Antitumor activity and molecular effects of the novel heat shock protein 90 inhibitor, IPI-504, in pancreatic cancer

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Abstract

Targeting Hsp90 is an attractive strategy for anticancer therapy because the diversity and relevance of biological processes are regulated by these proteins in most cancers. However, the role and mode of action of Hsp90 inhibitors in pancreatic cancer has not been studied. This study aimed to assess the antitumor activity of the Hsp90 inhibitor, IPI-504, in pancreatic cancer and to determine the biological effects of the agent. *In vitro*, we show that pharmacologic inhibition of Hsp90 by IPI-504 exerts antiproliferative effects in a panel of pancreatic cancer cells in a dose- and time-dependent manner. In pancreatic cancer xenografts obtained directly from patients with pancreas cancer, the agent resulted in a marked suppression of tumor growth. Although known Hsp90 client proteins were significantly modulated in IPI-504-treated cell line, no consistent alteration of these proteins was observed *in vivo* other than induction of Hsp70 expression in the treated xenografted tumors. Using a proteomic profiling analysis with isotope tags for relative and absolute quantitation labeling technique, we have identified 20 down-regulated proteins and 42 up-regulated proteins on IPI-504 treatment.tumor growth Identical changes were observed in the expression of the genes coding for these proteins in a subset of proteins including HSPA1B, LGALS3, CALM1, FAM84B, FDPS, GOLPH2, HBA1, HIST1H1C, HLA-B, and MARCKS. The majority of these proteins belong to the functional class of intracellular signal transduction, immune response, cell growth and maintenance, transport, and metabolism. In summary, we show that IPI-504 has potent antitumor activity in pancreatic cancer and identify potential pharmacologic targets using a proteomics and gene expression profiling. [Mol Cancer Ther 2008;7(10):3275–84]

Introduction

Pancreatic cancer is the fourth leading cause of cancer death in the United States (1). Patients with pancreatic cancer show the lowest survival rate due to the high incidence of metastatic disease at the time of diagnosis. Less than 5% of the patients with pancreatic cancer survive more than 5 years after first diagnosis (2, 3). Although there have been some advances in the diagnosis and treatment of this disease, no significant progress in survival rate and quality of life has been made. The development of early diagnostic tools, biomarkers, and therapeutic regimens for pancreatic cancer is an urgent need.

The heat shock protein 90 (Hsp90) is an ubiquitously expressed molecular chaperone responsible for ATP-dependent folding, stability, and function of client proteins that are involved in diverse biological processes such as cell growth and cell viability (4, 5). These client proteins, including ErbB2, Raf-1, cyclin-dependent kinase 4/6, hTERT, src, Bcr/Abl, Akt, HIF-1α, and estrogen/androgen receptors, play important roles in tumor development and progression (6). Due to its multifunctional roles in cells, targeting Hsp90 has been a very attractive strategy in anticancer drug development.

Geldanamycin and its analogue 17-allylamino,17-demethoxy-geldanamycin were shown to inhibit Hsp90 resulting in cell cycle arrest, differentiation, and apoptosis. Hsp90 inhibitors also exhibit antitumor activity with low incidence of drug resistance (7–9). However, solubility and toxicity issues with geldanamycin and its analogue are major obstacles for the clinical development of these drugs. Newly emerging Hsp90 inhibitors with improved water solubility and lower toxicity have been developed and examined in experimental animal models as well as clinical trials with encouraging results (10–12). IPI-504 is a novel Hsp90 inhibitor undergoing clinical development in patients with solid tumors (13–15).

Although it has been established that modulations of Hsp70, cyclin-dependent kinase 4, and c-Raf likely correlate with the clinical efficacy of Hsp90 inhibitors (16), the exact mode of action of this group of drugs remains to be determined. The discovery of pharmacodynamic markers of drug efficacy remains an intense area of research.

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Recently, liquid chromatography (LC)-tandem mass spectrometry (MS/MS)-based proteomic approach and gene expression profiling have been increasingly used for efficient identification and quantitation of proteins and mRNAs in response to therapeutic intervention in colon, ovarian cancer, lung, and head and neck cancer (17–20). Isotope tags for relative and absolute quantitation (iTRAQ) is an amine-specific isobaric tag for quantitation of proteins used in global quantitative proteomics (21).

Our group has focused in the development of patient-derived human xenograft tumors as a preclinical model to study the activity of novel anticancer agents and to discover biomarkers of drug action in pancreatic cancer (22). In this study, we assessed the preclinical antitumor activity of the Hsp90 inhibitor, IPI-504, and sought to identify global molecular signatures of the agent using integrated iTRAQ proteomic analysis and gene expression profiling in human pancreatic cancer.

Materials and Methods

Cell Proliferation Assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays were carried out to measure cell proliferation in response to Hsp90 inhibitor, IPI-504. Briefly, human pancreatic cells including Panc1, Panc430, and XPA3 were seeded in a 96-well plate at a density of 4,000 per well and allowed to adhere for 24 h in RPMI supplemented with 10% fetal bovine serum and penicillin/streptomycin. Cells were incubated with either vehicle control or various concentration of IPI-504 (0, 20, 50, 100, 200, 500, and 1,000 nmol/L) to assess a dose-dependent activity of the drug for 48 h. For the time course studies, cells were exposed in different time schedules (0.5, 4, 24, and 48 h) in the presence or absence of IPI-504 (1,000 nmol/L) treatment. After IPI-504 treatment for the corresponding times, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was done on a microplate reader at an absorbance of 595 nm. All experiments were done in triplicate to determine mean and SD.

Western Blot Analysis

Cells from *in vitro* experiments and tumor tissues from the xenografts before and after treatment with IPI-504 were prepared and lysed in lysis buffer containing 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 0.5% NP-40, phosphatase inhibitors (EDTA-free protease inhibitors), 20 mmol/L β-glycerophosphate, 20 mmol/L NaF, and 1 mmol/L $NaVO_3$. Equal amounts of protein (60 μg cell lysates and 100 μg tissue lysates) were separated on a 4% to 12% gradient SDS-PAGE (Bio-Rad) and transferred onto a nitrocellulose membrane. Membranes were incubated with 5% milk in TBST [20 mmol/L Tris-HCl (pH 7.5), 500 mmol/L NaCl, and 0.5% Tween 20] at room temperature for 1 h and probed with primary antibodies (1:1,000; p-Erb2, p-p70S6K, p-Akt, t-Akt, p-Erk, t-Erk, Hsp70, and actin from Cell Signaling Technology) in either 5% bovine serum albumin or nonfat dried milk in TBST overnight at 4°C. After washing three times with TBST for 5 min, membrane was incubated with secondary anti-rabbit or anti-mouse antibody for 1 h at ambient temperature and visualized by enhanced chemiluminescence (Amersham) detection.

Drug and Treatment Protocol

IPI-504 was provided by Infinity Pharmaceuticals. All the protocols and procedures related to drug administration followed the manufacturer's guideline. All the animal protocols and procedures strictly followed the Animal Care and Use Committee of the Johns Hopkins University regulations. Animal care was done under sterile condition throughout the study. The Viking Medical Vaporizer was used as an anesthesia.

Experimental Treatment

Primary cancer specimens obtained from patients with resected pancreatic cancer at Johns Hopkins Hospital as reported previously were used (22). In brief, tumor specimens obtained from patients at the time of resection were implanted in 18 to 22 mice, which were randomized when reaching a tumor size of about 200 mm^3 into two groups with 5 mice each in either untreated control or IPI-504 treatment (75 mg/kg, thrice per week for 6 weeks). Animals were checked daily for adverse effects. At 24 h after the end of last dosing, mice were sacrificed by CO_2 inhalation. For assessing tumor growth inhibition (TGI), tumor volumes were measured two times per week throughout the study and calculated using the formula: TGI: T / C = (mean tumor volume of drug-treated group / mean tumor volume of control group) × 100. TGI < 50% was considered as a criterion for significant activity.

iTRAQ Labeling and Strong Cation Exchange Fractionation

Tumor tissues from Panc253 and Panc410 untreated controls and IPI-504 treatment were collected, homogenized in 0.05% SDS, and sonicated on ice. The protein lysates were used for iTRAQ labeling experiment. Briefly, 100 μg protein from each tissue was reduced, alkylated, and digested using trypsin overnight. The tryptic peptides from control and drug-treated tissues were labeled with iTRAQ reagents having 114, 115, 116, and 117 reporter ions. The labeled samples were mixed, acidified, and subsequently fractionated using strong cation exchange chromatography on PolySULFOETHYL A column (PolyLC; 100 × 2.1 mm, 5 μA particles with 300 Å pores using LC packing systems). Forty-four strong cation exchange fractions (0.2 mL each) were collected using a gradient of 0 to 350 mmol/L KCl in 10 mmol/L potassium phosphate (pH 2.85) containing 20% acetonitrile with continuous monitoring UV absorption at 214 nm. The peak fractions were then dried and reconstituted in 10 to 20 μL of 2% trifluoroacetic acids depending on intensity of UV traces.

Microarray Gene Expression

Total RNA was prepared from tumor tissues of IPI-504-treated and untreated tumors according to manufacturer's instruction (Qiagen). Gene expression analysis was done at the Johns Hopkins Microarray Core Facility using the Affymetrix HG-U133 Plus 2.0 human gene chip according to the manufacturer guidelines. Each sample from IPI-504-treated and untreated groups was profiled in duplicates

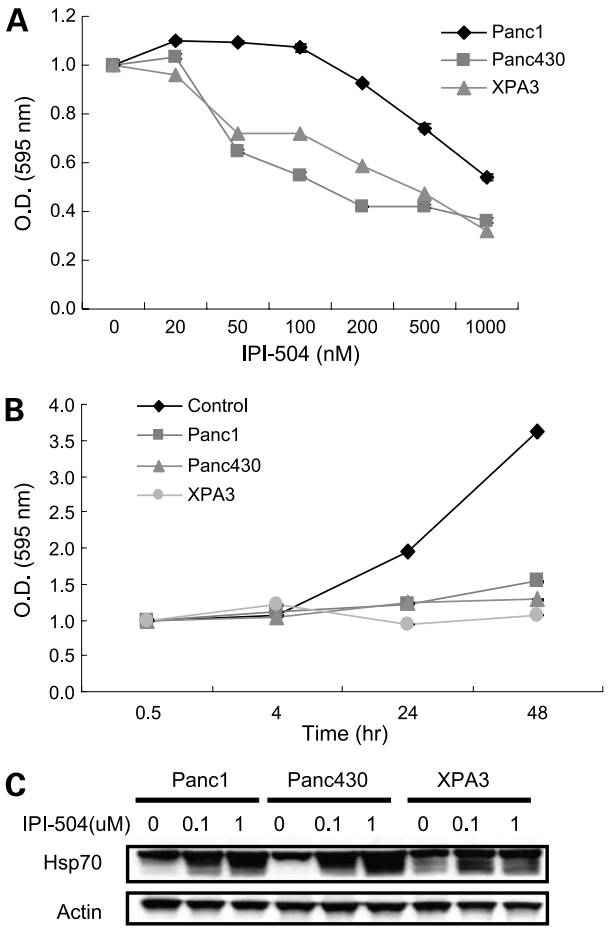

Figure 1. IPI-504 induces antiproliferation activity in pancreatic cancer cells in a time- and dose-dependent manner. **A** and **B**, Panc430, XPA3, and Panc1 cells were seeded and maintained with RPMI containing 10% fetal bovine serum for 24 h before drug treatment. Cells were treated with either indicated concentrations of IPI-504 for 24 h (**A**) or corresponding times at 1 μmol/L IPI-504 (**B**). At the given conditions, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was carried out at A595 nm on a microplate reader. Representative of a minimum of three independent experiments. **C,** cells were treated with IPI-504 in the indicated concentrations of IPI-504 for 48 h, harvested, and lysed for Western blotting to detect Hsp70. Actin was used as a loading control.

(GSE12113). The HG-U133 Plus 2.0 gene chip containing ~55,000 transcripts represent ~20,600 known human genes. Data extraction was done using Affymetrix MAS5.0 software.

Differential Gene Expression Analysis

Signal-to-noise ratio (*S2N*; ref. 23) was used to identify differential gene expression in IPI-504-treated versus untreated cases. Let μ_1 (μ_2) and σ_1 (σ_2) represent mean and SD for the treated (untreated) cases. The *S2N* is computed as $(\mu_1 - \mu_2) / (\sigma_1 + \sigma_2)$. A positive value of *S2N* indicates that the gene correlates with IPI-504-treated cases. Conversely, a negative value of *S2N* indicates that the gene correlates with untreated cases. With 2-fold of expression changes after IPI-504 treatment, a gene is considered up-regulated if the *S2N* > 1.4 and down-regulated if the *S2N* < -1.4. *P* values for the differentially expressed genes were calculated by paired Student's *t* test with $P < 0.05$ is considered significant.

Gene Set Enrichment Analysis

We employed Gene Set Enrichment Analysis (GSEA; ref. 24) version 2.0 in this study[7] to identify pathways in IPI-504-treated versus untreated cases. GSEA employs statistical significance tests to determine if a given gene set is enriched in a biological phenotype gene expression profile. We collapsed the probes into genes and did 500 gene set permutations on the gene expression profiles to assess the statistical significance of the pathways. Pathways with $P < 0.05$ were considered significant in this study. False discovery rate (q value) is computed by the GSEA program. Pathways that have less than 10 gene members in a set were excluded from the GSEA analysis.

Compilation of Pathway Gene Sets

We have analyzed 192 gene sets consisting of pathways defined by Kyoto Encyclopedia of Genes and Genomes database to stratify the altered gene expression on IPI-504 exposure into biological signaling pathways. Human pathway annotations were downloaded from Kyoto Encyclopedia of Genes and Genomes (August 2007 release) and mapped the pathway annotations to Affymetrix HG-U133 Plus 2.0 probe sets using the gene symbols available from Affymetrix Web site.

LC-MS/MS Analysis

The samples were analyzed by reverse-phase nano-LC MS/MS using quadruple time-of-flight MS (QSTAR, Applied Biosystems). Reverse-phase LC system consisted of desalting column (75 μm × 3 cm, C_{18} material 5-10 μm, 120 Å) and analytical column (75 μm × 10 cm, C_{18} material 5 μm, 120 Å). Solvent gradient containing acetonitrile (0-40% for 30 min) and 0.1% formic acid was used for peptide elution for LC-MS/MS analysis. MS were acquired in a data-dependent manner selecting three most abundant ions for MS/MS fragmentation and then excluded for 45 s to avoid the repeated MS/MS analysis. ProteinPilot software version 2.0 (Applied Biosystems MDS SCIEX) was used for searching MS data against RefSeq protein sequence database (version 26). The area of spectra of reporter ions in MS/MS spectra of peptides was used for quantitation. More than one peptide with >95% confidence score was selected for quantitation.

Immunohistochemical Labeling

Immunohistochemistry in formalin-fixed, paraffin-embedded tissue sections was used as a validation method for the MS results. The Envision kit (DAKO) was used according to the instructions. Staining for MHC class 1B, galactin 3, biglycan, and Golgi phosphoprotein 2 (GOLPH2) was done using anti-MHC class 1B (R&D Systems), anti-galactin 3 (R&D Systems), anti-biglycan (R&D Systems), and anti- GOLPH2 (Imgenex) antibodies at a dilution of 1:100 or as per the manufacturer's recommendations. The slides were first deparaffinized by xylene and rehydrated with ethanol. Antigen retrieval was done by heating the

[7] http://www.broad.mit.edu/gsea/

slides in 0.01 mol/L sodium citrate for 20 min on a streamer. Peroxidase activity was blocked by incubating in hydrogen peroxide for 5 min at room temperature. The signal was developed using DAKO chromogen supplied for peroxidase. Then, the slides were incubated with primary antibodies (p-HER-2 and p-MAPK, Cell Signaling Technology, dilution 1:200 and p-AKT, Cell Signaling Technology, dilution 1:50) and the corresponding horseradish peroxidase-conjugated secondary antibody. The reaction was detected by chromogen and hematoxylin staining.

Results

IPI-504 Inhibits Cell Proliferation in a Time- and Dose-Dependent Manner

A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was done in Panc1, Panc430, and XPA3 pancreatic cancer cells to assess whether IPI-504 inhibits cell proliferation in these cells. As shown in Fig. 1A, the agent induced growth inhibition with an IC_{50} that ranged from 0.14 μmol/L in Panc430 to 1.2 μmol/L in Panc1. Figure 1B shows the time-dependent effect of the drug. This activity correlated, as shown in Fig. 1C, with induction of Hsp70.

IPI-504 Shows Antitumor Activity in Pancreatic Xenografted Tumors

We next examined the antitumor activity of IPI-504 in six pancreatic cancer xenografts (Panc253, Panc185, Panc194, Panc410, Panc420, and Panc294). For comparison, the efficacy of gemcitabine, the agent used to treat pancreatic cancer in the clinic, is also presented. The drug was markedly active against the Panc420, Panc253, and Panc410 with TGI of 26%, 28%, and 36%, respectively, whereas Panc294, Panc185, and Panc194 with TGI of 46%, 51%, and 55%, respectively, exhibited modest growth inhibition (Fig. 2A). No sign of adverse events have been observed throughout the study.

Lack of Regulation of Classic Hsp Signaling Targets

Next, we evaluated the pharmacodynamic effects of IPI-504 in the xenograft model. It has been documented that geldanamycin and its derivatives regulate Hsp90 client

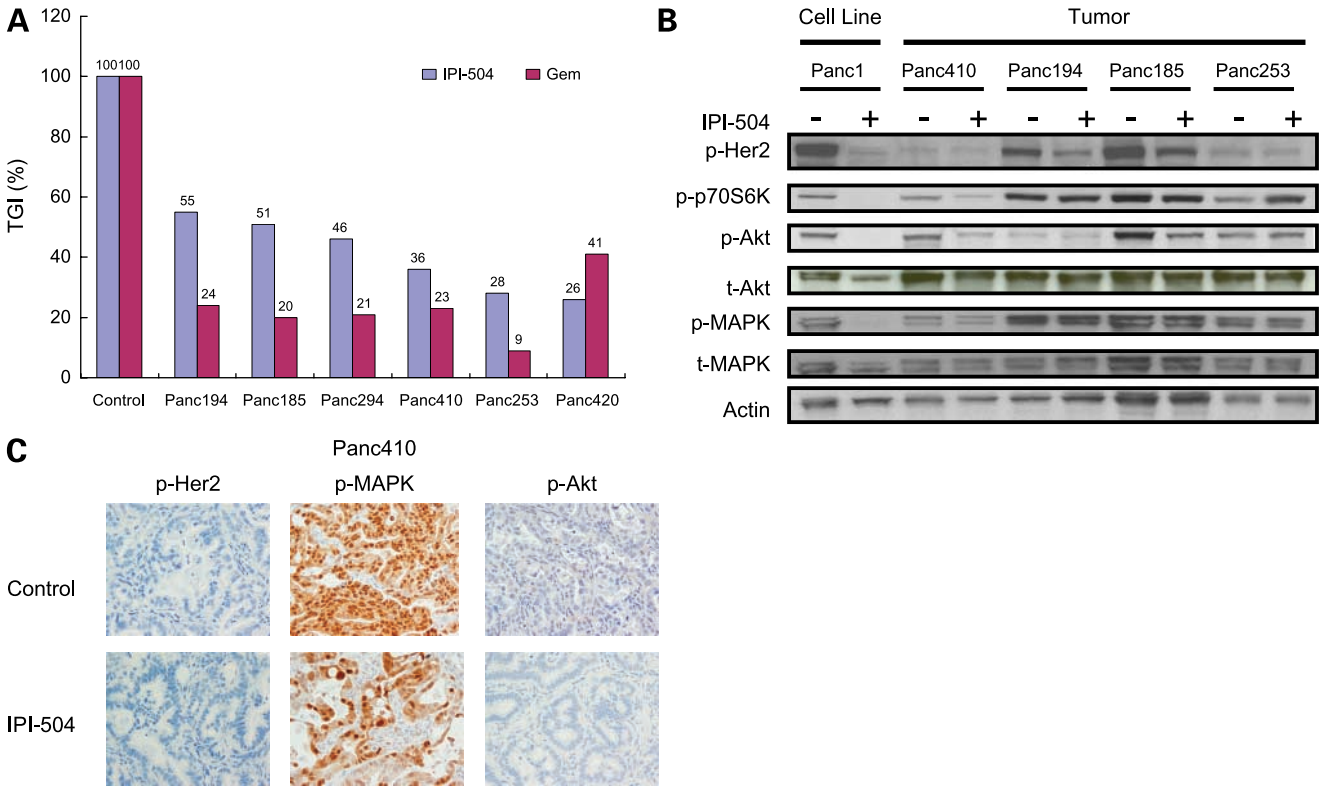

Figure 2. Antitumor effect of IPI-504 on pancreatic tumor xenografts. **A**, antitumor activity of Hsp90 inhibitor. Each tumor tissue from Panc253, Panc185, Panc410, Panc194, Panc294, and Panc420 was implanted into athymic nude mice (female; $n = 5$ per group) and randomized into two groups (untreated control versus treatment group) on reaching a tumor size of about 200 mm^3. IPI-504 was administered by i.p. injection at dose of 75 mg/kg thrice a week for 39 to 43 d. Tumors were measured using calipers two times a week. Tumor volumes were calculated: $[(\text{width})^2 \times \text{height}] / 2$. **B**, Hsp90 inhibitor reduces its client proteins solely *in vitro* cells but not in xenografted tumor tissues. Cell lysate from Panc1 was prepared 48 h after IPI-504 treatment (1 μmol/L). Xenografted tumor tissues from Panc410, Panc194, Panc185, and Panc253 were collected in both untreated control and IPI-504-treated groups. All lysates were prepared and used for the detection of indicated proteins by Western blotting. Actin was used as an internal control. Expression of client proteins in Panc1 cell line showed markedly decreased with IPI-504 exposure, whereas tumor patients exhibited no alteration of the proteins. **C**, immunohistochemical staining of client proteins in Panc410. Using DAKO staining kits, the paraffin-embedded tissues were stained with p-HER-2, p-MAPK, and p-Akt. There is no change of staining intensity in IPI-504 treatment cases compared with untreated control.

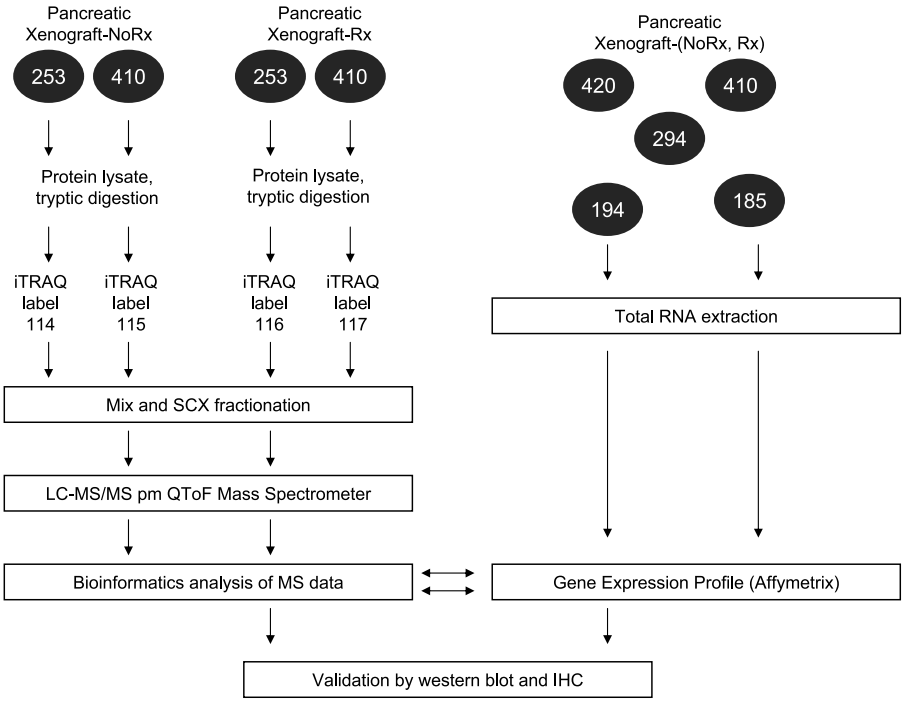

Figure 3. A schematic diagram of the strategy used for quantitative gene expression and proteomic analysis of Hsp90 inhibitor. Tissue homogenates were prepared from the drug-treated and untreated tumors. The protein samples were digested using trypsin and peptides were labeled with iTRAQ reagents. Labeled peptides were combined and fractionated by strong cation exchange chromatography and subsequently analyzed by LC-MS/MS. MS data were analyzed using public bioinformatic resources to identify a candidate proteins. For gene expression profile, total RNA from IPI-504-treated and untreated tumors was extracted and analyzed by Affymetrix gene expression assay. Altered proteins were validated by Western analysis and immunohistochemical staining.

proteins including Erb2, Akt, Erk, and p70S6K (6, 25). Although the drug, as predicted, down-regulated the majority of Hsp90 client proteins including Erb2, Akt, Erk, and p70S6K in tissue culture, no consistent effects were noted in xenograft tissues neither in Western blot nor in immunohistochemical studies (Fig. 2B and C). This indicates that IPI-504 may have an elusive molecular mechanism to abrogate the tumor growth in pancreatic cancer and that known client proteins may not be useful as pharmacodynamic markers of IPI-504. To further investigate this issue, we have conducted a gene expression and proteomics profiling tumor tissues from untreated control and IPI-504-treated groups to identify the molecular signatures and gene and/or protein expression patterns on drug exposure (Fig. 3).

iTRAQ-Labeled MS and Gene Expression Microarray Analysis

We chose two pairs of tumor tissue untreated and treated with IPI-504 for labeling using four iTRAQ tags. This is a promising multiplexing approach to study proteomic changes because all the tryptic peptides generated will be tagged at NH_2 terminus and ε-amine groups. LC-MS/MS analysis of 44 strong cation exchange fractions of iTRAQ-labeled peptides generated 65,472 MS/MS spectra. The fold changes were calculated from the ratio of intensity of iTRAQ reporter ions obtained from treated tumor peptides to those derived from untreated tumor peptides. In parallel with proteomic approach, gene expression was profiled in the xenografted tumor tissues from IPI-504 untreated and treated groups to investigate the concordance of these two agnostic system biology methods. The complete list of proteins modified by IPI-504 is listed in Supplementary Table S1.[8]

Proteins Down-regulated in Tumors Treated with IPI-504

With a fold change of 0.75, we observed 20 proteins down-regulated on IPI-504 treatment in tumor tissues when compared with the corresponding untreated control (Supplementary Table S2).[8] Interestingly, analysis of these proteins in human protein database[9] revealed that most of them are involved in macromolecule metabolism, cell localization, cell growth and maintenance, and transport (Fig. 4A). In addition, a majority of the down-regulated proteins were localized to the cytoplasm (70%) and organelle membrane (12%; Fig. 4B). Selected examples of these proteins included farnesyl diphosphate synthase, a peroxisomal protein overexpressed in colon cancer (26); epidermal growth factor receptor pathway substrate 8, a well-studied downstream signaling molecule in the epidermal growth factor receptor pathway known to participate in actin remodeling; GOLPH2, which is involved in transport function and is up-regulated in hepatocytes of patients with viral hepatitis (27) and has been reported as a potential serum biomarker for hepatocellular carcinoma (28); and MHC class IB, which plays a central role in antigen presentation in immune responses and is known to be involved in many types of cancer including breast,

[8] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).
[9] hprd.org

cervical, and lung (29–31). Other studies have shown that Hsp90 inhibitors suppress MHC class I antigen processing (32). To validate these findings, we show a decrease in the expression of GOLPH2 and MHC class IB proteins by immunohistochemistry (Fig. 5A and B) in treated tumors. In addition, we fragmented each peptide in GOLPH2 and MHC class IB. Figure 6C and D shows a representative spectra of a peptide identified by MS/MS analysis. The intensity of iTRAQ reporter ions decreased in IPI-504-treated groups, suggesting that these proteins were deregulated by the drug.

Proteins Up-regulated in Tumors Treated with IPI-504

With a 1.25-fold threshold, we found 42 proteins up-regulated in treated tumors (Supplementary Table S3).[8] Two of these proteins (biglycan and LGALS3) were further validated using immunohistochemical staining of tissue sections (Fig. 5A and B). The majority of the up-regulated proteins were involved in cellular metabolism and cell organization and biogenesis. Other important functions of up-regulated proteins were transport and biosynthesis (Fig. 4C). Localization data show many of these proteins resided in the cytoplasm and membrane-bound organelle (Fig. 4D). Biglycan, a proteoglycan closely resembles decorin and fibromodulin, is known to be involved in collagen fibrils interaction with transforming growth factor-β and function in connective tissue metabolism. We found 1.8-fold up-regulation of this proteins in pancreatic tumor treated with Hsp90 inhibitor. In addition, it is worth noting that biglycan is associated with the transforming growth factor-β pathway in pancreatic cancer (33). LGALS3, a member of the β-galactoside-binding gene family, has been related to cancer progression and maintenance in various cancer types including prostate, thyroid, and cervical cancer (34–36). The representative spectra from MS/MS analysis confirm the up-regulation of these proteins (Fig. 6A and B). Using immunoblotting, we have shown that induction of Hsp70 was observed in tumors on IPI-504 exposure (Fig. 5C). This is closely correlated with MS data on at least three isoforms of Hsp70 proteins (HSPA5, HSPA1B, and HSPA8). This finding is consistent with the results of the *in vitro* study as well as with earlier studies showing molecular cross-talk between other heat shock family proteins as result of Hsp90 modulation by 17-allylamino,17-demethoxy-geldanamycin (37, 38).

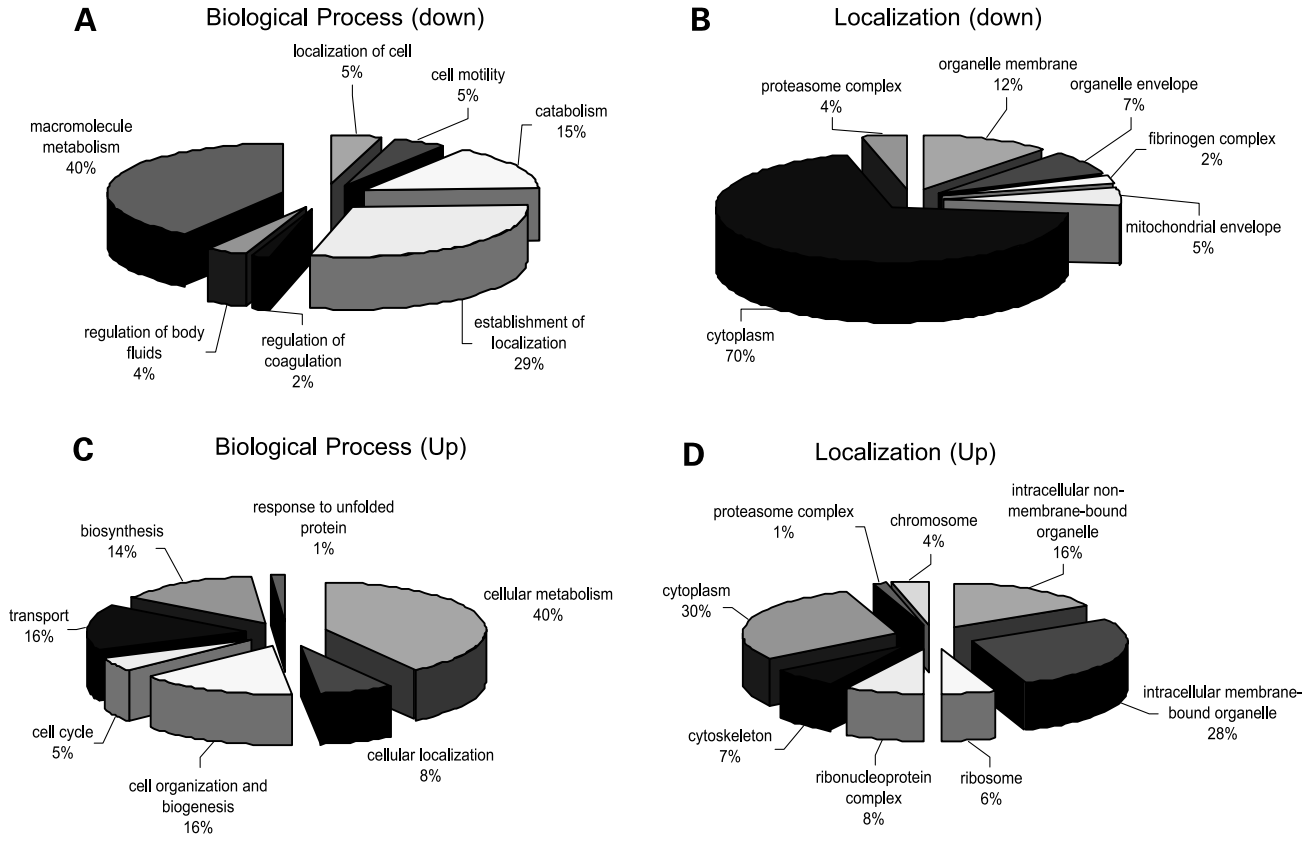

Figure 4. Functional and distributional diagrams of up-regulated and down-regulated proteins identified by MS/MS spectra. Based on the annotations of ProteinCenter and Genbank databases, down-regulated (**A** and **B**) and up-regulated (**C** and **D**) proteins by IPI-504 were categorized by its biological function and cellular localization. The major proportional functions of down-regulated proteins were closely related with macromolecule metabolism (40%) and establishment of localization (29%) and localized in cytoplasm (70%) and organelle membrane (12%; **A** and **B**). However, majority of up-regulated proteins were resided in cytoplasm (30%) and membrane-bound organelle (28%) and involved in cellular metabolism (40%) and cell organization and biogenesis (16%; **C** and **D**).

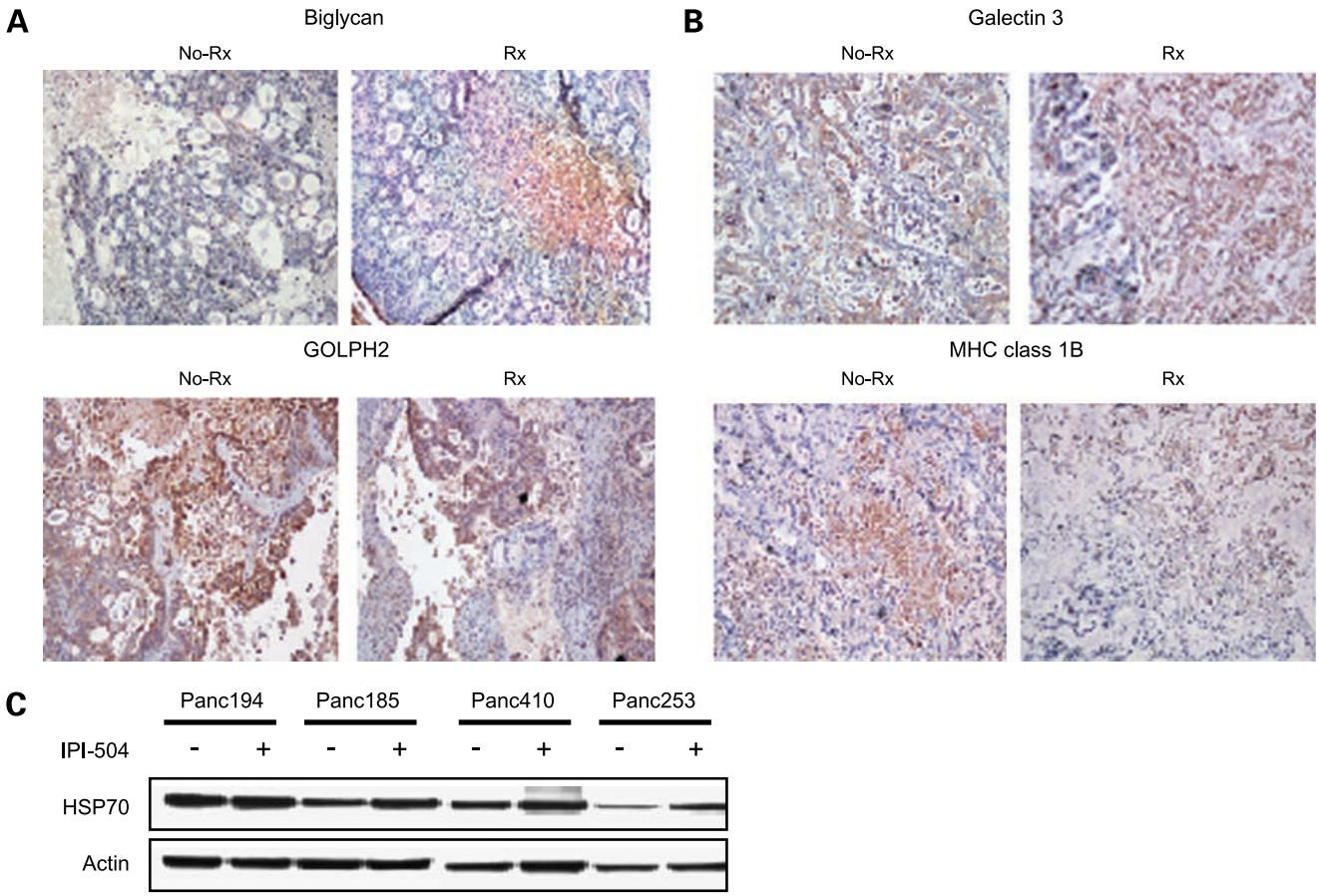

Figure 5. Confirmation of iTRAQ results using immunohistochemical labeling and Western analysis. **A** and **B**, overexpression of biglycan and LGALS3 and underexpression of MHC class IB and GOLPH2 in the drug-treated versus untreated tumor, which were used for iTRAQ analysis were confirmed. Wherever indicated, No-Rx and Rx represent the untreated tumor and treated tumor, respectively. **C**, Hsp70 was induced by IPI-504 in across the panel of xenografted tumors. Selected tumor tissues from Panc194, Panc185, Panc410, and Panc253 were prepared and analyzed by immunoblotting to assess the Hsp70 expression. Actin was used as a loading control. Wherever indicated, ''−'' and ''+'' represent in untreated control and IPI-504-treated groups, respectively.

Gene Expression Profile Showed Similar Patterns of MS/MS Analysis

To further explore new targets in response to IPI-504, we have determined changes in gene expression in the tumors after treatment with IPI-504 (Supplementary Tables S4 and S5).[8] There were 17 and 101 genes consistently up-regulated and down-regulated after treatment with IPI-504, respectively ($P < 0.05$). We next compared the gene expression and proteomic analysis. Two genes including HSPA1B and LGALS3 identified in the microarray study matched with the up-regulated proteins found in MS/MS result. In addition, eight down-regulated genes including CALM1, FAM84B, FDPS, GOLPH2, HBA1, HIST1H1C, HLA-B, and MARCKS were also observed to be down-regulated in the proteomics analysis (Supplementary Table S6).[8] Furthermore, we did GSEA analysis in all IPI-504-treated tumor tissues to identify a global molecular pathways affected by the drug (Supplementary Table S7).[8] Based on GSEA analysis per Kyoto Encyclopedia of Genes and Genomes annotations, we have observed that adherens junction and ribosome pathways were up-regulated by IPI-504, whereas the pathways involved in cell cycle, ABC transporters, lysine degradation, basal cell carcinoma, neuroactive ligand-receptor interaction, histidine metabolism, pyrimidine metabolism, selenoamino acid metabolism, naphthalene and anthracene degradation, glycine, serine, and threonine metabolism were deregulated (Supplementary Table S7).[8] These functional changes of signaling pathways in gene expression profile were consistent with the results of proteomic functional analysis (Fig. 4). Indeed, it has been reported that the molecular chaperone Hsp90 interacts with ribosomal proteins and thus prevents it from the ubiquitination and proteasome-derived degradation in mammalian cells (39).

Discussion

First-generation Hsp90 inhibitors, geldanamycin, and its derivatives including 17-allylamino,17-demethoxy-geldanamycin and radicicol have shown antitumor activity in various solid tumors (40–42). Blockade of the ATPase

activity of Hsp90 have effectively inhibited cell signaling molecules in proliferating cancer cells (43). However, geldanamycin and its derivatives have some significant limited pharmaceutical properties as well as toxicities in clinical trials (14, 44). For these reasons, the development of novel Hsp90 inhibitors has been an area of intense research. In this study, we aimed to investigate the antitumor and biological effects of the Hsp90 inhibitor IPI-504 in a mouse model of pancreatic cancer. The results show that the agent exerts antitumor efficacy both *in vitro* and *in vivo* with consistent up-regulation of Hsp70 expression. However, although in existing cell lines the drug induces well-known biological effects related to Hsp90 inhibition, we failed to observe such effects in the direct xenograft model. An unbiased proteomic and gene expression study identified a set of other novel markers that can be useful to further understand the biological effects of these drugs as well as to identify markers that can facilitate clinical development.

This preclinical work has been conducted in a direct pancreatic cancer xenograft model developed by our group. This model is based on implanting in nude mice pancreatic cancer tissues obtained at the time of surgery. These low-passage tumors are kept as a live bank in nude mice and are not developed as cell lines. Biologically, the xenograft tumors closely recapitulate the biological features of pancreatic cancer and resemble the features of the originator tumor. In our hands, these tumors are notoriously more resistant to multiple anticancer agents perhaps reflecting better the clinical characteristics of pancreas cancer (22). IPI-504 significantly inhibited the growth of the tumors tested and, although less effective than the cytotoxic gemcitabine, was more active than other targeted agents. Recent evidence suggests that Hsp90 inhibitors have shown antiproliferative effect in various cancers including colon, prostate, ovarian, and glioma cells (45–47). However, little has been done in pancreatic cancer. These results indeed support the clinical development of IPI-504 in pancreatic cancer.

One interesting observation in this study was the lack of expected pharmacodynamic effects in the xenograft tissues. The pharmacologic effects of inhibiting Hsp90 are well documented in preclinical models as well as in limited clinical samples but not in pancreatic cancer. These include up-regulation of Hsp70 and down-regulation of multiple signaling and cell cycle mediators such as HER-2, epidermal growth factor receptor, c-Raf, and cyclin-dependent

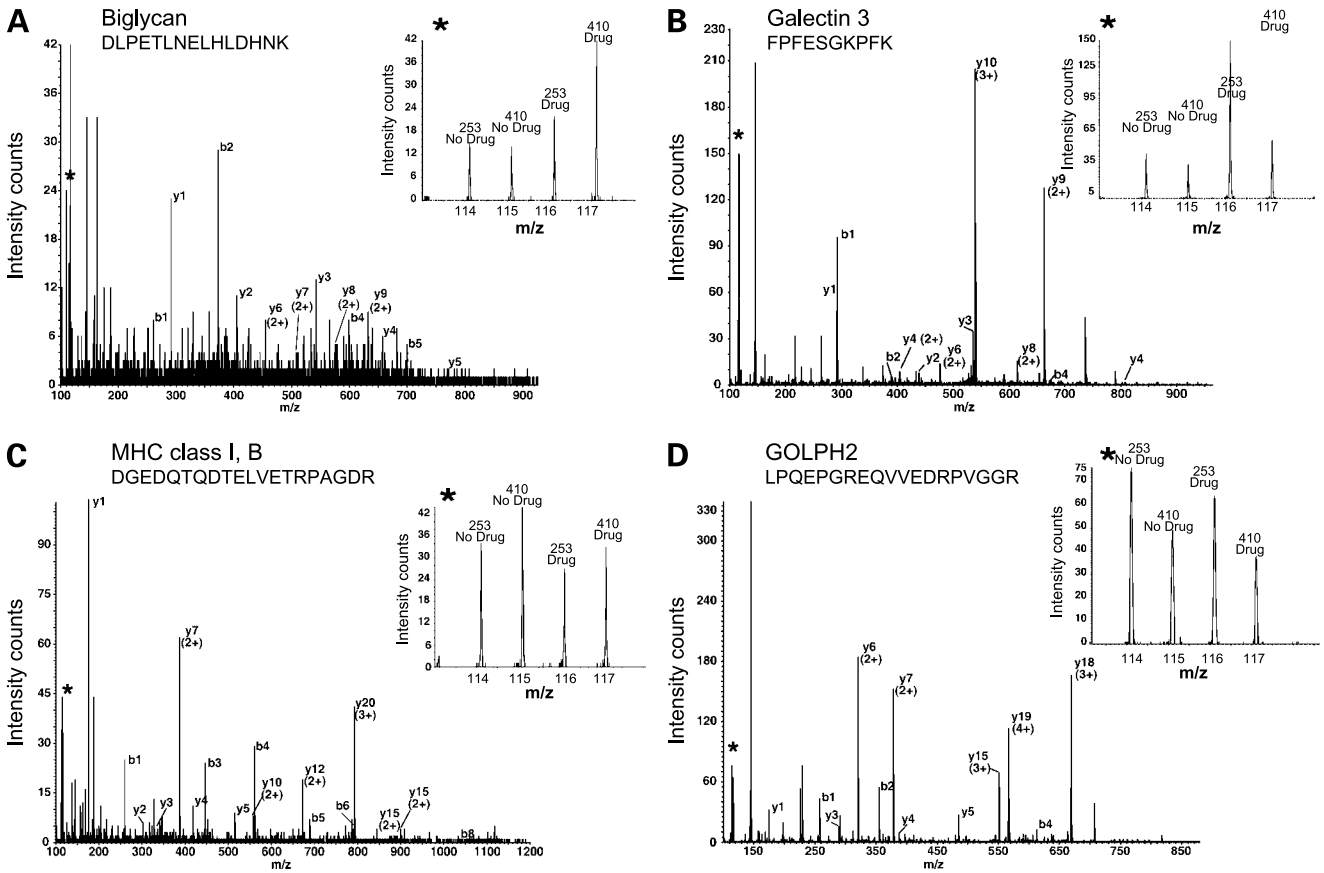

Figure 6. Peptide quantitation by iTRAQ from MS/MS spectra. MS/MS spectra of one representative peptide each from biglycan, LGALS3, MHC class IB, and GOLPH2 are shown. The inset in each case shows the corresponding relative intensity of reporter ions generated during MS/MS fragmentation. The labeled peptides from each protein represent *m/z* of the amino acid. A comprehensive list of y and b fragment ions are indicated.

kinase 4. We observed most of these effects in *in vitro* studies; however, no consistent effects, except up-regulation of Hsp70, were documented in the xenograft tumors regardless of their susceptibility to the drug by either Western blotting or immunohistochemistry. Results varied from one tumor to another and were not associated with less or greater susceptibility. Although technical issues cannot be totally ruled out, the concordance between Western blot and immunohistochemistry studies diminishes this possibility. The fact that the drug induced Hsp70 and resulted in TGI militates against a dose-response issue.

Puzzled by this observation, we conducted a global proteomic and gene expression analysis to further elucidate the biological effects of IPI-504 in pancreatic cancer. Using a cutoff of 0.75- to 1.25-fold, IPI-504 induces changes in the expression of a total of 62 proteins. As shown in Fig. 4, these proteins are widely distributed in different cell compartments including the cytoplasm, nuclei, and plasma membrane and are involved in a variety of cellular functions. Interestingly, none of the well-documented Hsp90 client proteins were found in this screen other than Hsp70 isoforms, although these may be influenced by issues of sensitivity of the methods employed. The results suggest that IPI-504 exerts an important perturbation in the cell proteome with changes in the levels of multiple proteins. Additional studies will need to be conducted to determine the contribution of these proteins, either individually or in groups, to the antitumor effects of IPI-504.

In a parallel effort to discover other molecular events on IPI-504 treatment, we have explored the effects of IPI-504 in global gene expression profile of pancreatic cancer. In some of the genes, there was significant concordance between the two methods. Most of the genes have not been reported in pancreatic cancer except 70 kDa heat shock protein isoforms. The complete list of gene expression affected by IPI-504 is provided as an additional data (Supplementary Tables S4 and S5).[8] In this study, we were unable to detect any alteration in genes coding for known Hsp90 client proteins including c-Raf-1, ErbB2, Akt, and cyclin-dependent kinase 4. Similarly, other investigators have found these phenomena in other cancer types (18, 19). Interestingly, when we compared our results with a recently published study in ovarian cancer, we found that only the 70 kDa heat shock protein isoforms and eukaryotic translation initiation factor 3 matched between these two tumor types. These discordant findings might be caused by the tumor type variation.

The reason why we did not observe the expected pharmacodynamic markers of IPI-504 in pancreatic cancer tumors is not known. We could postulate several potential explanations. First, we speculate that Hsp90 inhibitors have two sequential processes: client protein degradation as the primary effect of the drug action within 4 to 6 h followed by secondary alterations in the cell proteome after 12 h. For secondary processes, we might indeed observed changes in protein levels as a consequence of protein degradation and feedback transcriptional response. For primary effects, we would predict to observe changes related only to protein degradation. This is not to say that these two biological processes may not overlap. The samples in this study were collected at 24 h after the last dosing of Hsp90 inhibitor. Therefore, we could identify only the secondary effects of Hsp90 inhibitor in this model. Second, it could be that the known client proteins may not be responsible for Hsp90 inhibitor drug action in pancreatic cancer. Third, the client proteins may have a feedback mechanism of replenishing itself on prolonged drug exposure in the xenograft models. It should be emphasize, however, that IPI-504 is a different agent and this is a new model; therefore, results obtained with other drugs and models are not, necessarily, applicable.

In summary, this study shows that IPI-504 exerts significant antitumor activity in the direct pancreas xenograft model. Classic biology studies were not conclusive of any pharmacodynamic effects. Using a system biology approach, we have discovered new genes and proteins modulated by IPI-504. These data provide important candidates for subsequent pharmacologic and biological studies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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