Quantitative Proteomics Discloses MET Expression in Mitochondria as a Direct Target of MET Kinase Inhibitor in Cancer Cells*§

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Cancer cells with MET overexpression are paradoxically more sensitive to MET inhibition than cells with baseline MET expression. The underlying molecular mechanisms are incompletely understood. Here, we have traced early responses of SNU5, a MET-overexpressing gastric cancer cell line, exposed to sublethal concentration of PHA-665752, a selective MET inhibitor, using iTRAQ-based quantitative proteomics. More than 1900 proteins were quantified, of which >800 proteins were quantified with at least five peptides. Proteins whose expression was perturbed by PHA-665752 included oxidoreductases, transfer/carrier proteins, and signaling proteins. Strikingly, 38% of proteins whose expression was confidently assessed to be perturbed by MET inhibition were mitochondrial proteins. Upon MET inhibition by a sublethal concentration of PHA-665752, mitochondrial membrane potential increased and mitochondrial permeability transition pore was inhibited concomitantly with widespread changes in mitochondrial protein expression. We also showed the presence of highly activated MET in mitochondria, and striking suppression of MET activation by 50 nM PHA-665752. Taken together, our data indicate that mitochondria are a direct target of MET kinase inhibition, in addition to plasma membrane MET. Effects on activated MET in the mitochondria of cancer cells that are sensitive to MET inhibition might constitute a novel and critical non-canonical mechanism for the efficacy of MET-targeted therapeutics. Molecular & Cellular Proteomics 9: 2629–2641, 2010.

Recent improvements in survival of some malignancies owe much to advances in uncovering aberrantly active molecular pathways, against which molecularly targeted agents have been developed as new strategies to control cancers (1, 2).

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However, molecular mechanisms underlying the curious dependence of some cancer cells, which contain multiple genomic, genetic, and epigenetic abnormalities, on a single oncogenic molecule (the phenomenon of “oncogene addiction”) are incompletely understood (3–5).

Receptor tyrosine kinases are the most extensively studied oncogenic targets and receptor tyrosine kinase inhibitors have proven anticancer therapeutic efficacy. A receptor tyrosine kinase, MET, whose ligand is hepatocyte growth factor (HGF), is frequently amplified and overexpressed (6, 7) in gastric cancer, the second highest cause of cancer mortality globally (8, 9). Human gastric cancer cell lines harboring MET amplicons and overexpressing MET are readily induced to apoptosis by selective inhibitors of MET (10, 11), several of which are under active development for clinical use (12). One of the selective small molecular inhibitors, PHA-665752, designed chemically as (3Z)-5-[(2,6-dichlorobenzyl)sulfonyl]-3-[(3,5-dimethyl-4-[(2R)-2-(pyrrolidin-1-ylmethyl)pyrrolidin-1-yl]carbonyl]-1H-pyrrol-2-yl)methylene]-1,3-dihydro-2H-indol-2-one (molecule weight of 641.61), specifically suppresses MET kinase phosphorylation of MET. PHA-665752 has >50-fold higher selectivity for MET than for other tyrosine and serine/threonine kinases (13). The inhibition of MET kinase function by PHA-665752 on cancer cells had been confirmed with siRNA knockdown of MET, and a number of downstream effectors of MET signaling pathways were confirmed to be effectively abrogated by this compound (10, 13). PHA-665752 has been widely used as a potent and selective tool for the evaluation of MET-dependent cellular functions and signal transduction (10, 14–23).

The fact that only a subset of cancers is sensitive to killing by MET-directed therapeutics (hereafter referred to as sensitive cells) (12), raises an unexplained paradox. MET-overexpressing cancer cells could reasonably be expected to be more tolerant of MET kinase inhibition compared with cancer cells that do not overexpress MET. In reality, the opposite occurs. The underlying molecular mechanisms are incompletely understood.

To investigate this paradox we undertook a systematic exploration of responses of a MET-overexpressing gastric cancer cell line, SNU5, to sublethal MET inhibition using the iTRAQ-based quantitative proteomics approach. Our results unexpectedly showed a predominant perturbation of mitochondrial pro-
teins in response to MET inhibition. Next, we found that MET inhibition was rapidly associated with altered mitochondrial functions. These observations raised the possibility that mitochondria might be a direct target of MET inhibition. Both protein immunoblotting and confocal microscopy showed the presence of highly activated MET in the mitochondria of sensitive cancer cells. Furthermore, we observed that activating phosphorylation of tyrosine residues of mitochondrial MET was critically modulated by sublethal PHA-665752 treatment.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—All chemicals were purchased from Sigma-Aldrich unless otherwise stated. A selective MET inhibitor PHA-665752 (13) was from Pfizer Global Research and Development (La Jolla Laboratories, San Diego, CA). Stock solutions of this compound were prepared in DMSO, stored in −80 °C and diluted with fresh medium before use. In all experiments, the final concentration of DMSO was <0.1%.

**Cell culture**—Gastric cancer cell lines AGS, Kato III, SNU1, SNU5, SNU16, NCIN87, and Hs7467T, and a human fibroblast cell line, Hs68, were obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured as recommended. MKN7, and IM95 cells were from Japan Health Science Research Resource Bank and were cultured as recommended. YCC cells were a gift from Dr. Sun Young Rha (Yonsei Cancer Center, Seoul, Korea) and were grown in MEM supplemented with 10% fetal bovine serum (Hyclone, Thermo Fisher Scientific, Waltham, MA), 100 U penicillin, and 100 µg streptomycin per ml (Invitrogen).

**Gene expression profiling**—Total RNA was extracted from cell lines using the RNeasy Mini kit (Qiagen, Valencia, CA) and proﬁled using Affymetrix HG-U133 and HG-U133 Plus 2.0 GeneChip® (Affymetrix, Santa Clara, CA). Each RNA sample was ampliﬁed, labeled, and hybridized according to the manufacturer’s protocols. Normal gastric tissue RNA samples from two commercial sources were employed as controls. FirstChoice Human Stomach Total RNA (Ambion, Austin, TX) was RNA from a single individual. MVP Total RNA, Human Stomach (Stratagene, La Jolla, CA) was pooled RNA from two individuals. Four probe sets (203510_at, 211599_x_at, 213807_x_at and 213816_s_at) was RNA from a single individual. MVP Total RNA, Human Stomach (Stratagene, La Jolla, CA) was pooled RNA from two individuals. Four probe sets (203510_at, 211599_x_at, 213807_x_at and 213816_s_at) for MET were printed on the arrays.

**MTT assay**—Cell viability based on redox enzyme activity was quantiﬁed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, the MTT assay as described (10).

**iTRAQ protein sample preparation**—Four experimental groups of SNU5 cells were prepared in the absence or presence of PHA-665752. Three groups were exposed to 50 nM PHA-665752 for three time periods i.e. 4 h, 24 h, and 72 h. A parallel group without treatment served as the control. After treatment, proteins were extracted and three independent biological replicate ﬂasks for each experimental condition were pooled and quantiﬁed by bicinechonic acid protein assay kit as described previously (24).

**Isobaric labeling**—Two-hundred micrograms of protein from each experimental condition were tryptically digested and labeled with 4-plex iTRAQ reagents (Applied Biosystems, Foster City, CA) as follows: control, 114; 4 h, 115; 24 h, 116; 72 h, 117. The labeled samples were pooled and resolved into 20 fractions using strong cation exchange (SCX)(24). Eluted fractions were vacuum dried and desalted using SEP-PAK C18 cartridges (Waters, Milford, MA). Dried peptides were stored at −80 °C before MS analysis.

**Liquid chromatography (LC)**—MS/MS analysis—The LC-MS/MS analysis was performed as previously described (24, 25) with some modifications. Briefly, dried iTRAQ-labeled peptide samples were dissolved in HPLC grade water (Mallinckrodt Baker) acidiﬁed with 0.1% formic acid, and sequentially injected and separated in a home-packed nanobore C18 column with a picofrit nanospray tip (75 µm ID × 15 cm, 5-µm particles) (New Objectives, Woburn, MA) on a TempoTM nano-MLC system coupled with a QSTAR® Elite Hybrid LC-MS/MS system (Applied Biosystems). Each sample was divided into two equal aliquots and independently analyzed by the LC-MS/MS over a gradient of 120 min. The flow rate of LC system was set constantly at 300 nL/min. Data acquisition in QSTAR Elite was set to positive ion mode using Analyst® QS 2.0 software (Applied Biosystems). Precursors with a mass range of 300–2000 m/z and a calculated charge of +2 to +4 were selected for fragmentation. For each MS spectrum, a maximum of three most abundant peptides above a ﬁve-count threshold were selected for MS/MS. Each selected precursor ion was dynamically excluded for 30 s with a mass tolerance of 0.03 Da. Smart information-dependent acquisition was activated with automatic collision energy and automatic MS/MS accumulation. The fragment intensity multiplier was set to 20 and maximum accumulation time was 2 s.

**MS spectrum analysis**—Spectra acquired in LC-MS/MS system from the two independent runs were submitted in a batch to Protein-Pilot (v2.0.1, Applied Biosystems) for peak-list generation, as well as protein identiﬁcation and quantiﬁcation against the International Protein Index (IPI) human database (version 3.34; 67758 sequences) supplemented with porcine trypsin. The Paragon algorithm in Protein-Pilot software was conﬁgured as previously described (25) with some modiﬁcations. Briefly, default parameters including ﬁxed and variable modiﬁcations for tryptically digested samples labeled with 4-plex iTRAQ reagents (peptide labeled) were employed. The search was done thoroughly where all cleavage variants were considered. The conﬁdence threshold for both peptide and protein identiﬁcation was set to 70%. Default precursors and the fragments mass tolerance for QSTAR ESI MS instrument were adopted by the software. A concatenated target-decoy database search strategy was also employed to estimate the false discovery rate (FDR) (26). FDR was calculated as twofold of the percentage of decoy matches divided by the total matches. After stringent ﬁltering as described in Results, FDR of the reported iTRAQ data set was <1%. ProteinPilot software employed the peak area of iTRAQ reporters for quantiﬁcation. Details of the quantiﬁcation algorithm can be found in the supplier’s manual. Isoform-speciﬁc strategy was adopted to deal with quantiﬁcation of isoforms. Quality control of the data set is addressed in Results.

**Bioinformatics**—Gene IDs of the proteins of interest were searched in a batch using PANTHER classiﬁcation system (27) against NCBI (H. sapiens) dataset and the results were presented as genes. Most protein groups had more than one molecular function hit. Cellular localization information of the 50 proteins of interest was checked manually in Gene Ontology (28).

**Western blotting**—Western blotting was performed using primary antibodies at the dilutions indicated: 1:500 SDHB (clone 21A11), 1:500 NDUF53 (clone 17D95), 1:1000 VDAC1 (clone 20B112), 1:1000 MET (clone C-12), 1:1000 phospho-MET (Y1234/1235), 1:1000 phospho-MET (Y1349), 1:1000 E-cadherin (G-10), 1:2500 actin (Clone C4), 1:2000 α-tubulin (clone B-7). Phospho-MET antibodies were from Cell Signaling (Danvers, MA), actin antibody was from Millipore (Billerica, MA), whereas the other primary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against integrin β1 (1:500), MHM23, was kindly supplied by Dr Alex Law (School of Biological Sciences, Nanyang Technological University, Singapore).

**Mitochondrial membrane potential analysis**—Cells with or without PHA-665752 treatment were washed with ice-cold PBS and incubated with 5 µg/ml rhodamine 123 for 1 h, followed by flow cytometric analysis on FACs Calibur and CellQuest Pro software (Becton Dickinson, Franklin Lakes, NJ).
Mitochondrial permeability transition pore analysis—The activity of mitochondrial transition pore was evaluated by the MitoProbe™ Transition Pore Assay Kit (Becton Dickinson) following the manufacturer’s instruction. Briefly, cells were washed twice with ice-cold Hank’s balanced salt solution containing 1.3 mM calcium (Invitrogen) before incubation in the presence or absence of cobalt chloride at 37 °C for 15 min, followed by flow cytometry analysis as described earlier.

Confocal microscopy—SN5 cells were washed with HEPES twice, before incubating with 500 nM Mito Tracker Red CMXRos (Invitrogen) for 15 min. Cells were then fixed in 3% formaldehyde for 20 min and permeabilized with 0.1% Triton X-100 for 2 min. After blocking nonspecific antibody binding sites with 1% BSA for 1 h at 37 °C, cells were probed with primary antibodies (1:500) overnight at 37 °C and Alexa 488-conjugated goat-anti-rabbit secondary antibodies (Invitrogen) for 1 h at 37 °C. Finally the cells were washed with PBS and counterstained with Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA). Images were captured with a Zeiss LSM 710 confocal microscope.

Mitochondria isolation—Mitochondria isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) was employed to isolate mitochondria following the manufacturer’s protocol. Briefly, 5 × 10^7 SN5 cells with or without treatment were washed twice with PBS, and lysed in 2 ml of the provided lysis buffer supplemented with Complete Protease Inhibitor Mixture Tablets and phosSTOP (Roche, Basel, Switzerland). The crude cell lysate was incubated with anti-TOM22 MicroBeads for 1 h at 4 °C with gentle shaking. Subsequently, the suspension was loaded onto a pre-equilibrated MACS column, washed three times with separation buffer before removing the column from the magnetic field and eluting the mitochondria.

RESULTS

**MET expression and susceptibility of gastric cancer cells to PHA-665752**—As PHA-665752 is differentially cytotoxic in cancer cells depending on MET expression levels (10), we first evaluated MET expression data of a panel of 16 gastric cancer cell lines, (AGS, Kato III, SNU1, SNU5, SNU16, NCIN87, Hs746T, MKN7, IM95, YCC1, YCC2, YCC3, YCC6, YCC9, YCC11, and YCC16) in order to focus on a model cell line for systematic proteomics exploration. Our transcriptome data showed that SNU5 cells had markedly elevated levels of MET transcription (>40-fold compared with normal human stomach tissues), while MET expression of SNU1 cells was comparable to the controls (supplemental Fig. 1). MET protein expression levels of these two cell lines were compared by immunoblotting (supplemental Fig. 2). SN5 and SNU1 cells showed high and low expression of MET, respectively, in agreement with our transcriptome data as well as a previous study (10). We determined cytotoxic responses of the two gastric cancer cell lines to PHA-665752 using MTT assay (supplemental Fig. 3). The mean IC50 of PHA-665752 in SNU5 cells was ~77 nm, whereas SNU1 cells were relatively resistant to the compound (IC50 > 500 nm). SN5 was selected as the model cell line in subsequent temporal quantitative proteomics analyses because it was highly sensitive to PHA-665752. Conversely, SNU1 was chosen as being representative of gastric cancer cells resistant to MET inhibition in functional studies.

**Temporal quantitative proteomics analysis**—We treated SNU5 cells with PHA-665752 and analyzed the temporal dynamics of the proteome. First, we sought to determine an appropriate concentration of PHA-665752 for quantitative proteomic investigation of SNU5 cells, in order to trace early cellular responses of the cells to MET inhibition. The ideal treatment conditions with PHA-665752 should suppress phosphorylation of MET without causing substantial cell death. SNU5 cells were exposed for varying durations to two concentrations of PHA-665752 around its IC50 (determined at 72 h) and tested for viability using MTT assays. Our results showed that 50 nm PHA-665752 did not significantly impair cell viability, whereas 150 nm was rapidly cytotoxic (supplemental Fig. 4). As such, we regarded 50 nm as a sublethal concentration for SNU5 cells, and adopted these conditions for the subsequent proteomics study.

It is worth noting that it remains a daunting challenge that some small-molecule kinase inhibitors exhibit off-target effects that cannot be ignored (29). To minimize off-target effects in this study, we selected one of the most potent and specific MET inhibitors PHA-665752, which is >50 times more selective for MET than other protein kinases (13). Moreover, we applied it to a cell line SNU5 that overexpresses MET at unusually high levels i.e. >40 times higher than normal stomach tissue. In addition, we intentionally employed a low concentration of PHA-665752, i.e. 50 nm, which is sublethal to SNU5 cells but sufficient to inhibit MET activity. This further refined the data as arising from specific inhibition of MET because most off-target effects happen when inhibitors are used at high concentrations, such as >1 μM. We believe, in this scenario, the probability of inhibiting other proteins with even comparable or higher affinity than MET is low or negligible. Furthermore, previous work has documented that the differential effects of PHA-665752 are truly attributed to its effect on MET using small interfering (si) RNA targeting the MET receptor transcript in SNU5 cells (10). Finally, growth factor effectors in the downstream of MET signaling pathway, including ERK1/2, AKT, STAT3, and FAK, were effectively abrogated by 50 nm PHA-665752 (10). Thus, MET could reasonably be considered the main target of 50 nm PHA-665752 in SNU5 cells in this study.

We used iTRAQ reagents to label the tryptically digested proteome, coupled with shotgun multidimensional liquid chromatography and tandem mass spectrometry (30) to profile the temporal proteome responses (Fig. 1A). This approach allowed simultaneous comparison of the proteomes at four time points (0, 4 h, 24 h, and 72 h) after PHA-665752 treatment, to capture both the early and late responses of the SNU5 proteome.

**Quality control of quantitative MS data set**—To ensure the reliability of the quantitative datasets, three independent biological replicates of SNU5 cells were pooled for the proteomics study (Fig. 1A). Moreover, the iTRAQ-labeled samples were analyzed twice by LC-MS/MS to minimize technical variations. The ProteinPilot database search in a concatenated target and decoy strategy returned 26276 target proteins.
matches and only one decoy match. A total of 1908 target proteins were identified and quantified with estimated FDR of <1%. We next employed stringent inclusion criteria to filter the data set. A total of 806 proteins quantified with high confidence i.e. quantified from at least five peptides, of which there are at least two unique peptides, and having error factors <1.5, were advanced to the next phase of analysis (supplemental Table 1).

**Estimation of cutoff for confidently defining perturbed proteins**—The cutoff for defining perturbed and unperturbed pro-

**Fig. 1. Quantitative proteomic analysis shows dominant effects on mitochondrial proteins.** A, Schematic diagram of the iTRAQ-LC-MS/MS experimental workflow. B, Distribution of the relative expression levels of temporal proteomes. Ratios were calculated in log space before converting into linear space. C, Subcellular classification of the differentially expressed proteins based on gene ontology. Details of the proteins are shown in supplemental Table 2. Note that one protein may have >1 subcellular localization hits. D, Schematic display of the functions and localizations of PHA-665752-perturbed expression of mitochondrial proteins. *, mitochondrial proteins whose exact localization within the mitochondrion is uncertain; OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane; mPTP, mitochondrial permeability transition pore; ETC, electron transport chain.
tein expression in iTRAQ experiments depends on the characteristics of biological samples as well as MS instruments. To avoid setting the cutoffs arbitrarily, we examined the distribution of the expression levels of the 806 proteins (Fig. 1B). The three relative expression levels of these proteins i.e. 4 h/control, 24 h/control, and 72 h/control, were all normally distributed indicating that sublethal treatment of this compound only modulated a small percentage of the SNU5 proteome. Thus, we focused on the top 5% proteins whose expression was most perturbed by PHA-665752 treatment. With this criterion, protein ratios <0.774 were regarded as underexpressed, whereas ratios >1.181 were considered overexpressed, thereby narrowing the reliable differentially expressed proteins to a small number of 50 (supplemental Table 2), which reflected significant effects of MET inhibition in SNU5 cells.

Western blotting validation of iTRAQ ratios—To further evaluate the accuracy of iTRAQ ratios for the shortlisted 50 proteins, we examined the expression of three representative proteins, NDUFS3, SDHB, and VDAC1, by semiquantitative Western blot analysis. NDUFS3 and SDHB, proteins of the mitochondrial ETC, were quantified by MS with unique peptide numbers of six and three, respectively, whereas VDAC1, a component of mPTP, was quantified with 10 unique peptides. As shown in Fig. 2 and Table I, Western blot data showed similar trends corresponding iTRAQ ratios.

Quantitative proteomics dataset reveals perturbed cellular responses after sublethal PHA-665752 treatment—The iTRAQ dataset provided identification and quantification of 806 proteins with various molecular functions as classified by PANTHER (Fig. 3), including 50 proteins (supplemental Table 2) whose expression had been perturbed by PHA-665752-induced MET inhibition. These proteins represent various cellular responses as shown in Fig. 3. Consistent with the specificity of MET inhibition, although proteins involved in nucleic acid binding and the cytoskeleton were the two most abundant groups, very few (<3%) were perturbed in expression by PHA-665752. Remarkably, sublethal concentration of this compound mainly affected the expression of several other groups of proteins, including oxidoreductases, calcium-binding proteins, transfer/carrier proteins involved in transport of specific substances, and signaling proteins.

Our data showed that 7 (29%) of the 24 molecular function hits were associated with calcium-binding proteins, including annexins (i.e. annexin A2 (IPI00455315.4), annexin A4 (IPI00793199.1), annexin A5 (IPI00329801.12)), calmodulin-related proteins comprising hippocalcin-like protein 1 (IPI00219344.4), protein S100-A4 (IPI00032313.1), protein S100-A10 (IPI00183695.9), and mitochondrial aspartate-glutamate carrier protein (IPI00007084.2). Modulation of calcium-binding proteins by PHA-665752 treatment suggested that calcium signaling might be a downstream response to inhibition of MET kinase activity. Calcium signaling has been extensively documented to be tightly modulated e.g. by G protein-coupled receptors and tyrosine kinase receptors (31). It is worth noting that the link between calcium and MET signaling is supported by the recent observation that MET, when stimulated by hepatocyte growth factor, regulated calcium signals in human liver tumor cells (32).
| Mitochondrial function | Accessions     | Protein description                          | Gene symbol | % of sequence coverage | # of unique peptide | 4h/ctrl Ratio | 4h/ctrl N | 24h/ctrl Ratio | 24h/ctrl N | 72h/ctrl Ratio | 72h/ctrl N |
|------------------------|----------------|---------------------------------------------|-------------|------------------------|---------------------|---------------|------------|---------------|------------|---------------|------------|
| ETC Complex I          | IPI00025239.2  | NADH dehydrogenase iron-sulfur protein 2   | SLC25A13    | 26.1                   | 3                   | 0.838 ± 0.116  | 6          | 0.613 ± 0.058  | 6          | 0.772 ± 0.059  | 6          |
| ETC Complex I          | IPI00025796.3  | NADH dehydrogenase iron-sulfur protein 3   | NDUF3*      | 41.7                   | 6                   | 0.813 ± 0.070  | 5          | 0.712 ± 0.055  | 5          | 0.922 ± 0.054  | 5          |
| ETC Complex II         | IPI00294911.1  | Succinate dehydrogenase iron-sulfur subunit| SDHB*       | 29.6                   | 3                   | 0.921 ± 0.045  | 5          | 0.701 ± 0.041  | 5          | 0.922 ± 0.062  | 5          |
| ETC Complex III        | IPI00013847.4  | Ubiquinol-cytochrome-c reductase complex core protein 1 | UQCRC1 | 45.4                   | 8                   | 0.921 ± 0.045  | 16         | 0.612 ± 0.053  | 14         | 0.811 ± 0.040  | 15         |
| ETC Complex III        | IPI00305383.1  | Ubiquinol-cytochrome-c reductase complex core protein 2 | UQCRC2 | 42.8                   | 4                   | 0.863 ± 0.066  | 8          | 0.551 ± 0.032  | 8          | 0.802 ± 0.062  | 8          |
| ETC Complex IV         | IPI00017510.3  | Cytochrome c oxidase subunit 2             | COX2        | 23.3                   | 3                   | 0.691 ± 0.034  | 13         | 0.431 ± 0.029  | 13         | 0.772 ± 0.052  | 13         |
| ETC Complex IV         | IPI00025086.3  | Cytochrome c oxidase subunit 5A            | COX5A       | 64.7                   | 3                   | 0.784 ± 0.074  | 8          | 0.615 ± 0.080  | 8          | 0.894 ± 0.085  | 8          |
| ETC electron transporter | IPI00029264.3  | Cytochrome c1 heme protein                | CYC1        | 44.3                   | 4                   | 0.761 ± 0.044  | 6          | 0.603 ± 0.057  | 6          | 0.856 ± 0.104  | 6          |
| mPTP                   | IPI00007188.5  | Adenine nucleotide translocator 1          | ANT2        | 72.1                   | 10                  | 0.818 ± 0.114  | 6          | 0.563 ± 0.059  | 6          | 0.814 ± 0.085  | 6          |
| Others                 | IPI00064707.4  | Dihydrolipoamide S-acetyltransferase       | DLAT        | 40.0                   | 6                   | 1.621 ± 0.536  | 8          | 0.896 ± 0.169  | 5          | 1.083 ± 0.083  | 8          |
| Others                 | IPI00073772.5  | Fructose-1,6-bisphosphatase 1              | FBP1        | 50.6                   | 9                   | 0.942 ± 0.055  | 27         | 1.163 ± 0.079  | 33         | 1.483 ± 0.100  | 33         |
| Others                 | IPI00022793.4  | Trifunctional enzyme β subunit              | HADHB       | 45.9                   | 5                   | 0.784 ± 0.074  | 7          | 0.722 ± 0.055  | 6          | 0.926 ± 0.104  | 7          |
| Others                 | IPI00007242.1  | Galactin-2                                 | LGALS2      | 59.1                   | 6                   | 1.037 ± 0.117  | 15         | 0.986 ± 0.111  | 15         | 1.963 ± 0.221  | 15         |
| Others                 | IPI00017334.1  | Prohibitin                                 | PHB         | 58.1                   | 6                   | 0.844 ± 0.080  | 8          | 0.523 ± 0.054  | 8          | 0.766 ± 0.156  | 9          |
| Others                 | IPI00027252.6  | Prohibitin-2                               | PHB2        | 63.2                   | 6                   | 0.862 ± 0.058  | 11         | 0.502 ± 0.048  | 11         | 0.642 ± 0.049  | 11         |
| Others                 | IPI0183695.9   | Protein S100-A10                           | S100A10     | 70.1                   | 4                   | 0.661 ± 0.038  | 28         | 0.712 ± 0.048  | 28         | 1.062 ± 0.062  | 29         |
| Others                 | IPI00007084.2  | Mitochondrial aspartate-glutamate carrier protein | SLC25A13 | 29.1                   | 7                   | 0.856 ± 0.104  | 8          | 0.662 ± 0.057  | 9          | 0.903 ± 0.069  | 8          |
| Others                 | IPI00790115.1  | CDNA FLJ90278 fis, mitochondrial precursor | SLC25A3 | 39.6                   | 2                   | 0.681 ± 0.033  | 8          | 0.581 ± 0.039  | 8          | 0.892 ± 0.052  | 8          |
Of the 29 proteins classified as having transfer/carrier function, six proteins (21%) responded to PHA-665752 at nanomolar concentration. Aside from the three annexin proteins and mitochondrial aspartate-glutamate carrier protein that were also classified in the calcium-binding protein category, another two phosphate carrier proteins, i.e., adenine nucleotide translocator 2 (IPI00007188.5) and mitochondrial phosphate carrier protein (IPI00790115.1) were modulated by this compound.

Five of 17 (29%) proteins classified as signaling molecules, cytokine macrophage migration inhibitory factor (IPI00790382.1), myristoylated alanine-rich protein kinase C substrate (IPI00219301.7), COP9 constitutive photomorphogenic homolog subunit 8 (IPI00009480.1), galectin 2 (IPI00007242.1), and Rho GDP dissociation inhibitor α (IPI00794402.1), showed perturbed expression (Fig. 3), as a probable consequence of altered signal transduction induced by PHA-665752.

As shown in Table I, most of the mitochondrial proteins showed a similar trend of response to the compound. They were down-regulated at 4 h, further down-regulated at 24 h, and partially recovered at 72 h. This typical pattern of perturbed mitochondrial protein expression indicated a gradual but reversible effect of PHA-665752 on the SNU5 proteome.

**Dominant roles of mitochondrial proteins in PHA-665752-induced MET inhibition**—Interestingly, we found that many of the perturbed proteins in this dataset were associated with mitochondria. Hence, we performed a bioinformatics classification based on subcellular localization information from Gene Ontology (28) to determine how many perturbed proteins were mitochondrial. Not surprisingly, a significant number of the perturbed proteins were cytosolic. However, it was noteworthy that 19 proteins, i.e. 38% of the 50 proteins whose expressions were altered by PHA-665752, were localized in mitochondria (Fig. 1C), indicating a disproportionately dominant role of mitochondria in cellular responses to PHA-665752 treatment. Specifically, proteins from the two pivotal mitochondrial complexes i.e. electron transfer chain (ETC) and mitochondrial permeability transition pore (mPTP), were significantly perturbed by PHA-665752. Additionally, mitochondrial proteins involved in metabolism, signal transduction, survival, and apoptosis were also affected by PHA-665752 treatment (Fig. 1D).

**Mitochondrial ETC is perturbed by PHA-665752**—Eight (42%) of the 19 dysregulated mitochondrial proteins were components of the ETC in the inner mitochondrial membrane (IMM) (Fig. 1D, Table I). ETC is comprised of complex I (NADH dehydrogenase), complex II (succinate dehydrogenase), complex III (cytochrome bc1 complex), and complex IV (cytochrome c oxidase). Proteins from all four ETC complexes had decreased by 4 h after PHA-665752 treatment, were further inhibited at 24 h and had partially recovered at 72 h. These findings showed that PHA-665752 at a sublethal concentration rapidly modulated the expression of multiple ETC component proteins.

The ETC operates mainly through its constituent oxidoreductase enzyme activities. As the MTT assay measures cellular oxidoreductase enzyme activities, mainly of the ETC, we asked if oxidoreductase activity of the ETC was diminished by PHA-665752. By cross-referencing MTT assay data (supplemental Fig. 4) with iTRAQ data (Table I) at 24 h of treatment with 50 nM PHA-665752, significant decreases in ETC protein expression levels were associated with only a small decrease of ETC oxidoreductase enzyme activity at the same time point, suggesting that despite inhibition of ETC protein expression, mitochondria retained substantial oxidoreductase enzyme activities required for cell survival.

One of the most important functions of ETC is to maintain the MMP (33). The energy released by electron transport pumps protons across the IMM, generating the electrochem-
ical and pH gradients. We examined the MMP using rhodamine 123 staining and flow cytometry (34). Rhodamine 123, a cationic dye, has a strong emission at 529 nm that is quenched as it accumulates within mitochondrial intermembrane space, and then dequenched (with increased fluorescence) when released into the cytosol. Our data showed that rhodamine 123 fluorescence of SNU5 cells did not change upon treatment with nanomolar concentration of PHA-665752 until exposure was prolonged beyond 24 h (Fig. 4). The decrease in rhodamine 123 fluorescence at 48 h and 72 h was the evidence of an increase in MMP and hyperpolarization of the mitochondria (34). However, MMP of the PHA-665752-resistant cell line, SNU1, did not change with the same treatment (Fig. 4) and even at a higher concentration (300 nM, data not shown).

mPTP responses to PHA-665752 treatment—We observed inhibited expression of two core components of mPTP i.e. VDAC1 and ANT2 (35, 37), from the quantitative proteomics datasets (Table I, Fig. 1). The effect on VDAC1 was confirmed by Western blot analysis (Fig. 2). mPTP is generally regarded as a crucial channel that permits the exchange of metabolites and ions (38). Exchange of molecules is controlled by the flickering of mPTP between open and closed states (37). Channel opening or an increase in the frequency of mPTP flickering is extensively documented as an event tightly associated with both necrotic and apoptotic cell death (39). Therefore, we next asked whether mPTP was functionally associated with MET inhibition by PHA-665752. Flickering of mPTP was evaluated using the calcein AM/CoCl₂ method (40). Strikingly, mPTP was rapidly and effectively inhibited by 50 nM PHA-665752 in SNU5 cells within 30 min (Fig. 5). It is worth noting that inhibition of the mPTP was sustained for at least 72 h. The resistant gastric cancer line SNU1 did not show inhibition of mPTP, but only rapid and transient activation of mPTP (Fig. 5).

MET is present in mitochondria of SNU5 cells—The rapid effects of PHA-665752 on mitochondrial proteins and functions of SNU5 cells raised the possibility that this MET-selective inhibitor may act directly on mitochondria. Two reasons led us to hypothesize that MET might be present in mitochondria where it would be a target for PHA-665752. First, as PHA-665752 has high specificity for MET kinase (13), the rapid mitochondrial responses could well be mediated by MET. Second, cells in which MET gene is highly amplified overexpress MET proteins that may be constitutively activated. High intracellular levels of activated MET could facilitate its translocation or localization to intracellular organelles such as mitochondria. Indeed EGFR, another oncogenic receptor tyrosine kinase, was found to translocate to the mitochondria and nucleus when activated (41, 42). Moreover, MET has been reported to be translocate to the nucleus when stimulated by HGF (32, 43). However, no publication to date has reported the presence of MET in the mitochondria.
We employed two approaches to investigate whether MET is present in mitochondria of sensitive cells. Immunoblotting analysis could demonstrate the presence of MET in the isolated mitochondria fraction in a semiquantitative manner, although it may suffer from contamination of proteins from other organelles because no current technique is capable of enriching mitochondria to 100% purity. Confocal microscopic analysis provided additional complementary evidence visually.

Immunoblotting was used to probe MET and phospho-MET in SNU5 whole-cell lysate and isolated mitochondrial lysate. In order to maximize mitochondria enrichment efficiency and minimize contaminations from other organelles, we employed a newly developed method for mitochondria isolation based on superparamagnetic microbeads conjugated to anti-TOM22 antibody (44). The protocol is fast, reproducible, and standardized, resulting in mitochondria of high purity, with minimal contamination from cytoskeleton, cytosol, Golgi apparatus, endosome, endoplasmic reticulum, and nucleus (44). We employed several controls to further confirm the purity of isolated mitochondria in this study. Known plasma membrane proteins including E-calcium-dependent adhesion molecules (E-cadherin)/CD324 and integrin αL/CD18 were present in whole-cell lysate, but were almost absent in mitochondria fractions, indicating minimal plasma membrane contamination (Fig. 6A). The cytoskeleton protein, actin, displayed a similar pattern, whereas mitochondrial protein, SDHB, exhibited the opposite distribution, further proving the purity of isolated mitochondria (Fig. 6A). Equal loading of proteins for whole-cell lysate and mitochondria fraction was confirmed by Ponceau S staining (45).

In striking contrast to E-cadherin, integrin αL and actin that were present in whole-cell lysate but not in the mitochondrial fraction, MET was expressed at a high level in mitochondria of SNU5 cells (Fig. 6A). Remarkably, phosphorylated MET appeared to be enriched in mitochondria. Signals of pMET (Y1234/1235) and pMET (Y1349) were higher in mitochondria than in whole-cell lysate, indicating that mitochondrial MET was highly activated. SNU1 cells demonstrated minimal expression of MET compared with SNU5 cells.

To further confirm the presence of MET in mitochondria of SNU5 cells visually, we employed confocal microscopy to determine if MET and mitochondria were colocalized. Fig. 7B shows SNU5 cells fluorescently labeled with DAPI (blue channel), Mito Tracker Red CMXRos (red channel), and Alexa 488-conjugated antibodies against MET, pMET (Y1234/1235), and pMET (Y1349) (green channel). As expected, both MET and phosphorylated MET were found in high abundance in the plasma membrane of SNU5 cells. Remarkably, MET and phosphorylated MET also colocalized with mitochondria, as indicated by yellow colored areas in merged images. These results were consistent with immunoblotting experiments (Fig. 6A). In contrast, SNU1 displayed very weak fluorescence for MET and phospho-MET using the same staining protocol (data not shown).
Taken together, these data provided convincing experimental evidence for the presence of MET in mitochondria of SNU5 cells. PHA-665752 inhibits phosphorylation of mitochondrial MET in SNU5 cells—Next we asked whether mitochondrial MET that was highly phosphorylated would respond to treatment with PHA-665752 for 0.5 h and 2 h. As shown in Fig. 7, immunoblotting demonstrated that MET expression level in whole-cell lysates was not altered by the compound at both time points but phosphorylation of MET was inhibited, consistent with previous reports (10, 13). Importantly, phosphorylation of mitochondrial MET was inhibited in isolated mitochondrial lysates. Phosphorylation of MET at Y1234/Y1235 was suppressed by 50 nM PHA-665752 as early as 0.5 h after treatment. Phosphorylation of Y1234/Y1235 was further inhibited when the treatment was prolonged for 2 h. Phosphorylation of Y1349 in mitochondrial MET was inhibited by PHA-665752 at 2 h.

DISCUSSION

A curious observation in cancer research is that some cancers appear to be highly dependent for survival and progression on a single oncogene that is usually overexpressed (1, 4). MET has essential functions in both normal and malignant cells (46). It is not entirely clear why gastric cancer cells that overexpress MET are paradoxically much more sensitive to MET inhibition than cells whose MET expression is comparable to normal stomach (10).

In an effort to dissect the underlying molecular mechanisms, we have established a model to inhibit MET activity using a potent MET inhibitor PHA-665752 in a sensitive gas-
Accumulating evidence suggests that many mitochondrial proteins, including ETC and mPTP components, that maintain the functions of this organelle, are tightly modulated by protein kinases including protein kinase A (PKA), PI3K/Akt/PKB, Raf-MEK-ERK, and MAPK (50, 51). Phosphorylated mitochondrial MET may play active roles in modulating phosphorylation status of various mitochondrial proteins involved in mitochondrial functions. Deregulation of mitochondrial proteins and dysfunctional mitochondrial processes are likely consequences of inhibition of mitochondrial MET. Our findings also plausibly link receptor tyrosine kinase-targeted therapeutics with the Warburg effect (52). Mitochondria are key regulators of glycolysis. We have found expression of several glycolysis-associated mitochondrial proteins, for example, VDAC1 and ANT2, was concurrently regulated rapidly in response to PHA-665752 treatment in SNU5 cells (supplemental Table 2).

Our observations have uncovered novel mechanisms through which a kinase inhibitor directly acts on mitochondrial targets and influences mitochondrial functions in sensitive cells. This may explain the rapidly lethal effects of some targeted therapies and advance understanding of how these anticancer agents work. As localization of MET to mitochondria was not found in resistant cells, this may be a hallmark of sensitive cells and have potential implications in personalized cancer therapeutics.

Our findings do not contradict but rather enhance the conventional paradigm that MET inhibitors including small molecule inhibitors and monoclonal antibodies act on plasma membrane MET (12). As shown in Fig. 7, phosphorylation of MET in whole-cell lysate was rapidly inhibited by 50 nM PHA-665752, consistent with global suppression of MET molecules by the inhibitor as others have reported (10, 13). As reported previously, baseline phosphorylation of downstream signaling effectors, such as ERK1/2, AKT, STAT3, and FAK, were effectively abrogated by 50 nM PHA-665752, confirming that canonical MET signaling pathway was inhibited by this compound (10). However, this study provides evidence for an additional and novel locus of MET inhibition i.e. mitochondrial MET, that may be critical in contributing to the efficacy of MET inhibition in sensitive cells.

We are still unclear about the origin of the mitochondrial MET. One possibility is translocation of autophosphorylated plasma membrane MET to mitochondria. It is also possible that a fraction of synthesized MET is directly localized to mitochondria because of certain signal peptide sequence or posttranslational modification. Localization of MET to mitochondria may correlate with the high genetic expression of MET in sensitive cells. The substrates of mitochondrial MET remain to be determined. Future work is also needed to demonstrate whether the failure of MET to localize to mitochondria affects the behavior and response of sensitive cells.
MET Expressed in Mitochondria is a Direct Target for Kinase Inhibitor

CONCLUSION

In an effort to understand molecular mechanisms of the curious sensitivity of MET-overexpressing cancer cells to MET inhibition, we have uncovered novel mechanisms of MET inhibition in sensitive cells. In response to low concentration of MET inhibitor, sensitive cells displayed substantial deregulation of mitochondrial proteins and functions. This study is the first to show the presence of activated MET in mitochondria of sensitive cancer cells that might be a direct target of MET inhibitor.

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