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14. ABSTRACT

The main purpose of the study is to identify novel protein-protein interactions in various locations of cells to establish the molecular mechanisms of mutant p53 reactivation with PRIMA-1 in breast cancer cells. To achieve this goal, co-immunoprecipitation/mass spectrometry approaches are used to search for novel proteins that interact with p53 in the cytoplasmic and nuclear fractions of cells. The identity of interacting proteins are validated and confirmed by immunoblot analyses and protein translocation is detected by confocal microscopy. Our approach has identified hsp90 as a partner protein that is associated, in part, with the restoration of p53 transcriptional transactivation function of PRIMA-1.

15. SUBJECT TERMS
p53, proteomics, PRIMA-1, MALDI-TOF spectrometry, breast cancer

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Introduction:

Many clinical studies have shown that p53 mutations are strong predictor of relapse and are associated with resistance to several therapeutic regimens [1,2]. Studies by our laboratories and others, for example, showed that p53 mutations in human tumor cells were correlated with decreased sensitivity to DNA damaging agents [3-6]. Hence an improved understanding of the relationship between p53 and chemosensitivity may lay the groundwork for new cancer therapies. To better understand this relationship, we recently used a pharmacogenomic approach with cDNA microarrays to characterize gene expression profiles of wild-type p53 cells (p53+/+) and an isogenic p53 knockout counterparts (p53-/-) following treatment with topotecan, a specific topoisomerase I inhibitor and a DNA targeted agent [7]. Approximately 10% of the detected transcripts were differentially expressed in the p53+/+ cells in response to topotecan, whereas only 1% of the transcripts changed in the p53-/- cells [7]. These data clearly demonstrated the broad effect of p53 on the transcriptional response to DNA damage, which can lead to growth arrest or apoptosis.

Considering that p53 is the most commonly mutated gene in human cancers and that more than 50% of breast tumors are defective in p53 [8-10], extensive research effort are centered on restoring normal function to mutant p53 to promote tumor suppression. This effort includes the use of modifying peptides [11,12], antisense oligonucleotides [13], and small molecules [14,15]. Unfortunately, the problem of *in vivo* delivery and lack of selectivity to tumor cells has limited the practical application of the most of these efforts. Recently, PRIMA-1 has emerged from an *in vitro* screen of small molecules that reactivate the transcriptional activity of mutant p53 [16]. PRIMA-1 has the capability of restoring the transcriptional transactivation function to mutant p53 *in vitro* and *in vivo* with subsequent tumor regression. PRIMA-1 also has the ability to trigger apoptosis in tumor cells as a function of its mutant p53 reactivation response [17]. We recently reported [18] that PRIMA-1 (in a dose- and time-dependent effect) restored the transcriptional activity of p53 target genes such as $p21^{Waf1/cip1}$, in breast cancer cells with p53 mutation. However, the exact molecular mechanisms for mutant p53 reactivation by PRIMA-1 are not yet determined. A direct interaction between PRIMA-1

and p53 has not yet been demonstrated. It is possible that PRIMA-1 affects cellular chaperones resulting in the refolding of mutant p53 target genes. Alternatively, PRIMA-1 may block complex formation between mutant p53 and p73, leading to the release of active p73 that triggers proapoptotic target genes [15]. To identify the possible molecular candidates of mutant p53 reactivation by PRIMA-1 in breast tumor cells, we used in this study tools available for functional proteomics approach.

Body:

In this study, human MDA-231 breast carcinoma cells were treated with either 100 μM PRIMA-1 for 4 hr or DMSO (control). Cleared cell lysates were immunoprecipitated with DO-1 primary antibody directed against p53, washed, and resolved by SDS-PAGE (4 to 20% polyacrylamide). Figure 1 shows a Coomassie blue-stained gel of proteins co-immunoprecipitated with DO-1 antibody. The arrowhead indicates the band of stained proteins excised for enzymatic digestion by trypsin and subsequent mass fingerprinting with matrix-assisted laser desorption ionization-time-of-flight mass spectrometry. The protein bands were excised from the gels and after reduction and alkylation, proteins were dehydrated in acetonitrile and dried. The bands were digested in the gel with an excess of sequencing-grade trypsin (Progmega, Madison, WI). The digestion was performed overnight at 37°C. The resulting tryptic peptides were extracted from the gels, then desalted and concentrated with C_{18} ziptip (Millipore) before spotting for matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF). An OmniFlex MALDI-TOF mass spectrometer (Bruker Daltonics, Billerica, MA) was used for peptide mass fingerprinting. Desalted peptide solution (1 μl) was mixed with 1 μl of matrix solution (α-cyano-4-hyroxycinnamic acid in 0.1% trifluoroacetic acid and 50% acetonitrile) and was then spotted directly on the MALDI target. Figure 2 illustrates the MALDI mass spectrum acquired from the peptide mixture resulting from in-gel digestion of the 90 kDa bands shown in Fig. 1. On average, six peptide masses were identified in this spectrum that agreed with the expected peptide masses within a mass tolerance of ± 0.24 Da. The peak *m/z* values were used to search the SwissPort Database with the ProFound Program.

Identification of Hsp90 as a candidate target for p53 mutation reactivation.

The SwissPort Database search resulted in the identification of Hsp90 (Klenow fragment) with a probability score of 1.0 and Z score of 1.8, a strong identification of this protein. The sequence coverage of the matched protein candidate was 27%. These results were repeated with multiple co-immunoprecipitation experiments, which all resulted in the identification of hsp90α as a candidate protein that is differentially expressed after treatment of cells with PRIMA-1. To validate and confirmed the identity of this protein, we used immunoblotting assays. Cells were treated with 100 μM PRIMA-1 for 2, 4 or 8 hours, and protein samples from the whole cell extracts (WCE) and nuclear extracts (NE) were Western blotted with antibodies directed against both the α and β forms of Hsp90 protein. As shown in Figure 3, both MDA-231 and GI-101A cells exhibited interaction of p53 and hsp90α protein. The data indicate hat p53 interacts with hsp90α in both breast cancer cells. However, the p53 and hsp90α protein- protein interaction is different in both cell lines. For example, GI-101A cells show no noticeable changes in protein-protein interaction after treatment with PRIMA-1 (Fig. 3b), whereas more hsp90α is bound to p53 in MDA-231 cells after treatment with PRIMA-1 (Fig. 3a). This may reflect the phenotype of cells as well as differences in p53 mutations and polymorphism.

Nuclear translocation of hsp90α after treatment with PRIMA-1

To study nuclear translocation of the identified proteins, we used immunoblotting as well as immunocytochemistry techniques. Figure 4a shows Western blots of hsp90 proteins from control and PRIMA-1 treated cells. In this study the α isoform is expressed in greater amounts that the β-isoform in samples obtained from both WCE and NE. In addition, hsp90α was detected in the NE of protein samples obtained from both cell lines. The level of hsp90α in the NE was increased after treatment of cells with PRIMA-1 for 8 hours. In contrast, the hsp90β isoform was not detected in the NE of protein samples obtained from either cell line, suggesting that only the α isoform of hsp90 is translocated to the nucleus after treatment with 100 μM PRIMA-1. It is possible that the nuclear transport of hsp90α after treatment with PRIMA-1 constitutes part of a selective delivery of restored conformation of p53 to the nucleus. Therefore, we investigated whether the observed nuclear

accumulation of hsp90α after treatment with PRIMA-1 was correlated with any changes in p53 protein expression and/or localization. Figure 4b shows the nuclear accumulation of p53 after treatment of cells with 100 μM PRIMA-1 for 2, 4 or 8 hours. The ratio of the integrated absorbance of p53 bands to that of the actin band was used as an index of protein expression in both WCE and NE. It is clearly shown in Fig. 4b that the levels of p53 protein in the NE are much higher after treatment of both cell lines with PRIMA-1, especially at 8 hours, indicating that the observed increase in hsp90α protein levels in the NE (Fig. 4a) is correlated with that of p53 nuclear accumulation.

Immunocytochemistry to study nuclear localization of the hsp90α

MDA-231 cells treated with PRIMA-1 were subjected to nuclear isolation. A fraction of the isolated nuclear pellet was fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100 and incubated overnight with anti-p53 and anti-hsp90α monoclonal antibodies. Nuclear fractions were then immunostained with a secondary antibody conjugated with Oregon green to detect p53 (green) and a secondary antibody conjugated with Texas red to detect hsp90α (red) before detection by fluorescence microscopy. Nuclei were also stained with 4', 6-diamidino-2-phenylindole (blue). Figure 5 show that hsp90α is selectively localized in the nucleus of MDA-231 breast cancer cells treated with PRIMA-1 for 8 hours.

Conclusions.

This part of the study illustrates the use of a functional proteomics approach to identify target molecules that are associated with the reactivation of p53 mutation by PRIMA-1. Our initial approach has identified hsp90α as a partner protein hat is associated, in part, with the restoration of p53 transcriptional transactivation function by PRIMA-1. We also showed that the α isoform of hsp90 protein is associated with the nuclear translocation of p53 after treatment of breast cancer cells with PRIMA-1

Identification of other novel proteins in the nuclear the cytosolic fractions of cells treated with PRIMA-1

For this study we used chemical cross-linking with 1% formaldehyde to capture novel protein-protein interactions in the sub-cellular compartments of cells as shown in Figure 6. MDA-231 cells were incubated with 1% formaldehyde for cross-linking, and then the reaction was stopped by the addition of 1.25 M glycine and allowed to stand for 5 minutes at room temperature. Prior to purification, the cell lysates were precleared with 1 ml of protein-G agarose beads for 10 minutes at 4° C. The precleared lysates were loaded onto the immunoaffinity columns and allowed to incubate for 2 hours at 4°C. Columns were washed and bound material was eluted with 0.1M-glycine pH 2.5 for 15 minutes at 37°C and rebuffered with saturated Tris-base solution. Elutes were concentrated with an Amicon Ultra-4 centrifugal filter device (Millipore). Samples were boiled for 20 minutes at 115°C to reverse the formaldehyde cross-linking. Proteins were separated on 4-20% SDS-PAGE gels and visualized with silver staining. Figure 7 shows that the maximum cross-linking of proteins can be achieved by incubations of cells with 1% formaldehyde for 60 min. Thus all our cross-linking experiments were preformed by incubation with 1% formaldehyde for 60 min.

Figure 8 shows the reversal of formaldehyde cross-links of sub-cellular fractions. Formaldehyde-treated cell lysates of DMSO and PRIMA-1 treated cells for 24 hr were boiled in 6X sample buffer for 20 min at 95° prior to SDS-PAGE and analyzed by Western blotting with anti-p53 DO-1 antibody. The data indicate that cross-linked proteins associate with p53 mutation reactivation could be isolated in both the cytosol and the nuclear fractions of cells.

Key Research Accomplishments:

1) Performed co-immunoprecipitation assays with DO-1 primary antibody for p53 followed by separation of proteins on SDS-PAGE. Colloidal Coomassie blue was used to detect resolved protein bands. Repeated co-immunoprecipitation assays were performed to establish consistence separation of bands. Differentially separated bands were excised for in-gel protein digestion.
2) Performed mass fingerprinting of tryptic digests with LC/MS/MS/ESI and sequence analysis.
3) Performed protein validation and confirmation of identified proteins with Western blot analyses.
4) Identification of Hsp90 as a partner protein for the restoration of p53 transcriptional transactivation function by PRIMA-1.

Reportable outcomes:

1) Our functional proteomics approach to study protein-protein interaction as a result of p53 mutation reactivation with PRIMA-1 has proven to be very suitable to identify novel proteins involved in the molecular mechanisms of transcriptional reactivation of p53 with PRIMA-1.
2) The involvement of specific novel proteins in the cytosol and the nuclear compartments however requires the use of in vivo cross-linking approach to capture these transient protein-protein interactions to identify molecular targets.
3) Part of the outcomes was published as an abstract in 2005 Era of Hope Conference (16) and as a platform presentation in the same conference (17)

Conclusions

The use of a functional proteomics approach has proven to be very power tool to identify target molecules that are associated with the reactivation of p53 mutation by PRIMA-1. Our initial approach has identified hsp90α as a partner protein that is associated, in part, with the restoration of p53 transcriptional transactivation function by PRIMA-1. We also showed that the α isoform of hsp90 protein is associated with the nuclear translocation of p53 after treatment of breast cancer cells with PRIMA-1. However to establish in vitro protein-protein interactions (partner proteins) profiles in the cytosol and nuclear compartments of the cells, *in vivo* cross-liking approach was used to capture protein-protein interactions in the cytosol and the nucleus fractions of the cells. We have established the maximum conditions for the approach and we are in the process of identifying these proteins using MALDI-TOF spectrometry and validation of proteins with immunoblotting and immunocytochemistry.

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17. Sayed S. Daoud. **Biological pathway discovery and targeted interference.** Symposium 8. Era of Hoop, 2005

Appendices

Figure 1. Coomassie blue-stained gel of proteins co-immunoprecipitated with DO-1 primary antibody from MDA-231 cells. Cleared cell lysates were immunoprecipitated with DO-1 primary antibody directed against p53, washed, and resolved by SDS-PAGE (4 to 20% polyacrylamide). Two independent co-immunoprecipitated samples from untreated control and cells treated with PRIMA-1 were loaded. The gels were stained with Coomassie blue. Molecular masses of protein size markers are indicated (MW). The arrowhead indicates the band of stained proteins excised for enzymatic digestion by trypsin and subsequent mass fingerprinting with matrix-assisted laser desorption ionization-time-of-flight mass spectrometry.

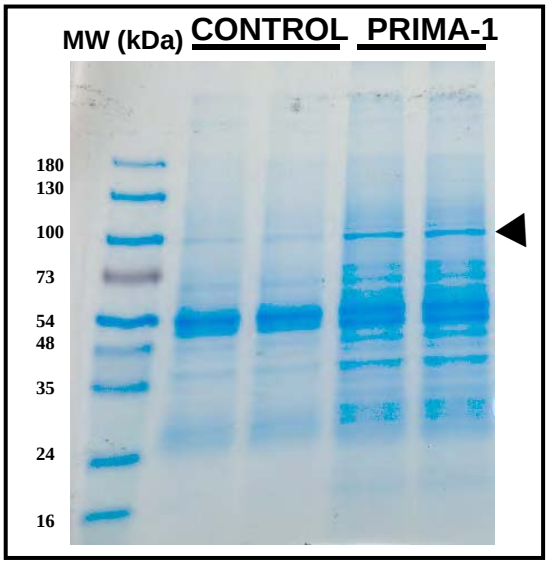


Figure 2. Peptide mass fingerprinting of in-gel tryptic digest of the 90 kDa band generated with a matrix-assisted laser desorption ionization-time-of-flight mass spectrometer. Protein bands indicated by the arrowhead in Fig. 1 were subjected to in-gel digestion and analyzed by mass spectrometry. The tryptic peptides from this band showed the presence of six peptides corresponding to heat shock protein 90 (hsp90) as one of proteins that were found in the altered protein-protein interaction pattern of p53 with and without PRIMA-1 treatment.

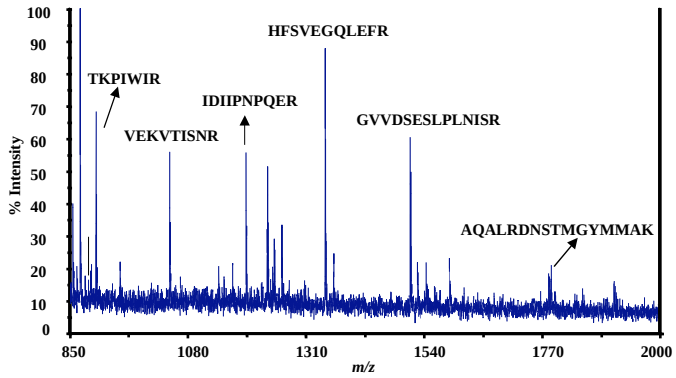


Figure 3. Protein-protein interaction analysis of p53 and the α isoform of heat shock protein 90 (hsp90α). **(a)** MDA-231 cell lysates from untreated cells (lanes 1 and 3) and cells treated for 4 hours with 100 μM PRIMA-1 (lanes 2 and 4) were immunoprecipitated (IP) with anti-hsp90α monoclonal antibody and subjected to Western blotting (WB) with anti-p53 (DO-1) monoclonal antibody (lanes 1 and 2)) in addition to reciprocal immunoprecipitation with DO-1 and Western blotting with anti-hsp90α (lanes 3 and 4). **(b)** GI-101A cell lysates from untreated cells (lanes 1 and 3) and cells treated for 4 hours with 100 μM PRIMA-1 (lanes 2 and 4) were immunoprecipitated (IP) with anti-hsp90α monoclonal antibody and subjected to Western blotting (WB) with anti-p53 (DO-1) monoclonal antibody (lanes 1 and 2)) in addition to reciprocal immunoprecipitation with DO-1 and Western blotting with anti-hsp90α (lanes 3 and 4).

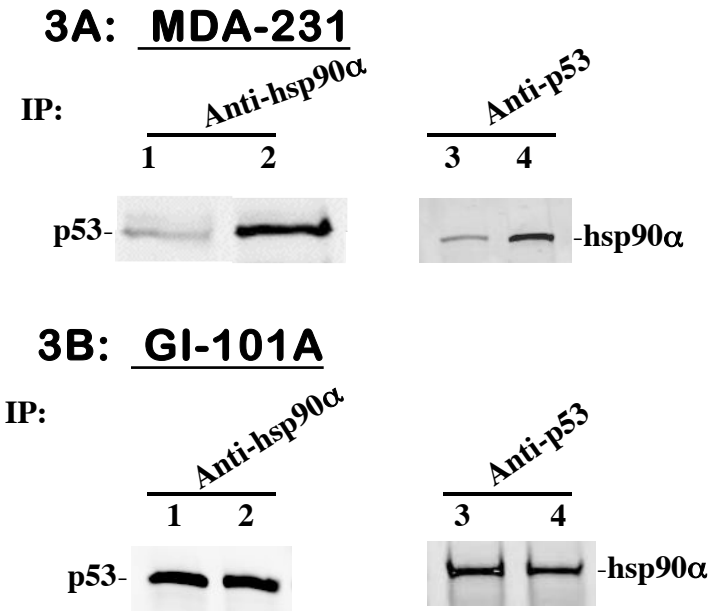


Figure 4. Western blots of heat shock protein 90 (hsp90) from control and PRIMA-1 treated cells. Cells were treated with 100 μM PRIMA-1 for 2, 4 or 8 hours. 20 μg of protein samples of cell lysates from the whole cell extracts (WCE) and nuclear extracts (NE) of the control (C) and treated samples were separated by SDS-PAGE (4 to 20% polyacrylamide) and Western blotted with antibodies directed against both the α and β isoforms of hsp90 **(a)** and against p53 **(b)**. β-actin was used as a loading control. The reactive bands were detected with the Odyssey™ Infrared Imaging System.

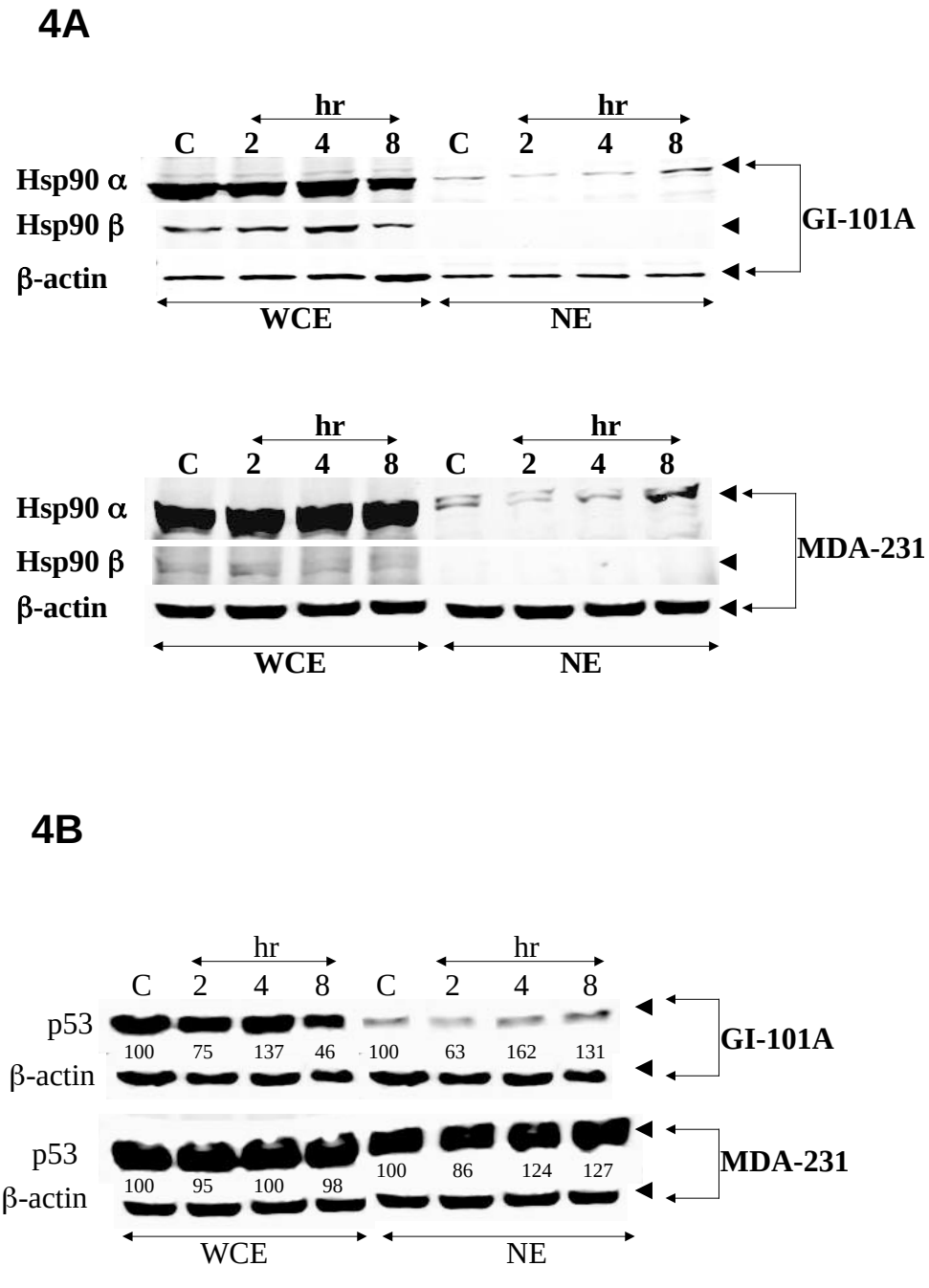


Figure 5. The nuclear localization of the α isoform of heat shock protein 90 (hsp90α) is enhanced by treatment of cells with PRIMA-1. MDA-231 cells treated with PRIMA-1 were subjected to nuclear isolation. A fraction of the isolated nuclear pellet was fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100 and incubated overnight with anti-p53 and anti-hsp90α monoclonal antibodies. Nuclear fractions were then immunostained with a secondary antibody conjugated with Oregon green to detect p53 (green) and a secondary antibody conjugated with Texas red to detect hsp90α (red) before detection by fluorescence microscopy. Nuclei were stained with 4', 6-diamidino-2-phenylindole, DAPI (blue). Normal mouse immunoglobulin G was used as a negative control (data not shown). Arrows mark nuclear staining of hsp90α. Scale bar, 5 μm.

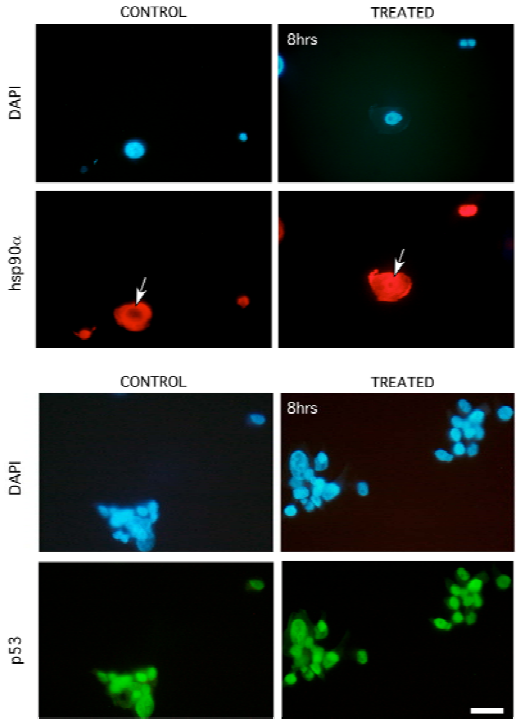


Figure 6. A schematic diagram of in vivo cross-linking approach to capture protein-protein interaction in both cytosol and nuclear fractions of cells.

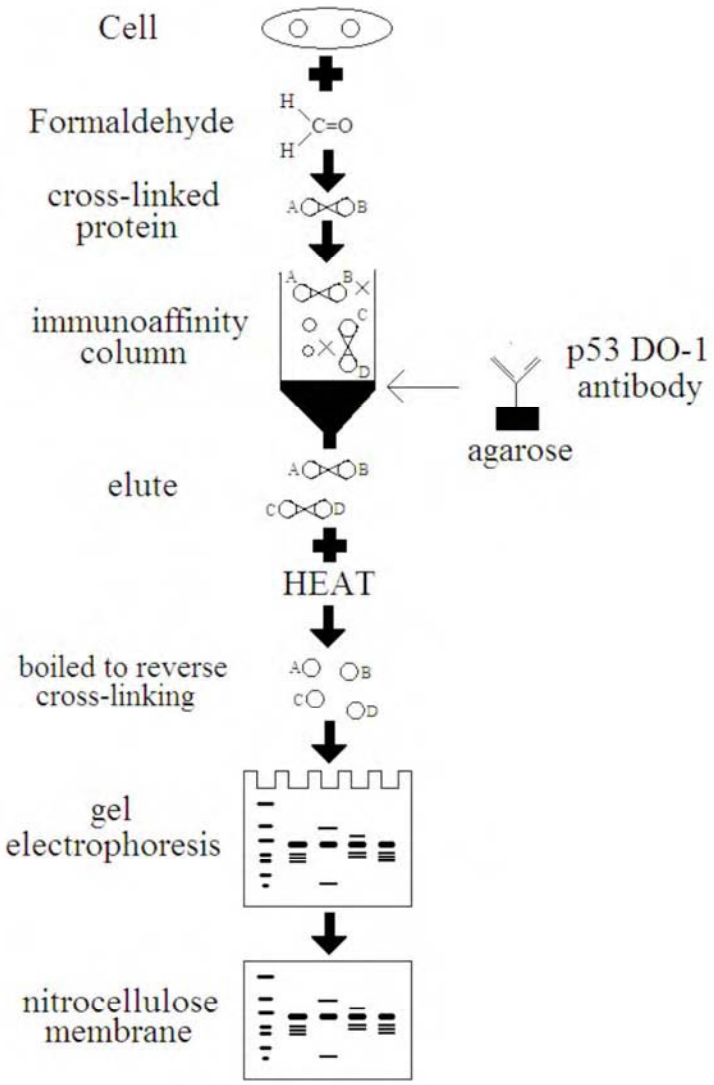


Figure 7. Optimization of in vivo cross-linking of p53 protein following treatment of MDA-231 cells with 100 μM PRIMA-1 for 24 hr. Cells were incubated with 1% formaldehyde for various times. Both X-link and X-link reversal lysates were analyzed by Western blotting with DO-1 primary antibody and bands were detected with the Odyssey™ Infra-red Imaging System.

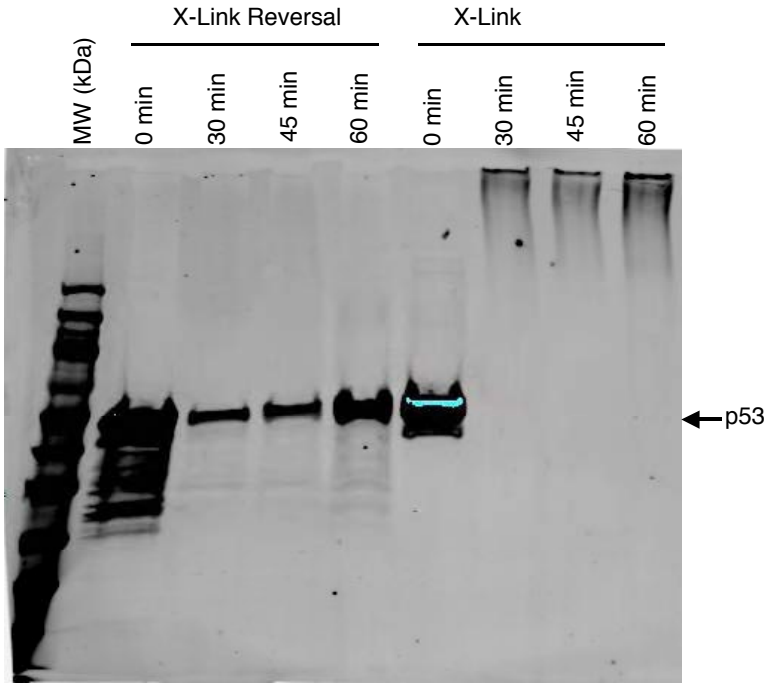
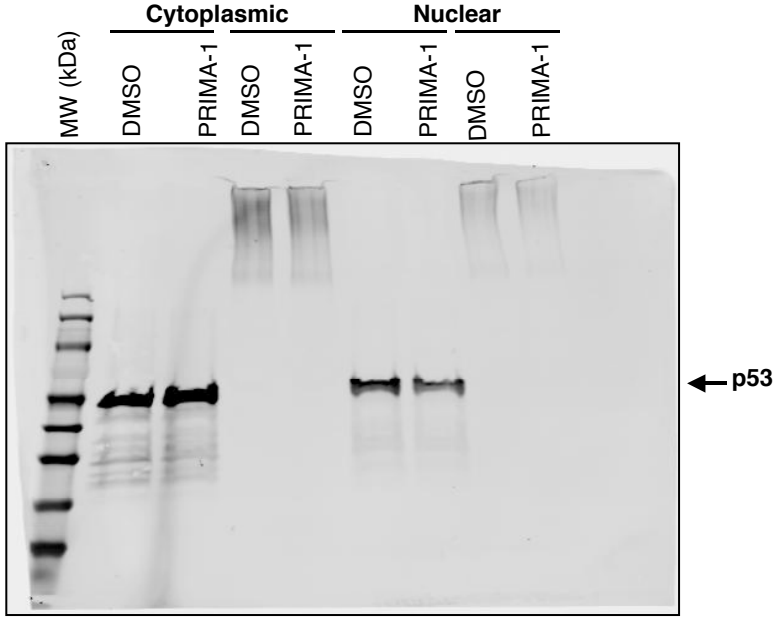


Figure 8. Reversal of formaldehyde cross-links of sub-cellular fractions. Formaldehyde-treated cell lysates of DMSO and PRIMA-1 treated cells for 24 hr were boiled in 5X sample buffer for 20 min at 95° prior to SDS-PAGE and analyzed by Western blotting with anti-p53 DO-1 antibody.



Meeting abstract: 2005 Era of Hop, P27-6

Proteomic Identification of Heat Shock Protein 90 (HSP90) As A Candidate Target for p53 Mutation Reactivation By PRIMA-1 in Breast Cancer Cells. Abdur Rehman, Manpreet S. Chahal, Xiaoting Tang, James E. Bruce, Yves Pommier, Sayed S. Daoud. Department of Pharmaceutical Sciences, Pharmacology and Toxicology Graduate Program, Department of Chemistry, Washington State University, Pullman, WA; Laboratory of Molecular Pharmacology, Center for Cancer Research, National Cancer Institute, NIH, Bethesda, MD.

Mutations in the p53 gene are the most frequent genetic alterations in cancer. Extensive efforts are presently aimed to develop new approaches to cancer therapy based on restoring mutant p53 function by small molecule drugs. We previously reported (Rehman *et al*. Proc. AACR 44: 4592, 2003) that PRIMA-1, a small molecule that is recently identified to reactivate mutant p53 protein, restored the transcriptional activity of p53 target genes such as *p21*, in breast cancer cells with p53 mutation. In an effort to identify the molecular targets that may be involved in restoring mutant p53 function by PRIMA-1, we used a functional proteomics approach based on MALDI-TOF spectrometry. In this approach, cell lysates of samples treated with 100 μM PRIMA-1 for 4 hr were co-immunoprecipitated with DO-1 primary antibody directed against p53. Upon subjecting the immunoprecipitated proteins to one-dimensional gel electrophoresis, we identified a protein of 90 kDa that is distinctly expressed in cells treated with PRIMA-1. After in-gel digestion by trypsin and analyzing samples by a MALDI, we identified this protein to b the heat shock 90 (hsp90). Immunoblot analysis were then used to validate and confirm the identity of hsp90 protein showed only the alpha-isoform of hsp90 accumulated in the nuclear extracts following the treatment of cells with PRIMA-1 for 8 hr. In parallel, we also observed the nuclear accumulation of p53 that led to the transcriptional activation of p21. These studies demonstrate that PRIMA-1 affects cellular chaperones such as hsp90 resulting in refolding of mutant p53 in cells.