, for example, β-2-microglobulin, cystatin C, and pro-platelet basic protein.

BRIEF DESCRIPTION OF THE DRAWINGS

The foregoing and other features and advantages of the present disclosure will be more fully understood from the following detailed description of the exemplary embodiments, taken in conjunction with the accompanying drawings.

Figure 1. **SELDI-MS analysis of CCM from p21-overexpressing HT1080 cells. (a)** Western immunoblot analysis of inducible p21 expression in HT-1080 p21-9 fibrosarcoma cells. Fifteen micrograms of total protein within whole cell lysates from induced and noninduced HT-1080 p21-9 cells was subjected to SDS-PAGE, and then probed with 1:1000 rabbit polyclonal anti-p21 antibody. Anti-β-actin is used as loading control. **(b)** CCM from p21-induced cells has a paracrine activity that promotes survival in low serum. Top graph shows the number of HT-1080 p21-9 cells remaining on the plate after the conditioned media were collected. Lower graph shows the survival of C8 cells after 2 days in 0.5% serum with CCM from either non-induced (CTRL) or p21-induced (IPTG) cells. Assay was performed, in triplicate, in 96-well plates, with cell viability being measured using the CellTiter-Glo kit. **(c)** Biomarker Wizard results with two different sample groups: Non-induced group including samples 1,2,5 and 6; Induced group including samples 3,4,7 and 8. The three potential biomarkers have p-values of 0.021.

Figure 2. **Differential binding of biomarkers from p21-overexpressing HT1080 cells over pH range on CM10 chips. (a)** Binding of the 10.2 kDa protein is observed on CM10 arrays using buffers from pH 3.5 to 6, the pI of this protein should be above 6. **(b)** Binding of the 11.7 kDa protein is observed on CM10 arrays using buffers from pH 3.5 to 5, the pI of this protein should be above 5. **(c)** Binding of the 13.4 kDa protein is observed on CM10 arrays using buffers from pH 3.5 to 6, the pI of this protein should be above 6. **(d)** Binding of the 10.2 and 13.4 kDa proteins is not observed on Q10 surfaces using buffers from pH 6 to 9:the pI of the protein should be above 9. Binding of the 11.7 kDa is observed on Q10 surfaces using buffers from pH7 to 9: the pI of this protein should be less than 7.

Figure 3. **Enrichment and identification scheme.** Optimal conditions from the profiling study carried out previously were used to enrich for the peaks found at 10.2, 11.7 and 13.4 kD in the p21-induced cells. Following Q Hyper D fractionation samples were run subject to 1DPAGE where enrichment allowed subsequent enzymatic digestion and passive elution for identification. Subsequent to identification, immunoassay and western blotting provided reconfirmation.

Figure 4. **Results of enrichment process.** The top spectrum of **(a)** shows the 72hr induced sample pre-Hyper Q fractionation where 11.7 and 13.4 peaks are evident. The lower spectrum of **(a)** shows this sample post-Q fractionation. Many contaminant peaks have been removed and the 11.7 and 13.4 peaks have been enriched (as measured by an increase in intensity). This sample was further purified on reverse-phase beads with **(b)** clearly showing the enrichment of the 3 peaks. **(c)** Reverse-phase bead fraction was run on 1D SDS-PAGE gel (Lane 1). Molecular weights are indicated in Lane 2.

Figure 5. **MS/MS and SELDI-TOF identification (a)** MS/MS analysis of ion with *m/z* of 1724 which was identified as a tryptic fragment of PPBP. This peptide was sequenced and matched pro-platelet basic factor. **(b)** The ion was fragmented by collision-induced dissociation and the MS/MS spectrum was submitted to MASCOT search tool for identification. The ion was identified as a fragment of pro-platelet basic factor with probability-based Mowse score of 47.5 (>30 indicates identity). **(c)** SELDI-TOF tryptic

digest of 11.7 band in Figure 4 (c). **(d) (i)** Amino acid sequence of β-2-microglobulin. Peptides directly identified by SELDI-TOF by searching Profound are underlined. **(ii)** Four ions were identified by SELDI-TOF as tryptic fragments of β-2-microglobulin. **(e)** SELDI-TOF tryptic digest of 13.4 band in Figure 4 (c). Profound database search identified Cystatin C with 66% minimum sequence coverage.

Figure 6. **Re-confirmation of the molecular weights of 10.2, 11.7 and 13.4 kDa bands.** Samples that were used for in-gel digestion and subsequent identification were also subject to passive elution. This technique "eluted" the whole proteins from the polyacrylamide gel, bound them to NP-20 surfaces and then re-confirmed their molecular weights of **(a)** the 11.7 kDa protein, and **(b)** the 13.4 kDa protein. (Compare Figure 2 and Figure 6) (± maximum 100 Da, 1%)

Figure 7. **On-chip immunoassay demonstrates accumulation in induced CCM compared to control CCM.** Polyclonal antibodies were coupled to pre-activated PS20 arrays with subsequent blocking, uninduced and induced CCM was incubated followed by washing and application of SPA. Arrays were read in PBS-IIc with laser settings of 180, detector sensitivity 8. **(a)** Antibody capture of 11.7 kDa protein using mouse anti-β-2-microglobulin antibody immobilised on preactivated PS20 ProteinChip. **(b)** Antibody capture of 13.4 kDa protein using mouse anti-cystatin C antibody immobilised on preactivated PS20 ProteinChip.

Figure 8. **Increased expression of extracellular and intracellular Cystatin C in p21- induced cells.** 15 μg of total protein within conditioned medium **(a)** and whole cell lysates **(b)** obtained from induced and non-induced HT1080 p21-9 cells was subjected to SDS-PAGE and immobilised to PVDF and probed with a 1:500 dilution of mouse monoclonal anti- Cystatin C antibody. An anti-BSA antibody and anti-actin antibody were used as loading controls for CCM and cell lysates respectively.

Figure 9. **Increased expression of extracellular and intracellular β-2-microglobulin in p21-induced cells.** Immunoblot analysis of extracellular and intracellular β-2-microglobulin. 15μg of total protein within conditioned medium and whole cell lysates obtained from induced and non-induced HT1080 p21-9 cells was subjected to SDS-PAGE, then probed with 1:1000 mouse monoclonal β-2-microglobulin antibody. An anti-BSA antibody and anti-actin antibody were used as loading controls for CCM and cell lysates respectively.

Figure 10. **Increased expression of intracellular PPBP in p21-induced human HT1080 fibrosarcoma cells.** 15 μg of total protein in whole cell lysates obtained from induced and non-induced HT1080 p21-9 cells was subjected to SDS-PAGE, and then probed with 1:1000 mouse monoclonal anti-PPBP antibody. An anti-actin antibody (AbCam) was used as loading controls for cell lysates.

DETAILED DESCRIPTION

Using SELDI-MS analysis of conditioned culture medium (CCM) taken from p21-overexpressing HT1080 fibrosarcoma cell lines, we have found a range of peaks across CM10, Q10 and IMAC surfaces that were increased or decreased in the CCM of p21-induced cells when compared to equivalent uninduced cells. Three of the peaks that were reproducibly increased in the p21-induced CCM were identified by SELDI-MS fingerprinting and tandem MS analysis and their identity was validated by onchip immunoassay and Western blot analysis.

β-2-microblobulin and cystatin C have been previously implicated in amyloid fibril formation [42, 43]. cystatin C is an endogenous cysteine proteinase inhibitor. Monomeric cystatin C is ubiquituous in human body fluids, being particularly abundant in cerebrospinal fluid, seminal plasma, and milk, and has been previously implicated as a modulator of proliferation. The glycosylated form, CCg, acts as an autocrine/paracrine factor required for the mitogenic activity of fibroblast growth factor 2 (FGF-2) on neural stem cells [44]. Treatment with chicken cystatin C results in growth stimulation of 3T3 fibroblasts as measured by radiolabelled thymidine incorporation [45] and secretion of cystatin C by rat

glomerular megengial cells creates an autocrine feedback loop with in vitro growth promoting activity [46]. CysC -/- homozygous null mice also show decreased metastatic spread of B16-F10 melanoma cells [47]. Compared with wild type mice, CysC -/- mice showed a seven-fold decrease in metastatic colonies.

β-2-microglobulin is constitutively secreted and found in normal serum. Its levels are elevated in urine in renal tubular disfunction and it has been implicated in a number of angiopathies. It is the most widely studied low-molecular-weight protein in end-stage renal disease and has been shown to cause dialysis-related amyloidosis, through its retention when renal function fails, its deposition in tissues, its aggregation into fibrils, and its ability to become glycosylated [48]. Previously, it has been found that β-2-microglobulin levels in the CCM of human prostate cancer cell lines and primary cultures derived from metastasis were higher than normal, through increased shedding from MHC I molecules [49]. However, β-2-microglobulin has been found to induce apoptosis in leukemia cell lines [50] and it suppresses the growth of isolated myeloma tumors and myeloma cell lines through inducing apoptosis and cell cycle arrest [51].

Pro-platelet basic protein ("PPBP"; also known as NAP-2 or CXCL7) is a member of the ELR+ CXC family of chemokines and is known as a major granular protein of platelets [52]. It is maintained as an inactive precursor and requires proteolytic cleavage to obtain receptor affinity [53]. PPBP (upon activation) binds to the CXCR2 receptor and is a powerful inducer of neutrophil adhesion and transmigration [54]. ELR+ CXC chemokines have been shown to be potent mediators of angiogenesis, whereas CXC chemokines lacking the ELR motif were found to be angiostatic [55]. Indeed, expression of the ELR+ CXC chemokine receptor, CXCR2, has been associated with increased angiogenesis and decreased necrosis of renal cell carcinomas in a syngeneic mouse model [56].

There are several possibilities to explain the observed increased levels of these three proteins in the CCM from p21-induced cells. There may be enhanced secretion of intracellular stores or alternatively increased synthesis upon p21-induction at the time points examined. Western blot analysis of cell lysates from the 48 and 72hr time points showed that there were concomitant intracellular increases of cystatin C, β-2-microglobulin and PPBP when compared to non-induced cells (Figures 8b, 9b and 10). RT-PCR analysis also

indicated that the levels of cystatin C mRNA increase in response to p21 induction (data not shown), and based on earlier transcriptomic studies it would appear that there is a degree of de novo protein synthesis involved in the accumulation of these three proteins. However, there may also be intracellular accumulation from earlier time points that we have not examined yet.

p21-induction leads to a secretory phenotype whereby neighboring cell growth is affected, is based on the evidence that the CCM taken from p21-overexpressing cells had mitogenic and anti-angiogenic activity on 2 cell line models. CCM from induced cells increased radiolabelled thymidine incorporation three-fold in HS 15T fibroblast fibrosarcoma cells [27]. In addition, induced CCM decreased apoptosis in the mouse embryo fibroblast cell line C8 [27]. The three secreted factors that we have identified in this study have all been shown previously to have growth promoting effects and as such may contribute to this observed anti-apoptotic and mitogenic paracrine activity of p21-expressing cells.

Example 1: Discovery of p21-regulated Factors in CCM by SELDI-MS Analysis

Table I summarizes the number of peaks identified by profiling CCM on the three different ProteinChip arrays (CM10, Q10 and IMAC). Samples profiled on the ion-exchange array surfaces (CM10 and Q10) produced the highest numbers of peaks, with a total of 340 peaks either increased or decreased in the CCM from p21-induced cells. On the CM10 surface, 151 peaks were obtained with 26 of those being significant ($p<0.05$); 20 peaks were found to be increased in CCM from p21-expressing cells, with 6 peaks being decreased. On the Q10 surface, 189 peaks were found to have altered expression, with 48 significant ($p<0.05$). In this case, 31 of these peaks were increased and 17 were found to be decreased in CCM from p21-induced cells.

Profiling on IMAC surfaces (metal affinity arrays) produced a lower number of peaks compared to the ion exchange arrays (Table I). Two metal ions were loaded on the IMAC surfaces (nickel and copper). Proteins with exposed histidine, tryptophan, or cysteine typically bind to metals immobilised on this array surface. Nickel is mainly used to bind proteins with multiple histidine residues [35], while copper is known to bind proteins that have a mitogenic effect [36]. Profiling the CCM on copper surfaces produced more peaks

than the nickeladsorbed arrays (92 peaks as compared to 52 peaks, Table I). Moreover, there were also more statistically significant peaks decreased in CCM from p21-induced cells in the case of the copper-adsorbed arrays. We also noticed a trend towards low to mid-mass peaks in the total number of peaks found (data not shown), which is a common phenomenon of SELDI-MS analysis [37]. There was also an apparent bias towards peaks that were increased in the CCM of p21-induced cells on the three surfaces (Table I).

In evaluating the combined results, it was apparent that 3 peaks, with approximate molecular weights of 10.2 kDa, 11.7 kDa and 13.4 kDa respectively, were reproducibly and consistently found to be increased in CCM from p21-induced cells compared with CCM from control cells. These 3 peaks were found on CM10, Q10 and IMAC surfaces. Figure 1a shows a representative spectrum obtained on a CM10 array, binding at pH4, for these three putative p21-responsive proteins. Also shown is a gel representation view of their expression levels in CCM from induced versus non-induced cells (Figure 1b) and the Biomarker Wizard (Ciphergen Biosystems) determination of their statistical significance (Figure 1c).

With a view to investigating further the properties and ultimately the identity of these putative p21-responsive proteins, we used the differential binding capacities of the ion exchange arrays, depending on the pH range, to estimate their respective pI's (Figure 2). Using CCM from p21-induced cells, the three peaks were present on CM10 arrays at pH 3.5, 4, and 5. The 11.7 peak was not present on CM10 arrays at pH6. Thus, the pI of the 10.2 and 13.4 kDa peaks should be over 6 and the pI of the 11.7 kDa peak should be over 5. When profiling the CCM from p21-induced cells on Q10 surfaces, only the 11.7 kDa peak was present at pH 7, 8, and 9 (Figure 2d), whereas the 10.2 and 13.4 kDa peaks were not present over this pH range. (data not shown). Thus, we concluded that the pI of the 11.7 kDa peak should be less than 7 and the pI of the 10.2 and 13.4 kDa peaks should be over 9. These binding characteristics are consistent with the 11.7 kDa peak having a pI of between 5 and 7 while the 10.2 and 13.4 kDa peaks having pI's of over 9. These biochemical properties were further confirmed with the identification of these peaks.

TABLE I

Array Type	Cation-exchange	Anion-exchange Q10	Copper-loaded IMAC	Nickel-loaded IMAC

	CM10			
Total peaks identified	151	189	92	52
Total significant peaks*	26	48	21	12
Peaks increased in induced CCM**	20	31	7	11
Peaks decreased in induced CCM**	6	17	14	1

* Signal-to-Noise >/= 3.5 (first pass); Signal-to-Noise >/= 3.0 (second pass)

** with statistical significance of $p<0.05$

Spectra were analyzed individually in the mid- and high mass ranges. Prior to statistical analysis spectra were manipulated as follows: Using Ciphergen ProteinChip software the baseline was subtracted, noise values were calculated from the minimum of 2500Da (medium mass range) and 10000Da (high mass region) to 100% spectrum size. All spectra were then normalized using total ion current normalization with any outliers removed before proceeding to statistical analysis.

Samples were then assigned the sample groups of "non-induced" or "induced", regardless of 48 or 72 hr collection interval i.e. 4 non-induced and 4 induced samples.

To generate peak clusters with the software, qualified mass peaks (signal-to-noise ratio) >/=3.5 were detected. Peak clusters were completed using second pass peak filter (signal-to-noise ratio >/=3), and estimated peaks were added. Comparison of the 2 sample groups over the different array surfaces gave a total number of peaks that were increased/decreased in induced media. Overlapping peaks found between mid- and high mass spectra were removed and statistically significant peaks were those considered to be those with $p<0.05$.

Example 2: Enrichment and Identification of the 10.2, 11.7, 13.4 kDa Proteins.

As the peaks at 10.2, 11.7 and 13.4 kDa were consistently altered in the CCM from p21-induced cells, they may represent putative paracrine mediators, so we developed strategy of enrichment and purification. Using information gained from the profiling study regarding

the binding characteristics of each protein and our estimation of their respective pI's, we used a combination of anion exchange chromatographic fractionation, SDS-PAGE and tryptic digestion followed by SELDI-MS and hybrid quadrupole-TOF (Q-STAR) MS. Our enrichment and identification strategy of the 3 unknown proteins is summarized in Figure 3.

Firstly, CCM from p21-induced cells was subjected to Q Hyper D fractionation (Figure 4a). This initial enrichment step increased the peak intensity of the 11.7 kDa peak from 1.5 to 6 (relative intensity), while the peak intensity of the 13.4-kDa peak was not altered but an adjacent contaminant peak (13.8 kDa) was removed. At this stage, the 10.2 kDa peak was not visible in the spectrum. The flow-through from the Q Hyper D fractionation was further enriched by selection with reverse phase beads (Figure 4b). After this step, the 10.2 kDa peak was clearly visible on our spectrum, while the 11.7 and 13.4 kDa peaks demonstrated increased intensities. Most of the contaminating peaks were also removed by this selective enrichment strategy. This enriched sample was then subjected to 1D-SDS-PAGE (Figure 4c). Following enrichment, we sought to identify the three peaks of interest. We used traditional in-gel enzymatic digestion with subsequent peptide database searching, in combination with passive elution and 2 different immunoassays (antibody capture and Western blot analysis), to conclusively identify the 3 proteins.

Firstly, the enriched 3-day induced CCM sample was separated by 1D-PAGE and bands at 10.2, 11.7 and 13.4 kDa were excised and subject to enzymatic digestion. Identification of the 10.2 kDa peak was carried out by sequencing tryptic digest peptides prepared as above, by interfacing the SELDI source to a hybrid quadrupole-TOF (Q-STAR) MS. The MS/MS spectrum was submitted to the Mascot search tool which returned proplatelet basic factor (UniProt entry P02775), with a probability-based Mowse score of 47.5 (>54 indicates identity) (Figures 5b, c, d). To profile peptides generated by in-gel tryptic digest of the 11.7 kDa band (Figure 5e), the Profound protein sequence database was searched with the following entries: protein mass range: 0-30 kDa; pI range 7 ± 6; organism classification: human; with a mass tolerance of 1.00Da. This search returned β-2-microglobulin (Uniprot entry P61769) with 50% minimum sequence coverage, molecular weight of 13.71 kDa and pI of 6.1. The latter agrees with our estimation of the pI of this peak being between 5 and 7 (Figure 2). The discrepancy in molecular weight is due to the fact that

mature β-2-microglobulin is secreted constitutively after synthesis and also possesses a signal peptide. The tryptic digest of the 13.4 kDa protein (Figure 5e), with the same search criteria as above, returned cystatin C (UniProt entry P01034) with 66% minimum sequence coverage. The molecular weight of cystatin C is 13.33 kDa, with a pI of 9.3, both of which agree with our data above (Figures 2 and 6). This form of cystatin C is the mature secreted non-glycoslyated form with signal peptide cleaved.

Half of each band excised for enzymatic digestion was retained for passive elution and re-confirmation of the size of each protein. Figure 6 depicts the SELDI spectra for each of the eluted proteins, demonstrating that the proteins eluted from the excised bands had matching mass/charge ratios (± maximum 100 Da, 1%) to the proteins identified by the original profiling study.

Example 3: Validation of Identified p21-responsive Secreted Factors

Following the identification of the proteins, we sought to validate their identity by on-chip immunoassay (antibody capture) and Western blot analysis. Antibodies directed against β-2-microglobulin (Figure 7a) and cystatin C (Figure 7b) were loaded onto PS20 pre-activated arrays and used to capture their respective protein targets from CCM. The anti-β-2-microglobulin antibody successfully captured a protein of the same mass (11.7 kDa) as previously identified in the p21-induced CCM samples. An increased level of protein was captured from 48 and 72hr p21-induced HT-1080 cells (Figure 7a). Similar results were observed using an anti-cystatin C antibody (Figure 7b). A control bovine antibody did not capture any proteins from the CCM (data not shown).

In addition to SELDI-based validation approaches, we also examined the induction of expression of PPBP, β-2-microglobulin and cystatin C in CCM from p21-expressing HT1080 cells by Western blot analysis. Induction of p21 expression led to the accumulation of cystatin C and β-2-microglobulin in CCM, when examined 48 and 72 hrs post-induction, compared with non-induced cells (Figures 8a and 9a). In addition, we examined intracellular levels of all three proteins, again by Western blot analysis, and found a corresponding accumulation of all 3 proteins inside induced compared to non-induced cells (Figures 8b, 9b and 10).

Materials and Methods

Cell culture and sample preparation.

The HT-1080 human fibrosarcoma cell line p21-9, that carries p21 in an isopropy-β-D-thiogalactoside (IPTG)-inducible stably integrated transgene [27], was propagated in DMEM supplemented with 10% FC2 serum (HyClone, Logan, UT, USA). CCM was prepared by plating 1x106 HT-1080 p21-9 cells per 15cm plate, adding 50μM IPTG the following day and replacing the medium 3 days later with medium containing IPTG and 0.5% FC2. Conditioned medium was then collected at 2 and 3 days post-replacement. Control IPTG-free conditioned medium containing 0.5% FC2 was collected from untreated cells grown to the same density as the IPTG-treated cells. Harvested cell pellets were resuspended in 500μl of lysis buffer (50mM Tris-HCl, pH 7, 150mM NaCl, 1% Triton-X100). Whole cell lysates were obtained by rotation at 4°C for 30min, and then centrifuged at 10,000g to remove insoluble debris. Total protein concentration in CCM and lysates was determined by BCA assay (Pierce, Rockford, IL, USA). All samples were stored at -80°C.

Profiling of CCM Using SELDI-MS.

Optimization studies on three chromatographic surfaces were carried out prior to full profiling analysis. In more detail, we used an IMAC30 immobilised metal ion chromatography surface (a nitriloacetic acid surface for transition metal binding) and two ion-exchange surfaces, CM10 (carboxymethyl weak cation exchange) and Q10 (strong anion exchanger) (Ciphergen Biosystems, Palo Alto, CA, USA). On IMAC30 and CM10 surfaces, the optimal conditions were found to be a 1:10 dilution of untreated CCM (1.5mg total protein/ml) with the appropriate buffers. On Q10 ProteinChip arrays, optimal conditions were a 1:2 dilution of CCM (0.3mg total protein/ml) with the appropriate buffer. CCM samples were then profiled on CM10, Q10 and IMAC30 arrays as follows: CM10 arrays were equilibrated with 100mM sodium acetate buffers of varying pH range (pH 3-7) with 0.5% Triton X-100; Q10 arrays with 100mM Tris-HCl of varying pH range (pH6-9) with 0.5% Triton X-100; IMAC arrays with 100mM copper sulphate/nickel sulphate buffers, in a bioprocessor module (Ciphergen Biosystems) on a Micromix orbital shaker. Ten μl aliquots

(15μg) of CCM from induced and non-induced HT-1080 cells were diluted into 90μl binding buffer and incubated for 30min at room temperature with agitation. The solution was removed and the spots were washed in the appropriate buffer and finally with distilled water. After air drying, sinapinic acid (SPA) (50% acetonitrile, 0.5% trifluoroacetic acid (TFA)) was applied to each spot, the array was air-dried and then analyzed by SELDI using a linear TOF analyser, PBS IIc (Protein Biology Systems IIc, Ciphergen Biosystems). All spectra were acquired in the positive ion mode. Ions were extracted using a 3kV ion extraction pulse and accelerated to a final velocity using 20kV of acceleration potential. Time-lag focusing delay times were set to 750ns for mid-mass scans and 1420ns for high-mass scans. For accurate molecular weight measurements, external mass calibration was performed with using Hirudin BHVK (7034 Da), cytochrome c (12230 Da) and equine myoglobin (16951 Da) as calibrants in the mid-mass range and hirudin, cytochrome C, myoglobin, bovine carbonic anhydrase (29023 Da), enolase (46671 Da), bovine albumin (66433 Da) and bovine IgG (147300 Da) in the high mass range.

Enrichment of Biomarker Candidates

CCM from induced and non-induced HT1080 p21-9 cells was adjusted with 1M Tris-HCl to give a final concentration of 100mM Tris-HCl, pH 9.0 and then bound to Q Ceramic HyperD F columns (Ciphergen Biosystems) (pre-equilibrated in above buffer). Flow-through fractions were collected at pH 9.0, followed by elutions at pH 4.0 and an organic acetonitrile wash. pH 9.0 fractions were purified further on reverse phase beads (Polymer Labs Ltd., Shropshire, UK) followed by elution of the enriched biomarkers using a discontinuous gradient (10%, 50%, 60%) of acetonitrile. The latter fractions were combined and speed vacuum dried.

In-gel Tryptic Digestion and Passive Elution of Biomarkers

Post Q-fractionated CCM samples were separated by SDS-PAGE gel electrophoresis using a Novex 4-20% Tris-Glycine gradient gel (Invitrogen, Carlsbad, CA, USA) and subsequently stained with Simply Blue Coomassie Stain (Invitrogen) in accordance with the manufacturer's recommendations. Gel pieces containing the target protein were excised. For

tryptic digestion, the gel pieces were washed in 50mM ammonium bicarbonate/50% acetonitrile, dehydrated in 100% acetonitrile, the supernatant removed and the samples vacuum dried. Samples were rehydrated with 100µl 100mM ammonium bicarbonate at room temperature for 5min then incubated for 1hr at 56°C. The ammonium bicarbonate solution was removed and, following a final dehydration step, modified porcine trypsin (10ng/µl) (Promega, Madison, WI, USA) in 25mM ammonium bicarbonate/10% acetonitrile was added and incubated at room temperature. After 1hr, 4hr, and overnight incubations, 0.8µl was analyzed on Normal Phase (NP20) array with 20% □-cyano-4-hydroxycinnamic acid (CHCA), dissolved in 50% acetonitrile/0.5% TFA. Identification of PPBP was carried out by sequencing tryptic digest peptides prepared as above by interfacing the SELDI source to a hybrid quadrupole-TOF (QSTAR) MS (Applied Biosystems/MDS SCIEX, Foster City, CA, USA). For passive elution, gel pieces were washed with 50% acetonitrile/50mM ammonium bicarbonate then dehydrated with 100% acetonitrile. The gel pieces were heated to 50°C before addition of a 45% formic acid/30% acetonitrile/10% isopropanol solution and incubation in a sonicating waterbath for 30 min at room temperature. Gel pieces were then incubated for 3hr at room temperature. The passively eluted material was then spotted on an NP-20 array followed by addition of saturated SPA.

On-chip Immunoassay

Rabbit polyclonal anti-human cystatin C antibody (0.2mg/ml; 3µl per spot; R & D Systems Inc, Minneapolis, MN, USA) and mouse monoclonal anti-human β-2-microglobulin (1 mg/ml; 2 µl per spot; R & D Systems Inc) in PBS pH 8.0 were added to the spots of a pre-activated PS-20 array. The extent of non-specific binding was tested using a bovine IgG antibody (Ciphergen Biosystems) instead of the specific antibody. The arrays were incubated in a humidity chamber for 1hr at room temperature to allow covalent binding of the antibody. Antibody solution was removed and ethanolamine (0.5M; 4µl per spot) at pH 8.0 was added to block remaining activated coupling sites and incubated for 20min at room temperature. The whole array was then washed twice in 10ml PBS for 5min and then transferred to a bioprocessor module. Antigen solution (75µg conditioned medium from induced and noninduced HT1080 p21-9 cells and 50µl PBS) were added to the spots

containing the bound antibodies and incubated with agitation for 1hr at room temperature. The solution was removed and the spots washed with PBS pH 7.2/0.05% Triton X-100, PBS without Triton X-100 and then finally with doubly distilled water. After air-drying, SPA was applied to each spot, the array was air-dried and then analyzed by SELDI-MS.

Immunoblot Analysis

CCM and whole cell lysates (15 μg of each sample in both cases) from induced and noninduced HT1080 p21-9 cells were loaded on 10% NuPage Bis-Tris gels (Invitrogen) and run under non-reducing conditions in 2-morpholinoethanesulfonic acid (MES) SDS buffer. Resolved proteins were transferred to polyvinylidenedifluoride (PVDF) membrane (BioRad, Hurcules, CA, USA) using a Mini-Protean II blotting system. Proteins were detected by incubation with primary antibodies in 5% non-fat dried milk in TBS containing 0.1% Tween-20, followed by incubation with a 1:7500 dilution of appropriate secondary antibody (Promega). Differences in protein loading were monitored by re-probing stripped membranes with either 1:1000 mouse monoclonal anti-BSA antibody (Sigma, St. Louis, MO, USA) for CCM samples or 1:2000 mouse monoclonal anti-actin antibody for cell lysates. Immunoreactive complexes were detected using Enhanced Chemiluminescence Plus reagents (Amersham Pharmacia Biotech, Uppsala, Sweden) and recorded by exposure to Fuji Medical SuperRX film (Fuji Photo Film Co. Ltd., Tokyo, Japan).

References

[1] Campisi, J., *Cell* 2005, *120*, 513-22.

[2] Krtolica, A., Parrinello, S., Lockett, S., Desprez, P., Campisi, J., *Proc. Natl. Acad. Sci. USA* 2001, *98*, 12072–12077.

[3] Roninson, I. B., *Cancer Lett.* 2002, *179*, 1-14.

[4] Dilley, T. K., Bowden, G. T., Chen, Q. M., *Exp Cell Res* 2003, *290*, 38–48.

[5] Stevens, C., La Thangue, N. B., *Arch Biochem Biophys.* 2003, *412*, 157-69.

[6] el-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R. *et al.*, *Cell* 1993, *75*, 817-25.

[7] Nevins, J. R., *Cell Growth Differ.* 1998, *9*, 585-93.

[8] Rossig, L., Jadidi, A. S., Urbich, C., Badorff, C., Zeiher, A. M. *et al.*, *Mol Cell Biol.* 2001, *21*, 5644-57.

[9] Kitaura, H., Shinshi, M., Uchikoshi, Y., Ono, T., Iguchi-Ariga, S. M. *et al.*, *J Biol Chem.* 2000, *275*, 10477-83.

[10] Coqueret, O., Gascan, H., *J Biol Chem.* 2000, *275*, 18794-800.

[11] Delavaine, L., La Thangue, N. B., *Oncogene* 1999, *18*, 5381-92.

[12] Harris, T. E., Albrecht, J. H., Nakanishi, M., Darlington, G. J., *J Biol Chem.* 2001, *276*, 29200-9.

[13] Perkins, N. D., Felzien, L. K., Betts, J. C., Leung, K., Beach, D. H. *et al.*, *Science* 1997, *275*, 523-7.

[14] Fritah, A., Saucier, C., Mester, J., Redeuilh, G., Sabbah, M., *Mol Cell Biol.* 2005, ,*25*, 2419-30.

[15] Gregory, D. J., Garcia-Wilson, E., Poole, J. C., Snowden, A.W., Roninson, I. B. *et al.*, *Cell Cycle* 2002, *1*, 343-50.

[16] Dupont, J., Karas, M., LeRoith, D., *J Biol Chem.* 2003, *278*, 37256-64.

[17] Zhang, C., Kavurma, M. M., Lai, A., Khachigian, L. M., *J Biol Chem.* 2003, *278*, 27903-9.

[18] Dong, Y., Chi, S. L., Borowsky, A. D., Fan, Y., Weiss, R. H., *Cell Signal.* 2004, *16*, 263-9.

[19] Zhou, B. P., Liao, Y., Xia, W., Spohn, B., Lee, M. H. *et al.*, *Nat Cell Biol.* 2003, *3*, 245-52.

[20] el-Deiry, W. S., *Nat Cell Biol.* 2001, *3*, E71-3.

[21] Winters, Z. E., Hunt, N. C., Bradburn, M. J., Royds, J. A., Turley, H. *et al.*, *Eur J Cancer.* 2001, *37*, 2405-12.

[22] Zhang, H., Hannon, G. J., Beach, D., *Genes Dev.* 1994, *8*, 1750-8.

[23] Weiss, R. H., Joo, A., Randour, C., *J Biol Chem.* 2000, *275*, 28340.

[24] Beuvink, I., Boulay, A., Fumagalli, S., Zilbermann, F., Ruetz, S. *et al.*, *Cell* 2005, *120*, 747-59.

[25] Biankin, A. V., Kench, J. G., Morey, A. L., Lee, C. S., Biankin, S. A. *et al.*, *Cancer Res.* 2001, *61*, 8830-7.

[26] Li, Y., Dowbenko, D., Lasky, L. A., *J Biol Chem.* 2002, *277*, 11352-61.

[27] Chang, B. D., Watanabe, K., Broude, E. V., Fang, J., Poole, J. C. *Et al.*, *Proc Natl Acad Sci U S A* 2000, *97*, 4291-6.

[28] Zhang, Y., Fujita, N., Tsuruo, T., *Oncogene* 1999, *18*, 1131-8.

[29] Suzuki, A., Tsutomi, Y., Yamamoto, N., Shibutani, T., Akahane, K., *Mol Cell Biol.* 1999, *19*, 3842-7.

[30] Wouters, B. G., Giaccia, A. J., Denko, N. C., Brown, J. M., *Cancer Res.* 1997, *57*, 4703-6.

[31] Fan, S., Chang, J. K., Smith, M. L., Duba, D., Fornace, A. J. Jr. *et al.*, *Oncogene* 1997, *14*, 2127-36.

[32] Shim, J., Lee, H., Park, J., Kim, H., Choi, E. J., *Nature* 1996, *381*, 804-6.

[33] Asada, M., Yamada, T., Ichijo, H., Delia, D., Miyazono, K. *et al.*, *EMBO J.* 1999, *18*, 1223-34.

[34] Perkins, N. D., *Cell Cycle* 2002, *1*, 39-41.

[35] Crowe, J., Dobeli, H., Gentz, R., Hochuli, E., Stuber, D., Henco, K., *Methods Mol Biol.* 1994, *31*, 371-87.

[36] Herlyn, M., Mancianti, M. L., Jambrosic, J., Bolen, J. B., Koprowski, H., *Exp Cell Res.* 1988, *179*, 322-31.

[37] Hodgetts, A., Bosse, J. T., Kroll, J. S., Langford, P. R., *Vet Microbiol.* 2004, *99*, 215-25.

[38] Roessler, M., Rollinger, W., Palme, S., Hagmann, M. L., Berndt, P. *et al.*, *Clin Cancer Res.* 2005, *11*, 6550-7.

[39] Guo, J., Yang, E. C. C., DeSouza, L., Diehl, G., Rodrigues, M. J. *et al.*, *Proteomics* 2005, *5,* 1953 –1966.

[40] Ruetschi, U., Zetterberg, H., Podust, V. N., Gottfries, J., Li, S. *et al.*, *Exp Neurol. in press.*

[41] Poon, T. C., Hui, A. Y., Chan, H. L., Ang, I. L., Chow, S. M. *et al.*, *Clin Chem.* 2005, *51*, 328-35.

[42] Ivanova, M. I., Sawaya, M. R., Gingery, M., Attinger, A., Eisenberg, D., *Proc Natl Acad Sci U S A* 2004, *101*, 10584-9.

[43] Maruyama, K., Kametani, F., Ikeda, S., Ishihara, T., Yanagisawa, N., *Neurosci Lett.* 1992, *144*, 38-42.

[44] Taupin, P., Ray, J., Fischer, W. H., Suhr, S. T., Hakansson, K. *et al.*, *Neuron* 2000, *28*, 385-97.

[45] Sun, Q., *Exp Cell Res.* 1989, *180*, 150-60.

[46] Tavera, C., Leung-Tack, J., Prevot, D., Gensac, M. C., Martinez, J. *et al.*, *Biochem Biophys Res Commun.* 1992, *182*, 1082-8.

[47] Huh, C. G., Hakansson, K., Nathanson, C. M., Thorgeirsson, U. P., Jonsson, N. *et al.*, *Mol Pathol.* 1999, *52*, 332-40.

[48] Floege, J., Ehlerding, G., *Nephron* 1996, *72*, 9-26.

[49] Abdul, M., Hoosein, N., *Urol Oncol* 2000, *5*, 168-172.

[50] Gordon, J., Wu, C. H., Rastegar, M., Safa, A. R., *Int J Cancer* 2003, *103*, 316-27.

[51] Min, R., Li, Z., Epstein, J., Barlogie, B., Yi, Q., *Br J Haematol.* 2002, *118*, 495-505.

[52] Walz, A., Baggiolini, M., *J Exp Med.* 1990, *171*, 449-54.

[53] Van Damme, J., Struyf, S., Opdenakker, G., *Semin Cancer Biol.* 2004, *14*, 201-8.

[54] Schenk, B. I., Petersen, F., Flad, H. D., Brandt, E., *J Immunol.* 2002, *169*, 2602-10.

[55] Strieter, R. M., Polverini, P. J., Kunkel, S. L., Arenberg, D. A., Burdick, M. D. *et al.*, *J Biol Chem.* 1995, *270*, 27348-57.

[56] Mestas, J., Burdick, M. D., Reckamp, K., Pantuck, A., Figlin, R. A. *et al.*, *J Immunol.* 2005, *175*, 5351-7.

Other Embodiments

All publications mentioned in this specification are herein incorporated by reference to the same extent as if each independent publication was specifically and individually indicated to be incorporated by reference.

Although the foregoing present disclosure has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this present disclosure that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method for inhibiting apoptosis in a cell, said method comprising administering to said cell an effective amount of a p21-regulated protein.

2. The method of claim 1, wherein said cell is at increased risk for undergoing apoptosis.

3. The method of any of claims 1-2, wherein said administering comprises contacting said cell with a p21-regulated protein.

4. The method of any of claims 1-2, wherein said administering comprises (i) introducing into said cell a nucleic acid encoding a p21-regulated protein, wherein said nucleic acid is operably linked to a transcriptional promoter and (ii) inducing the transcription of said nucleic acid.

5. The method of any of claims 2-4, wherein said increased risk is caused by exposure to at least one of ionising radiation, a chemotherapeutic or radiomimetic agent, a toxin, hypoxia, traumatic injury, neurodegenerative disease, and an immunological reaction.

6. The method of any of claims 2-5, wherein said cell is a mammalian cell.

7. The method of claim 6, wherein said cell is a human cell.

8. The method of any of claims 1-7, wherein said p21-regulated protein is selected from the group consisting of β-2-microglobulin, cystatin C, and pro-platelet basic protein.

9. A method for inducing apoptosis in a cell, said method comprising reducing the biological activity of a p21-regulated protein in said cell or neighboring cells.

10. The method of claim 9, wherein said method comprises administration of, or immunodepletion with, an antibody that specifically binds to said p21-regulated protein.

11. The method of claim 9, wherein said method comprises administering to said cell a p21-regulated protein-specific RNAi, or other method of suppression of p21-regulated protein gene expression.

12. The method of claim 9, wherein said method comprises (i) introducing into said cell a nucleic acid encoding a sequence complementary to the nucleic acid sequence of a p21-regulated protein, wherein said nucleic acid is operably linked to a transcriptional promoter and (ii) inducing the transcription of said nucleic acid.

13. The method of claim 12, wherein said nucleic acid comprises at least 12 nucleotides.

14. The method of any of claims 9-13, wherein said cell is a cancer cell.

15. The method of claim 14, wherein said cancer is selected from the group consisting of breast cancer, ovarian cancer, skin cancer, lung cancer, cervical cancer, colon cancer, prostate cancer and bladder cancer.

16. The method of any of claims 9-15, wherein said p21-regulated protein is selected from the group consisting of β-2-microglobulin, cystatin C, and pro-platelet basic protein.

17. A method for assessing the effectiveness of an anti-neoplastic treatment in a patient, said method comprising:

(i) assessing the level of a p21-regulated protein prior to an anti-neoplastic treatment regimen;

(ii) assessing the level of said p21-regulated protein following said anti-neoplastic treatment regimen; and

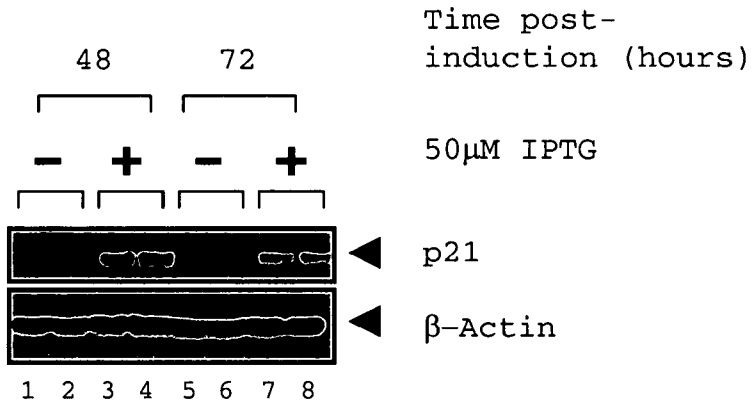
(iii) comparing the results of said assessing step (ii) with the results of said assessing step (i), wherein a decrease in said level of said p21-regulated protein is indicative of efficacy, or an increase of said p21-regulated protein is indicative of residual cellular senescence and the potential for tumor recurrence.

18. The method of claim 17, wherein at least one of assessing step (i) and assessing step (ii) comprises assessing RNA levels of said p21-regulated protein.

19. The method of claim 17, wherein at least one of assessing step (i) and assessing step (ii) comprises assessing protein of said p21-regulated protein.

20. The method of any of claims 17-19, wherein said p21-regulated protein is selected from the group consisting of β-2-microglobulin, cystatin C, and pro-platelet basic protein.

Figure 1a



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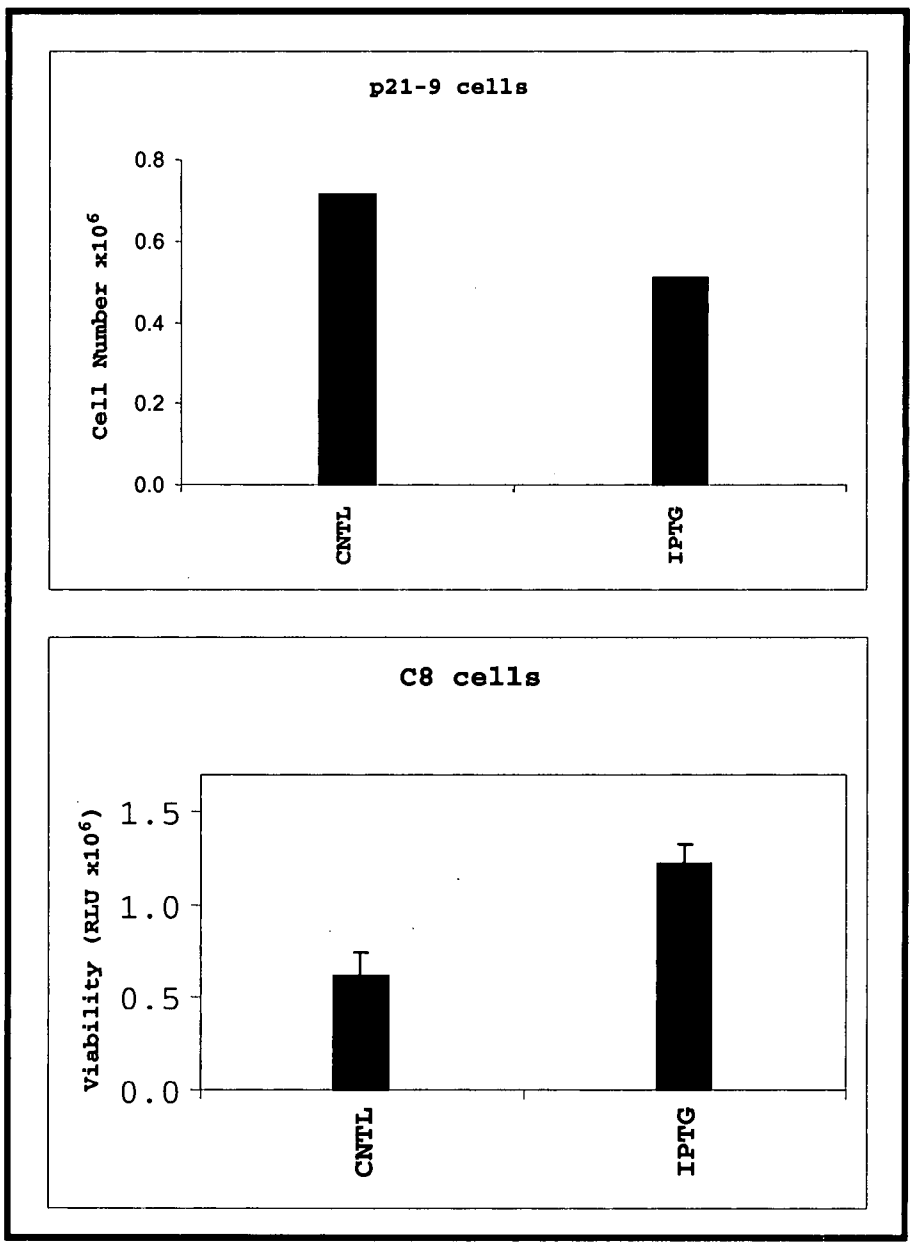

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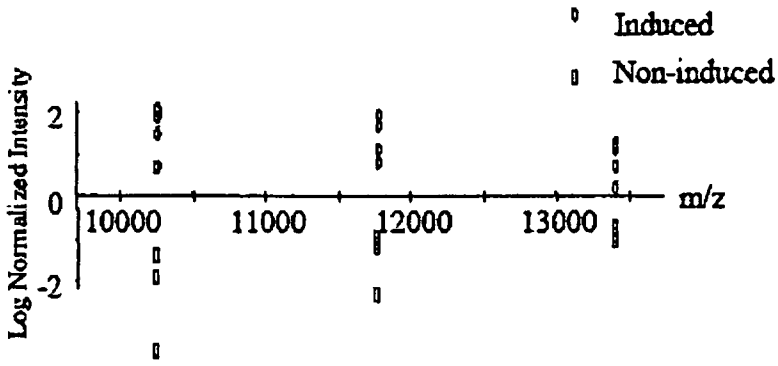
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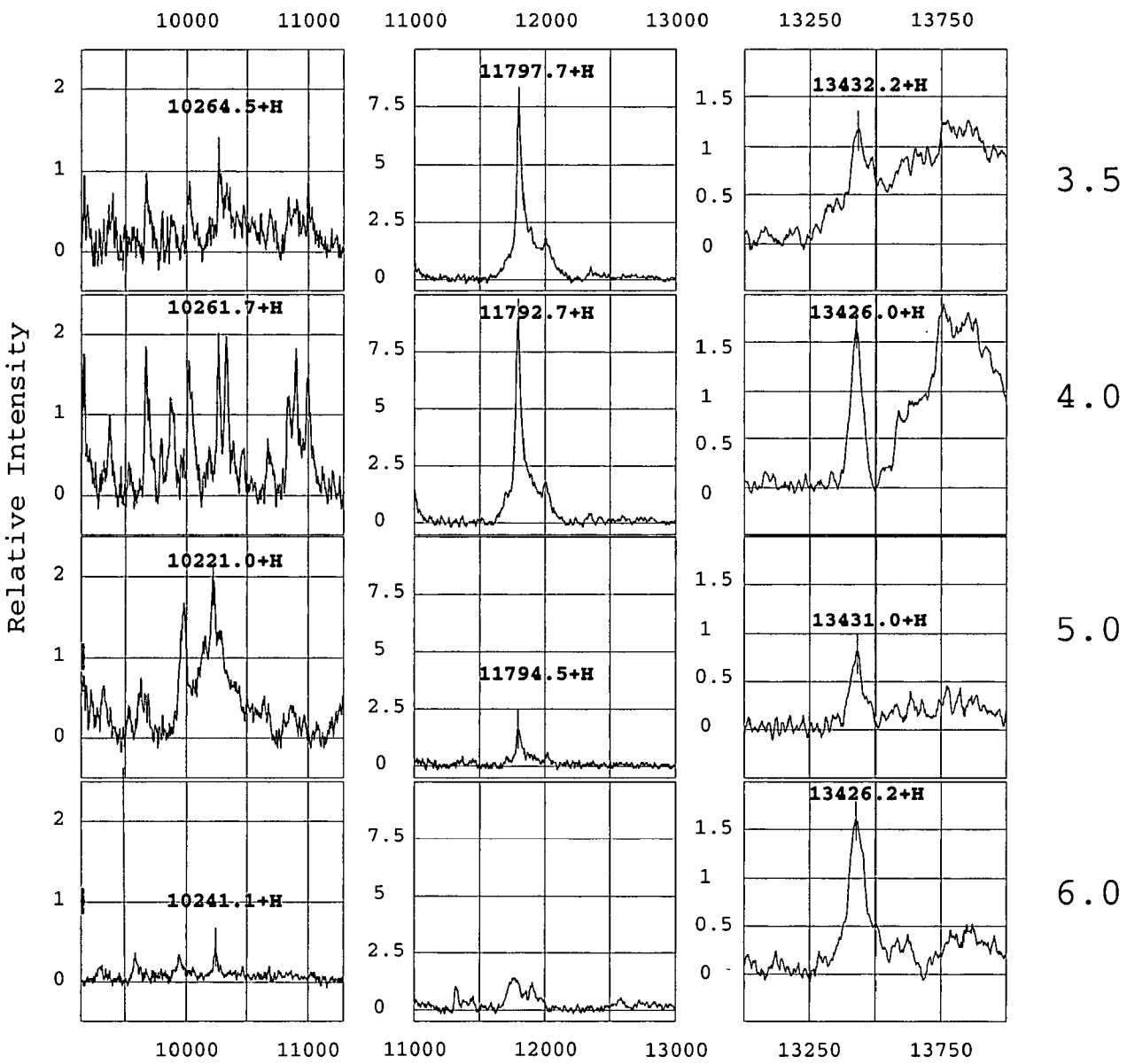
Figure 2a Figure 2b Figure 2c



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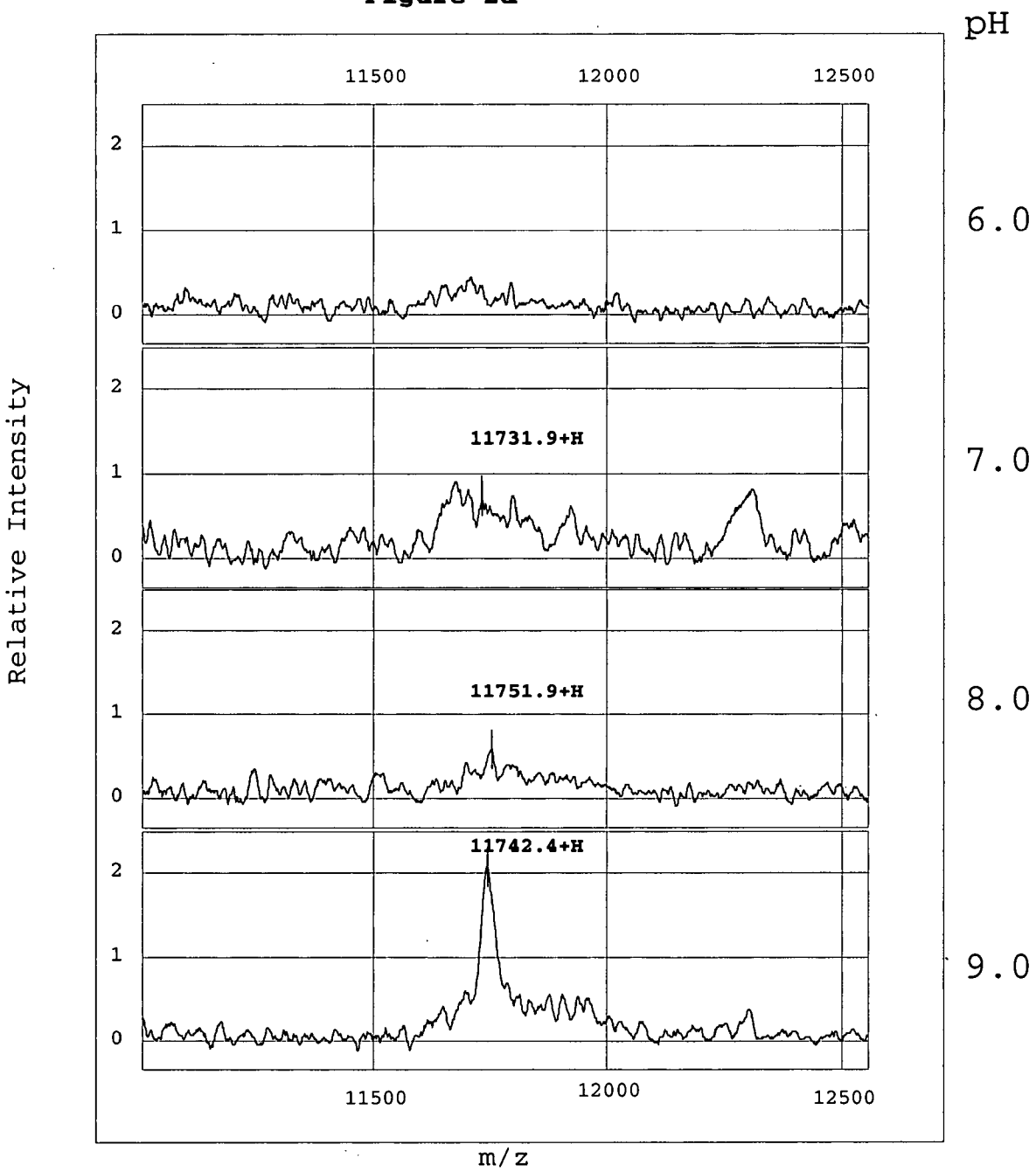
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12500
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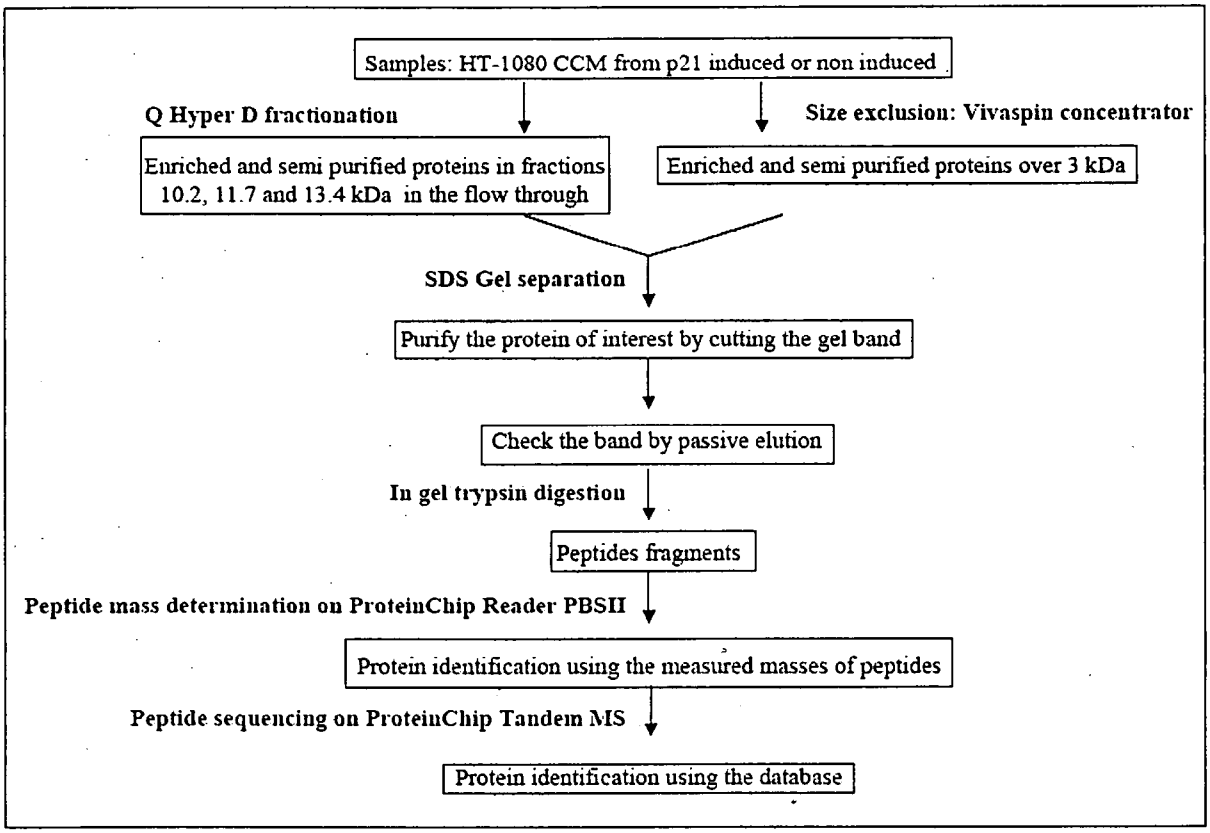
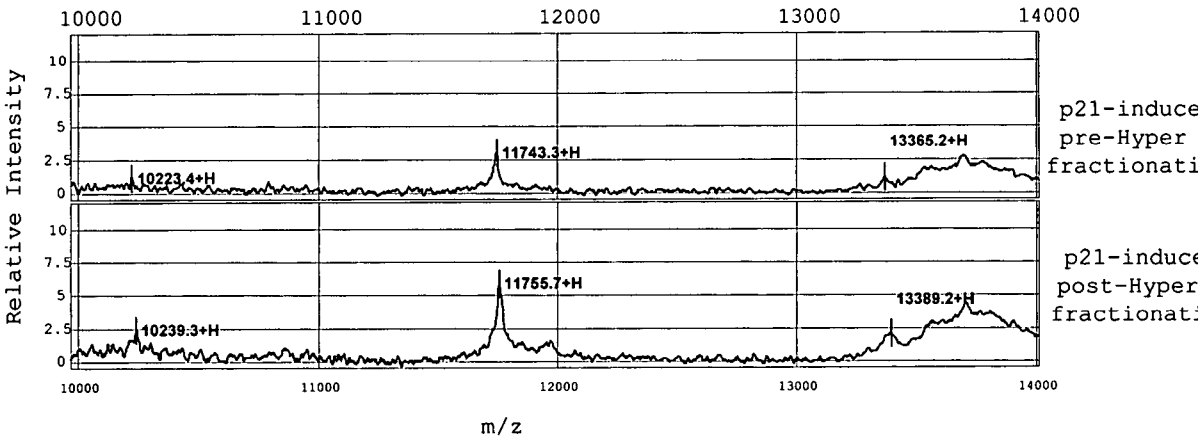
9.0
Relative Intensity
m/z

Figure 3: Purification and identification scheme





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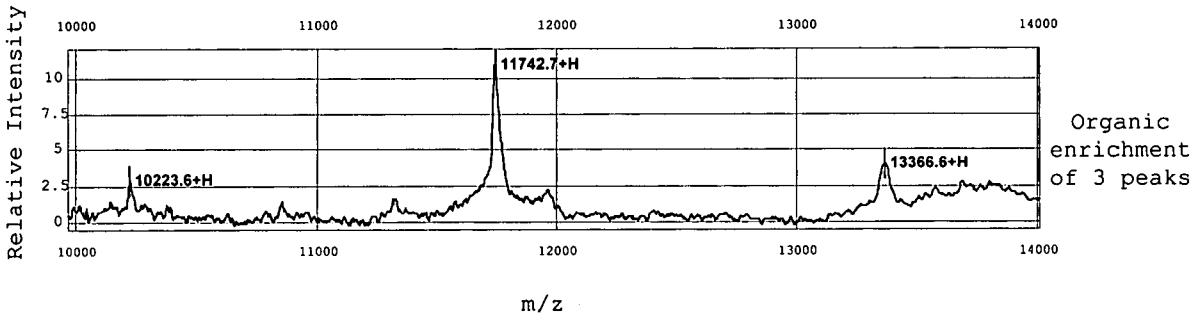
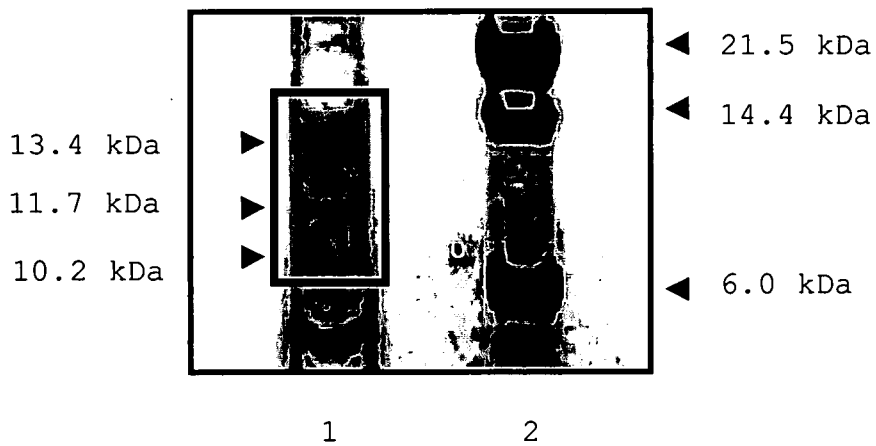


Figure 4c



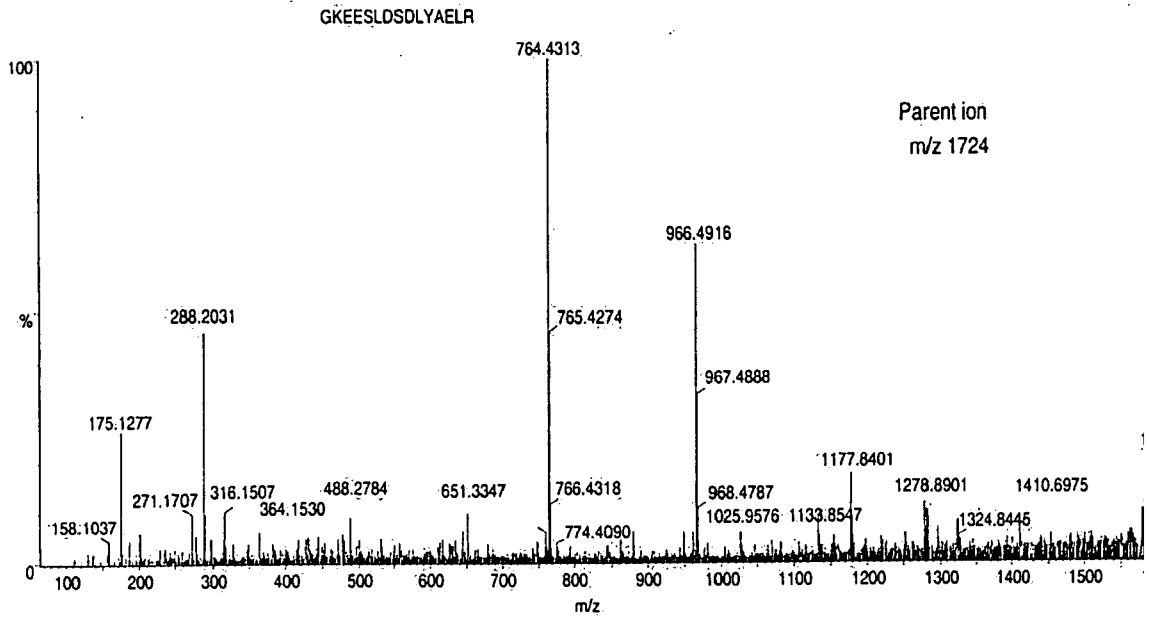


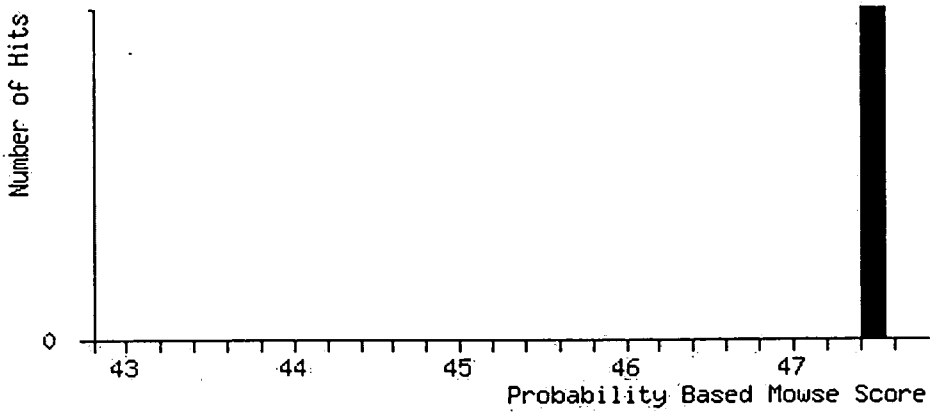
Figure 5b

1. P02775 **Mass:** 13885 **Score:** 47 **Queries matched:** 1
(SZ07_HUMAN) Platelet basic protein precursor (PBP) (Small inducible cytokine B7)

☐ Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
☑ 1	1724.78	1723.77	1723.83	-0.05	1	47	0.00094	1	GKEESLDSDLYAEL

Ions score is -10*Log(P), where P is the probability that the observed match is a random event
Individual ions scores > 15 indicate peptides with significant homology.
Individual ions scores > 30 indicate identity or extensive homology ($p \leq 0.05$).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



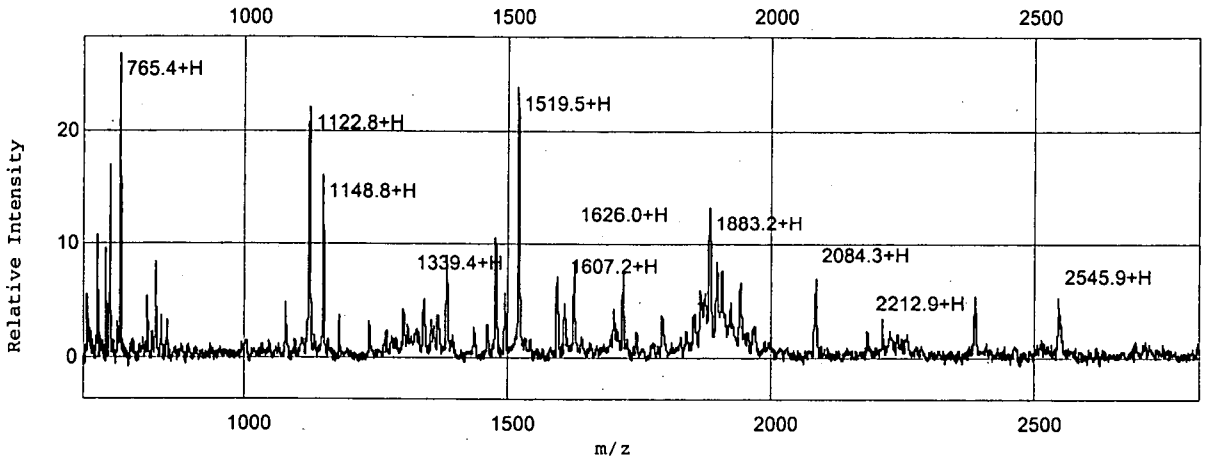


Figure 5d

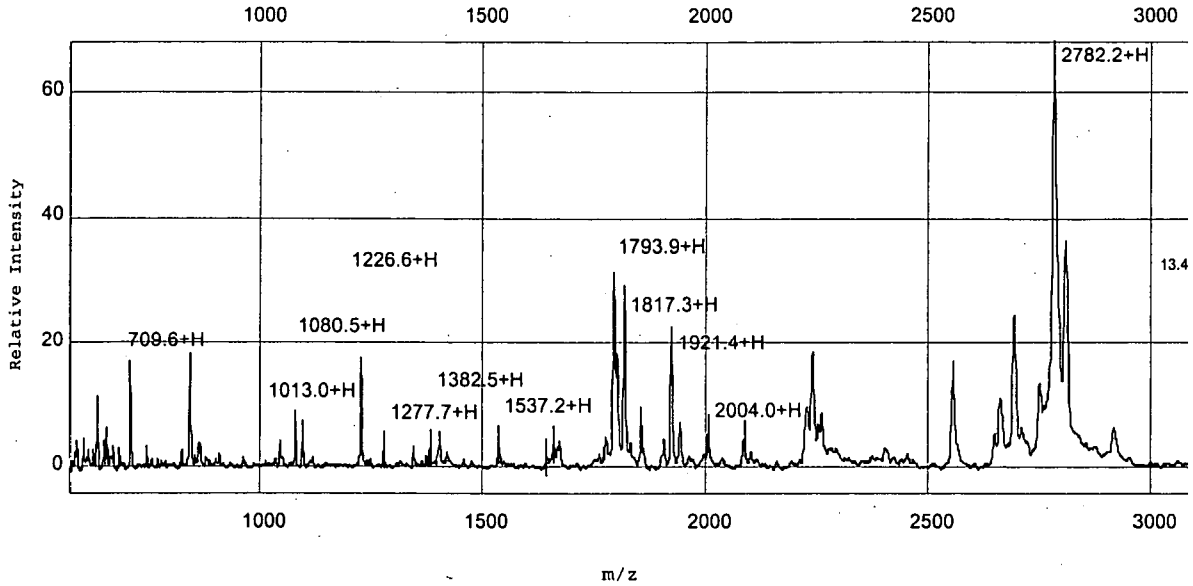
(i)

10	20	30	40	50	60
MSRSVALAVL	ALLSLSGLEA	IQRTPK**IQVY**	**SR**HPAENGKS	NFLNCYVSGF	HPSDIEVDLL

70	80	90	100	110	
KNGER**IEKVE**	**HSDLSFSK**DW	SFYLLYYTEF	TPTEKDEYAC	R**VNHVTLSQP**	**K**IVK**WDRDM**

(ii)

m/z	**amino acids**		**sequence**
722.190	115	119	WDRDM
1122.760	102	111	VNHVTLSQPK
1519.460	66	78	IEKVEHSDLSFSK
765.360	27	32	IQVYSR


1000
1500
2000
2500
3000
60
40
20
0
Relative Intensity
2782.2+H
1793.9+H
1226.6+H
1817.3+H
1080.5+H
709.6+H
1921.4+H
1013.0+H
1382.5+H
1277.7+H
1537.2+H
2004.0+H
13.4
m/z

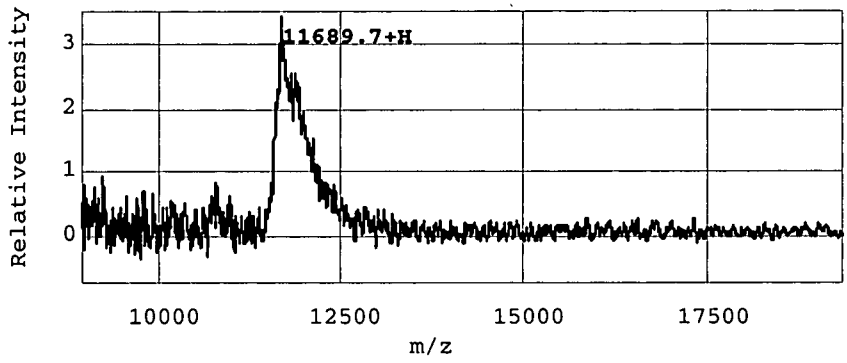

11689.7+H
Relative Intensity
3
2
1
0
10000
12500
15000
17500
m/z

Figure 6b

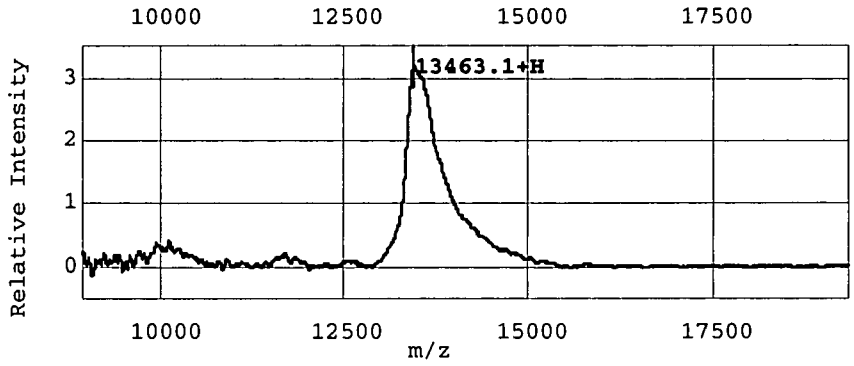
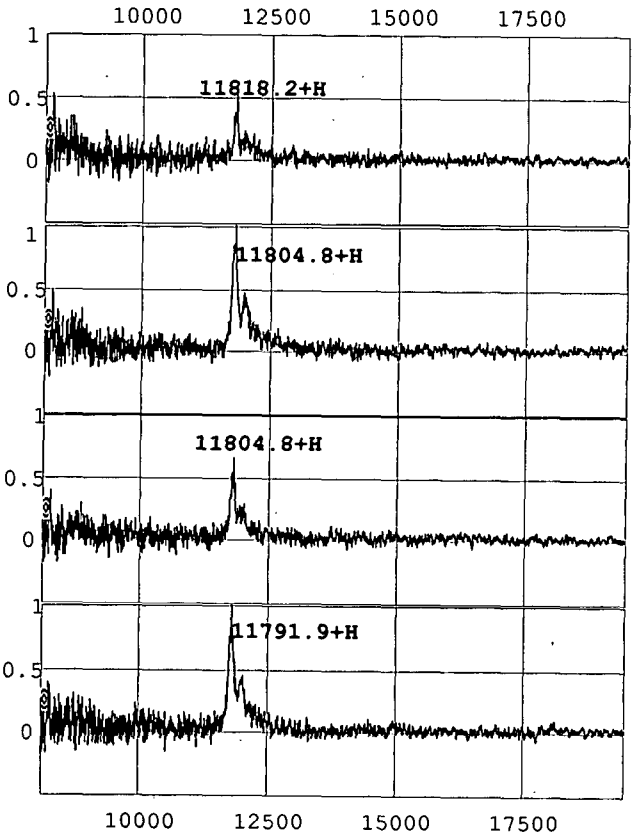

10000
12500
15000
17500
13463.1+H
Relative Intensity
3
2
1
0
10000
12500
15000
17500
m/z

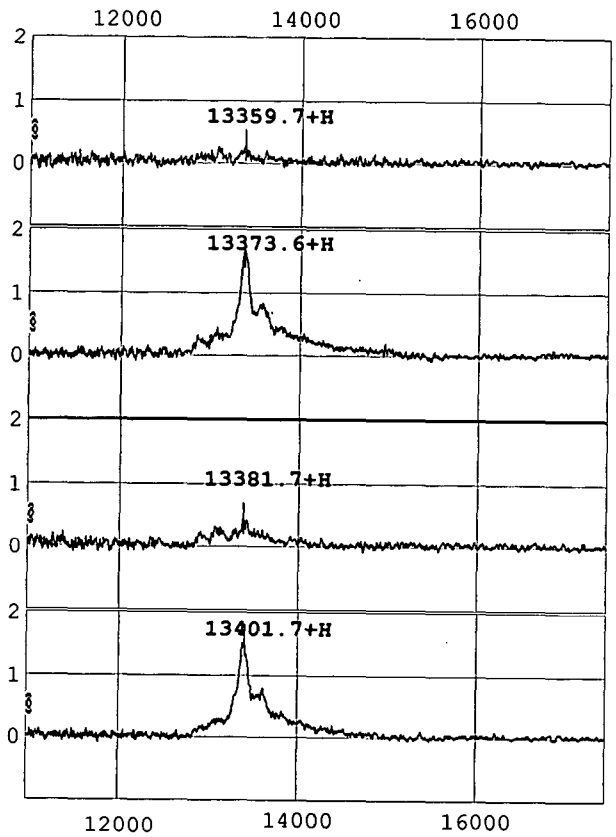
Figure 7a



50μM IPTG | Time post-induction (hours)

50μM IPTG	Time post-induction (hours)
−	48
+	48
−	72
+	72

Figure 7b



50μM IPTG | Time post-induction (hours)

50μM IPTG	Time post-induction (hours)
−	48
+	48
−	72
+	72

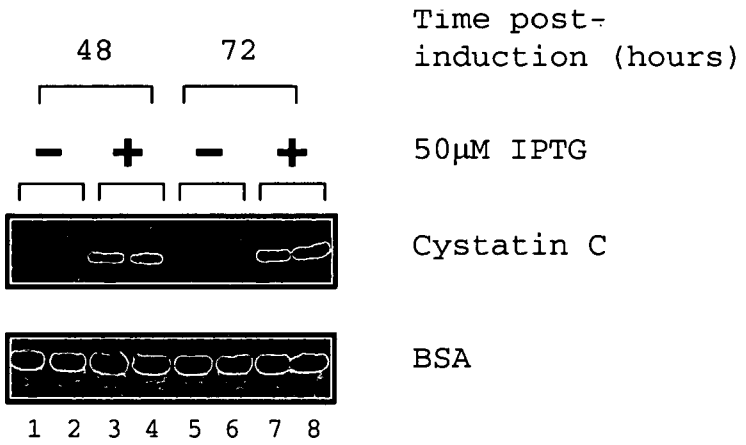
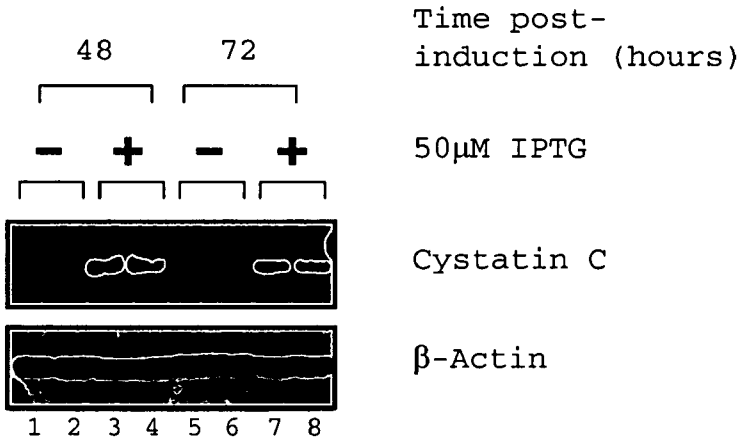

48
72
Time post-induction (hours)
- + - +
50μM IPTG
Cystatin C
BSA
1 2 3 4 5 6 7 8

Figure 8b


48
72
Time post-induction (hours)
- + - +
50μM IPTG
Cystatin C
β-Actin
1 2 3 4 5 6 7 8

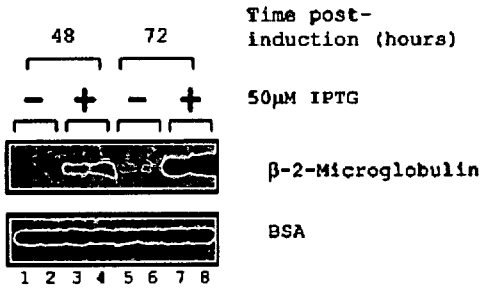
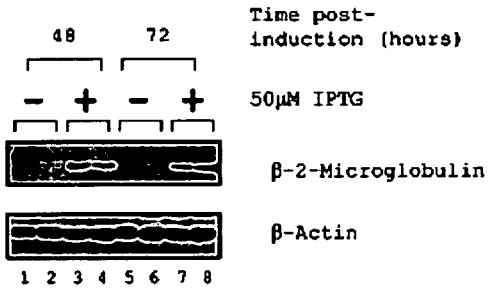
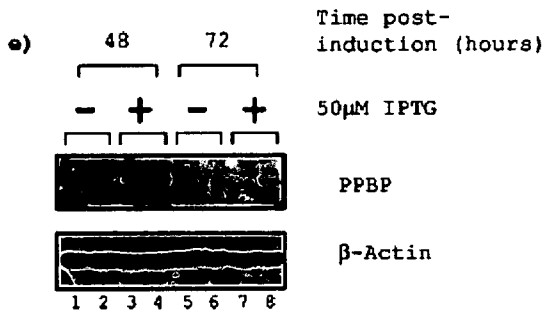

48
72
Time post-induction (hours)
- + - +
50μM IPTG
β-2-Microglobulin
BSA
1 2 3 4 5 6 7 8

Figure 9b


48
72
Time post-induction (hours)
- + - +
50μM IPTG
β-2-Microglobulin
β-Actin
1 2 3 4 5 6 7 8


a)
48
72
Time post-induction (hours)
- + - +
50μM IPTG
PPBP
β-Actin
1 2 3 4 5 6 7 8