Strong anti-tumor effect of NVP-AUY922, a novel Hsp90 inhibitor, on non-small cell lung cancer

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ABSTRACT

The anti-tumor activity of a newly developed Hsp90 inhibitor, NVP-AUY922 (AUY922), against non-small cell lung cancer (NSCLC) was examined. Twenty-one NSCLC cell lines were used, the somatic alterations of which were characterized. Cell proliferation was analyzed using a modified MTS assay. Expression of the client proteins was assessed using Western blotting. The cell cycle was analyzed using flow cytometry. The IC_{50} value of AUY922 for the NSCLC cell lines ranged from 5.2 to 860 nM (median, 20.4 nM). Based on previous data, cells with an IC_{50} of less than 50 nM were classified as sensitive cells and 19 of the 21 NSCLC cell lines were judged to be sensitive. The IC_{50} of five malignant pleural mesothelioma (MPM) cell lines revealed that the MPM cells had a significantly higher IC_{50} value (median, 89.2 nM; range, 22.2-24,100 nM) than the NSCLC cells (p=0.015). There was significant depletion of both the total and phosphorylated client proteins - EGFR, MET, HER2 and AKT - at low drug concentrations (50-100 nM) in drug-sensitive cell lines. Cell-cycle analysis was performed for two sensitive cell lines, H1975 and H838. Following AUY922 treatment, an increase in the sub-G_0-G_1 cell population, as well as appearance of cleaved PARP expression, indicated the induction of apoptosis. In conclusion, AUY922 was effective against most NSCLC cell lines, independent of the type of known molecular

alteration, and appears to be a promising new drug for the treatment of NSCLC.

1. Introduction

Lung cancer is associated with various types of molecular alteration, including epidermal growth factor receptor (EGFR) mutation, *K-ras* mutation, *HER2* amplification and, as recently found, *EMK4-ALK* gene fusion.[1-3] Improvements in our understanding of the molecular alterations involved in lung cancer have brought significant advancements in molecular-targeted therapy.[4] Among these alterations, *EGFR* mutations, which are frequent alterations in lung adenocarcinoma, are a predictive factor for the efficacy of EGFR-tyrosine kinase inhibitors (EGFR-TKIs), such as gefitinib and erlotinib.[1, 2] These EGFR-TKIs have a marked anti-tumor effect on NSCLCs with common *EGFR* mutations. However, acquired resistance from, for example, a secondary *EGFR* T790M mutation or *MET* amplification is a major problem that is responsible for treatment failure.[5-7]

The heat-shock protein 90 (Hsp90) complex is a chaperone protein that facilitates the refolding of unfolded or misfolded proteins. It plays a pivotal role in cancer cell survival, as it stabilizes a large set of proteins, so-called client proteins, many of which are essential for apoptosis, cell-cycle regulation, proliferation, and other characteristic properties of cancer cells.[8, 9] In NSCLC, Hsp90 stabilizes oncogenic proteins such as EGFR, MET, HER2 and AKT.[9, 10] We and some other studies have

shown that geldanamycin (GM) and its analogues, the benzoquinone ansamycin class (17 allylamino-17demethoxygeldanamycin [17-AAG] and 17 dimethylaminoethylamino -17-demethoxygeldanamycin [17-DMAG]), are effective against *EGFR*-mutated cell lines, even those that contain the *EGFR* T790M mutation that causes resistance to EGFR-TKI.[11-14] However, the results of clinical trials for 17-AAG and 17-DMAG were somewhat disappointing [15-19] and new potent Hsp90 inhibitors have therefore been pharmacologically designed and synthesized to offer improved efficacy and acceptable toxicity. NVP-AUY922 (AUY922) is one of these newly designed small-molecule Hsp90 inhibitors based on the 4,5-diarylisoxazole scaffold; it has a much higher affinity for Hsp90 than previous GM analogues.[20] AUY922 is bound to the ATP binding site of Hsp90α at the N-terminal domain, and its X-ray crystal structure confirms a crucial network of hydrogen bonding interactions. It exhibits the tightest binding of any small-molecule Hsp90 ligand because the entropy of binding to Hsp90 is almost negligible. Indeed, preclinical data from various types of human cancer have shown an anti-proliferative effect of AUY922, with low nanomolar potency both *in vivo* and *in vitro*, with no major adverse effects being observed in mice.[20-24] In these studies, AUY922 suppressed the client proteins (EGFR, MET, HER2 and AKT) that participate in the progression of various cancer cells, and AUY922 is considered to

be a promising agent for NSCLC. However, to our knowledge, the efficacy of AUY992 has been reported in only one NSCLC cell line (A549) to date,[25] although Phase II clinical trials for patients with advanced NSCLC have recently started.

In this study, we examined the anti-tumor effect of AUY922 against NSCLC cell lines containing several known genetic alterations, including *EGFR* mutations.

2. Materials and methods

2.1. Drugs and cell lines

AUY922 was obtained from Novartis (Nuremberg, Germany) and dissolved in dimethyl sulfide (DMSO) at stocked concentrations of 10 mM and stored at -20°C. Working dilutions were always freshly prepared. Most of NSCLC and MPM cell lines used in this study were established at two institutions. The prefix NCI-H- (abbreviated as H-) indicates cell lines established at the National Cancer Institute-Navy Medical Oncology Branch, National Naval Medical Center, Bethesda, MD and the prefix HCC- indicates lines established at the Hamon Center for Therapeutic Oncology Research, the University of Texas Southwestern Medical Center at Dallas, Dallas, TX. These cell lines were kindly provided by Dr. Adi F. Gazdar (University of Texas Southwestern

Medical Center at Dallas, Dallas, TX, USA). A549 was purchased from American Type Culture Collection (Manassas, VA). NCI-H3255 was provided from Dr. Bruce Johnson (Lowe Center for Thoracic Oncology, Dana-Farber Cancer Institute, Boston, MA). PC-9 was provided from Immuno-Biological Laboratories (Takasaki, Gunma, Japan). Gefitinib-resistant PC-9 cell line (RPC-9) were provided from the Department of Hematology, Oncology, and Respiratory Medicine, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Japan.[26] All the cancer cell lines were maintained in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum. All cell lines were incubated at 37°C in a humidified atmosphere with 5% CO_2.

2.2. Determination of cell proliferation

Cell proliferation was determined by a modified MTS assay with CellTiter 96® AQueous One Solution Reagent (Promega, Madison, WI). Cells were seeded on 96-well flat-bottomed tissue culture plates (Becton Dickinson, San Jose, CA) at a concentration of 3×10^3 cells/well with complete culture medium and allowed to adhere to the plate for 24 hours. Then the cells were incubated in the presence of the drug of each concentration ranging from 0 (control) to 10 μM of for another 72 hours at 37°C in a

humidified atmosphere of 5% CO_2 in air. After the treatment, 20 µl of CellTiter 96® AQueous One Solution Reagent were dropped into each well of plates. After the incubation of another 60 minutes, the optical densities (OD) of these samples were directly measured using an Immuno Mini NJ-2300 (Nalge Nunc International, Rochester, NY). A reference wavelength at 490 nm was used to subtract background contributed by excess cell debris, fingerprints and other nonspecific absorbance. The OD of control samples was regarded as 100 and others were compared to the control. Each drug concentration was distributed in 4-replicate wells and each experiment was repeated thrice. The anti-proliferative activity of AUY922 was shown as IC_{50}, which is the concentration of the drug required to inhibit cell proliferation by 50%.

2.3. Western blot analysis and immunoprecipitation

Protein expression analysis was assessed by Western blotting. The lysate was extracted and 20 µg of total protein were then separated by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membrane. The membranes were incubated with anti-EGFR, anti-phospho-EGFR (Ty1068), anti-Met (25H2), anti-phospho-Met (3D7, Tyr1234/1235), anti-HER2, anti-phospho-HER2 (Tyr877), anti-Akt, anti-phosphor-Akt (Ser473), anti-p44/42 mitrogen-activated protein kinases (MAPK),

anti-phosphor-MAPK (Thr202/Tyr204), anti-Cyclin D1, anti-cdc2 and anti-cleaved poly (ADP-ribose) polymerase (PARP) (Asp214) (19F4) antibodies (Cell Signaling Technology, Beverly, MA), anti-Hsp90 (Novocastra, Newcastle, UK), anti-Hsp70 (Stressgen Bioreagents, Ann Arbor, MI), anti-CDK4 (C-22) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Actin (used as loading control, Millipore Billerica, MA) and then with goat anti-rabbit and goat anti-mouse IgG-HRP coupled to horseradish peroxidase conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). After the incubation with antibodies, the membranes were developed by ECL Plus Western Blotting Detection Reagents (Amersham Biosciences UK Limited, Buckinghamshire, UK).

2.4. Flow cytometric analysis

Cells were harvested and resuspended in PBS containing 0.2% Triton X-100 and 1 mg/mL RNase for 5 min at room temperature and then stained with propidium iodide at 50 μg/mL to determine subdiploid DNA content using a FACScan. Doublets, cell debris, and fixation artifacts were gated out, and cell cycle analysis was done using CellQuest version 3.3 software.

3. Results

3.1. Anti-proliferative effect of AUY922 in NSCLC cell lines

The concentrations of AUY922 at IC_{50} in each cell line are shown in Table I and Fig. 1. The molecular characteristics of NSCLC cell lines are also described (Table I). The IC_{50} values in the NSCLC cell lines ranged from 5.2 to 860 nM, whereas those in the MPM cell lines ranged from 22.2 to 24,100 nM ($p=0.015$), indicating a significant difference in AUY922 sensitivity between NSCLC and MPM cell lines. For NSCLC, AUY922 exhibited a strong anti-proliferative effect in cell lines with *EGFR* mutations that were either sensitive to EGFR-TKI or that had acquired resistance to EGFR-TKI, similar to the effects of GM analogues. Furthermore, AUY922 also exhibited anti-proliferative effects on cell lines with wild-type *EGFR*, a *K-ras* mutation, *EML4-ALK* fusion gene, or other genetic alterations.

We also determined the IC_{50} value of the SKBR3 breast cancer cell line to validate the IC_{50} value determined with our MTS assay by comparing it with published data.[22] Our and previously published IC_{50} values were 9.7 ± 3.5 nM and 3.3 ± 0.9 nM, respectively, suggesting that the IC_{50} value measured using our system was not remarkably different from the published data.[22] Thus, in accordance with the

published criteria, an IC_{50} value of less than 50 nM was regarded as being a sensitive cell line.[22] On the basis of this criteria, 19 of the 21 NSCLC cell lines and two of the five MPM cell lines were classified as being sensitive (p=0.034).

Two cell lines, H1395 and Calu3, were considered to be resistant. H1395 contains a *B-raf* mutation as a known molecular alteration, while Calu3 has a strong amplification of *HER2* and increased copy numbers of *EGFR* and *PIK3CA*. However, the H2170 cell line, which also exhibited strong *HER2* amplification and an increased copy number of *EGFR*, was classified as a sensitive cell line (IC_{50} = 9.1 $\pm$ 0.3), suggesting that amplification of *HER2* or *EGFR* is not the factor that causes resistance to AUY922.

3.2. Effects of AUY922 on molecular signature in NSCLC cell lines

Subsequent experiments focused on NSCLC. The effect of AUY922 on protein expression was examined according to concentration and exposure time in three sensitive cell lines (H1975, A549 and H838) and two resistant cell lines (H1395 and Calu3). Cells were harvested 24 hours after drug treatment in a concentration gradient experiment (Fig. 2 and Supplementary Fig. 1). In sensitive cell lines, the depletion of both the total and the phosphorylated client proteins, such as EGFR, MET, HER2, AKT,

and Cyclin D1 (CCND1), was observed after treatment with 50 nM of AUY922. Suppression of phospho-MAPK (p-MAPK) but not total-MAPK (t-MAPK) may be caused by down-regulation of its upstream molecules, which are the client proteins of Hsp90. Although inhibition of Hsp90 activity with drugs is generally correlated with Hsp70 protein levels after treatment,[22, 27] Hsp70 expression increased in both sensitive and resistant cell lines. In terms of the resistant cell lines, although expression of the client proteins was not depleted after treatment with a high concentration of AUY922 in Calu3 (IC_{50} = 248 nM), H1395-another resistant cell line (IC_{50} = 850nM)-showed depletion of client proteins after treatment with AUY922 at a low concentration (Fig. 2 and Supplementary Fig. 1).

For exposure time analysis, each cell line except H1395 was treated with the AUY922 concentration, which was five times as high as each IC_{50}. H1395, the IC_{50} of which was 850 nM, was exposed to 100 nM of AUY922. Although variation of protein depletion and recovery was observed according to proteins or cell lines, decreased expression of the majority of proteins was observed from 12 to 72 hours (Fig. 3 and Supplementary Fig. 2). Of note, there was no major difference in the pattern of the protein expression profile time course between sensitive cell lines and H1395-resistant cell lines.

3.3. Effects of AUY922 on cell cycle and apoptosis

We analyzed the cell cycle in two sensitive cell lines (H1975 and H838) to examine the impact of AUY922 on cell-cycle distribution, especially induction of apoptosis. Whereas the pattern of cell-cycle distribution after treatment of AUY922 was different between two cell lines, sub-G_0-G_1 DNA content increased in a time-dependent manner for both cell lines. Cleaved PARP also increased with AUY922 treatment, indicating that AUY922 induced apoptosis in these two cell lines (Fig. 4).

4. Discussion

In this study, we found that AUY922 had a strong anti-proliferative effect on most NSCLC cell lines. Previous studies have indicated that GM analogue Hsp90 inhibitors have an anti-tumor effect on *EGFR* mutant NSCLC cell lines, including acquired TKI-resistant NSCLC. This suggests that Hsp90 inhibitors are promising agents for resistance to EGFR-TKI in the treatment of NSCLC.[12] However, a recent clinical trial for IPI-504, an analog of 17-AAG, failed to show its significant effectiveness for *EGFR* mutant NSCLC patients.[17] On the other hand, IPI-504

showed response to 2 of 3 NSCLC patients with *EML4-ALK* fusion gene. One of the reasons is that enrolled patients with *EGFR* mutation had been treated at least two prior EGFR-TKI agents, suggesting that the biological features of these *EGFR* mutant tumors might be different from those of untreated tumors with single oncogene addicted status. In addition, cancer cell lines with *EML4-ALK* might be more sensitive for 17-AAG than those with *EGFR* mutation.[17] Unlike GM analogues including 17-AAG, AUY992 exhibited similar anti-tumor effect not only in *EGFR* mutant tumors, but also in wild-type *EGFR* tumors with various molecular alterations including *K-ras* mutation, *EML4-ALK* fusion gene, or *MET* or *HER2* amplification. One reason is that AUY922 has a much higher affinity for the N-terminal nucleotide-binding site of human Hsp90 than other Hsp90 inhibitors and can strongly suppress the expression of many client proteins at low concentrations.[20]

Cell-cycle distribution was examined in two cell lines to assess the induction of apoptosis, but the pattern of distributions was not identical. Many client proteins of Hsp90 are thought to be involved in the pathogenesis of cancers. The degree and manner of involvement of each client protein should vary according to the cancer type, resulting in variation of the cellular response, such as cell-cycle distribution and degree of apoptosis. This would account for the difference in pattern of cell distribution and

degree of apoptosis even in the sensitive cell lines.

In our series, the two cell lines Calu3 and H1395 were regarded as being resistant to AUY922. The client proteins in Calu3 were not depleted with AUY922 treatment as much. The fact that expression of Hsp70 was induced in Calu3 confirmed the inhibition of Hsp90 with AUY922, which suggested that drug transporters or metabolic activity might not be responsible for the resistance of Calu3. The cause of preserved expression of client proteins is unclear. In contrast, H1395 showed decreased expression of the client proteins at a low concentration of AUY922, which was similar to the response in sensitive cell lines. As early recovery of client proteins under AUY922 treatment was related to drug resistance in glioblastoma,[28] we examined whether there was a difference in the recovery time of depleted proteins between sensitive and resistant cell lines. However, there was no difference between them in NSCLC and the mechanism of resistance was unclear. One possible explanation for the observed resistance is that although Hsp90 has many client proteins that are generally essential for tumor proliferation and survival in the majority of cancers, when cancer cells do not depend on these client proteins for survival, the inhibition of Hsp90 may not be effective. Of clinical relevance, this point may suggest that the selection of patients suited to AUY922 treatment based on molecular properties is difficult. Further

investigation to identify the factors that can predict sensitivity or resistance to AUY922 is necessary.

Our results suggest that AUY922 is not effective in MPM compared to NSCLC. Although the precise mechanism of resistance is not clear, the molecular characteristics of MPM are different to those of NSCLC.[29, 30] Regarding the clinical use of AUY922, Phase I/II trials of intravenously administered AUY922 are currently ongoing (http://clinicaltrials.gov/) for patients with various types of cancer. From February 2011 to present, two interesting clinical trials have begun for advanced NSCLC. The NCT01124864 trial is for patients who have received at least two lines of prior chemotherapy, and the patients are stratified according to *K-ras* and *EGFR* mutation status. The NCT01259089 trial is for patients with lung adenocarcinoma with "acquired resistance" to EGFR-TKI. It is noteworthy that our data strongly support the use of AUY922 for the treatment of NSCLC patients with various somatic alterations or with acquired resistance to EGFR-TKI.

In conclusion, our study suggests that AUY922 is a potent candidate for the treatment of the majority of NSCLCs, independent of the major known genetic alterations.

Conflict of interest statement

None

References

[1] Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, Harris PL, Haserlat SM, Supko JG, Haluska FG, Louis DN, Christiani DC, Settleman J, Haber DA. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med 2004; 350: 2129-2139.

[2] Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S, Herman P, Kaye FJ, Lindeman N, Boggon TJ, Naoki K, Sasaki H, Fujii Y, Eck MJ, Sellers WR, Johnson BE, Meyerson M. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 2004; 304: 1497-1500.

[3] Soda M, Choi YL, Enomoto M, Takada S, Yamashita Y, Ishikawa S, Fujiwara S, Watanabe H, Kurashina K, Hatanaka H, Bando M, Ohno S, Ishikawa Y, Aburatani H, Niki T, Sohara Y, Sugiyama Y, Mano H. Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. Nature 2007; 448: 561-566.

[4] Toyooka S MT, Soh J, Aokage K, Yamane M, Oto T, Kiura K, Miyoshi S. Molecular oncology of lung cancer. Gen Thorac Cardiovasc Surg In press.

[5] Kobayashi S, Boggon TJ, Dayaram T, Janne PA, Kocher O, Meyerson M, Johnson

BE, Eck MJ, Tenen DG, Halmos B. EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. N Engl J Med 2005; 352: 786-792.

[6] Pao W, Miller VA, Politi KA, Riely GJ, Somwar R, Zakowski MF, Kris MG, Varmus H. Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. PLoS Med 2005; 2: e73.

[7] Engelman JA, Zejnullahu K, Mitsudomi T, Song Y, Hyland C, Park JO, Lindeman N, Gale CM, Zhao X, Christensen J, Kosaka T, Holmes AJ, Rogers AM, Cappuzzo F, Mok T, Lee C, Johnson BE, Cantley LC, Janne PA. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. Science 2007; 316: 1039-1043.

[8] Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011; 144: 646-674.

[9] Solit DB, Chiosis G. Development and application of Hsp90 inhibitors. Drug Discov Today 2008; 13: 38-43.

[10] Neckers L. Heat shock protein 90: the cancer chaperone. J Biosci 2007; 32: 517-530.

[11] Kobayashi N, Toyooka S, Soh J, Yamamoto H, Dote H, Kawasaki K, Otani H,

Kubo T, Jida M, Ueno T, Ando M, Ogino A, Kiura K, Miyoshi S. The anti-proliferative effect of heat shock protein 90 inhibitor, 17-DMAG, on non-small-cell lung cancers being resistant to EGFR tyrosine kinase inhibitor. Lung Cancer 2011.

[12] Shimamura T, Lowell AM, Engelman JA, Shapiro GI. Epidermal growth factor receptors harboring kinase domain mutations associate with the heat shock protein 90 chaperone and are destabilized following exposure to geldanamycins. Cancer Res 2005; 65: 6401-6408.

[13] Shimamura T, Li D, Ji H, Haringsma HJ, Liniker E, Borgman CL, Lowell AM, Minami Y, McNamara K, Perera SA, Zaghlul S, Thomas RK, Greulich H, Kobayashi S, Chirieac LR, Padera RF, Kubo S, Takahashi M, Tenen DG, Meyerson M, Wong KK, Shapiro GI. Hsp90 inhibition suppresses mutant EGFR-T790M signaling and overcomes kinase inhibitor resistance. Cancer Res 2008; 68: 5827-5838.

[14] Sawai A, Chandarlapaty S, Greulich H, Gonen M, Ye Q, Arteaga CL, Sellers W, Rosen N, Solit DB. Inhibition of Hsp90 down-regulates mutant epidermal growth factor receptor (EGFR) expression and sensitizes EGFR mutant tumors to paclitaxel. Cancer Res 2008; 68: 589-596.

[15] Nowakowski GS, McCollum AK, Ames MM, Mandrekar SJ, Reid JM, Adjei AA, Toft DO, Safgren SL, Erlichman C. A phase I trial of twice-weekly 17-allylamino-demethoxy-geldanamycin in patients with advanced cancer. Clin Cancer Res 2006; 12: 6087-6093.

[16] Sharp S, Workman P. Inhibitors of the HSP90 molecular chaperone: current status. Adv Cancer Res 2006; 95: 323-348.

[17] Sequist LV, Gettinger S, Senzer NN, Martins RG, Janne PA, Lilenbaum R, Gray JE, Iafrate AJ, Katayama R, Hafeez N, Sweeney J, Walker JR, Fritz C, Ross RW, Grayzel D, Engelman JA, Borger DR, Paez G, Natale R. Activity of IPI-504, a novel heat-shock protein 90 inhibitor, in patients with molecularly defined non-small-cell lung cancer. J Clin Oncol 2010; 28: 4953-4960.

[18] Holzbeierlein JM, Windsperger A, Vielhauer G. Hsp90: a drug target? Curr Oncol Rep 2010; 12: 95-101.

[19] Ramanathan RK, Egorin MJ, Erlichman C, Remick SC, Ramalingam SS, Naret C, Holleran JL, TenEyck CJ, Ivy SP, Belani CP. Phase I pharmacokinetic and pharmacodynamic study of 17-dimethylaminoethylamino-17-demethoxygeldanamycin, an inhibitor of heat-shock protein 90, in patients with advanced solid tumors. J Clin Oncol 2010;

28: 1520-1526.

[20] Brough PA, Aherne W, Barril X, Borgognoni J, Boxall K, Cansfield JE, Cheung KM, Collins I, Davies NG, Drysdale MJ, Dymock B, Eccles SA, Finch H, Fink A, Hayes A, Howes R, Hubbard RE, James K, Jordan AM, Lockie A, Martins V, Massey A, Matthews TP, McDonald E, Northfield CJ, Pearl LH, Prodromou C, Ray S, Raynaud FI, Roughley SD, Sharp SY, Surgenor A, Walmsley DL, Webb P, Wood M, Workman P, Wright L. 4,5-diarylisoxazole Hsp90 chaperone inhibitors: potential therapeutic agents for the treatment of cancer. J Med Chem 2008; 51: 196-218.

[21] Eccles SA, Massey A, Raynaud FI, Sharp SY, Box G, Valenti M, Patterson L, de Haven Brandon A, Gowan S, Boxall F, Aherne W, Rowlands M, Hayes A, Martins V, Urban F, Boxall K, Prodromou C, Pearl L, James K, Matthews TP, Cheung KM, Kalusa A, Jones K, McDonald E, Barril X, Brough PA, Cansfield JE, Dymock B, Drysdale MJ, Finch H, Howes R, Hubbard RE, Surgenor A, Webb P, Wood M, Wright L, Workman P. NVP-AUY922: a novel heat shock protein 90 inhibitor active against xenograft tumor growth, angiogenesis, and metastasis. Cancer Res 2008; 68: 2850-2860.

[22] Jensen MR, Schoepfer J, Radimerski T, Massey A, Guy CT, Brueggen J, Quadt C,

Buckler A, Cozens R, Drysdale MJ, Garcia-Echeverria C, Chene P. NVP-AUY922: a small molecule HSP90 inhibitor with potent antitumor activity in preclinical breast cancer models. Breast Cancer Res 2008; 10: R33.

[23] Lee KH, Lee JH, Han SW, Im SA, Kim TY, Oh DY, Bang YJ. Antitumor activity of NVP-AUY922, a novel heat shock protein 90 inhibitor, in human gastric cancer cells is mediated through proteasomal degradation of client proteins. Cancer Sci 2011.

[24] Okui T, Shimo T, Hassan NM, Fukazawa T, Kurio N, Takaoka M, Naomoto Y, Sasaki A. Antitumor Effect of Novel HSP90 Inhibitor NVP-AUY922 against Oral Squamous Cell Carcinoma. Anticancer Res 2011; 31: 1197-1204.

[25] Stingl L, Stuhmer T, Chatterjee M, Jensen MR, Flentje M, Djuzenova CS. Novel HSP90 inhibitors, NVP-AUY922 and NVP-BEP800, radiosensitise tumour cells through cell-cycle impairment, increased DNA damage and repair protraction. Br J Cancer 2010; 102: 1578-1591.

[26] Ogino A, Kitao H, Hirano S, Uchida A, Ishiai M, Kozuki T, Takigawa N, Takata M, Kiura K, Tanimoto M. Emergence of epidermal growth factor receptor T790M mutation during chronic exposure to gefitinib in a non small cell lung cancer cell line. Cancer Res 2007; 67: 7807-7814.

[27] Beere HM. "The stress of dying": the role of heat shock proteins in the regulation of apoptosis. J Cell Sci 2004; 117: 2641-2651.

[28] Gaspar N, Sharp SY, Eccles SA, Gowan S, Popov S, Jones C, Pearson A, Vassal G, Workman P. Mechanistic evaluation of the novel HSP90 inhibitor NVP-AUY922 in adult and pediatric glioblastoma. Mol Cancer Ther 2010; 9: 1219-1233.

[29] Toyooka S, Pass HI, Shivapurkar N, Fukuyama Y, Maruyama R, Toyooka KO, Gilcrease M, Farinas A, Minna JD, Gazdar AF. Aberrant methylation and simian virus 40 tag sequences in malignant mesothelioma. Cancer Res 2001; 61: 5727-5730.

[30] Toyooka S, Kishimoto T, Date H. Advances in the molecular biology of malignant mesothelioma. Acta Med Okayama 2008; 62: 1-7.

Table I. IC_{50} inhibition values for NVP-AUY922 in NSCLC and MPM cell lines

Cancer type	Cell lines	Histological subtypes	AUY922		Genetic alterations	
			*Sensitivity	IC_{50} (nM)		
NSCLC	PC-9	AD	Sensitive	8.6 ± 0.5	*EGFR* mutation	Exon19 del.
	HCC2935	AD	Sensitive	9.2 ± 0.1		Exon19 del.
	HCC827	AD	Sensitive	16.9 ± 0.4		Exon19 del.
	HCC2279	AD	Sensitive	26.3 ± 3.6		Exon19 del.
	HCC4011	AD	Sensitive	17.9 ± 0.1		L858R
	H3255	AD	Sensitive	29.5 ± 5.8		L858R
	RPC-9	AD	Sensitive	20.4 ± 1.4		Exon19 del. + T790M
	H1975	AD	Sensitive	5.2 ± 0.3		L858R + T790M
	H1650	AD	Sensitive	23.5 ± 2.9		Exon19 del. + PTEN del.
	H1299	LC	Sensitive	32.4 ± 0.1	*N-ras* mutation	
	A549	AD	Sensitive	16.3 ± 0.6	*K-ras* mutation	
	H2009	AD	Sensitive	21.4 ± 0.8	*K-ras* mutation	
	H358	AD	Sensitive	28.1 ± 4.1	*K-ras* mutation	
	H2170	SQ	Sensitive	9.1 ± 0.3	*HER2* amplification	
	H1648	AD	Sensitive	9.6 ± 0.1	*HER2* amplification	
	H1819	AD	Sensitive	23.9 ± 1.0	*HER2* amplification	
	Calu3	AD	Resistant	248 ± 8.5	*HER2* amplification	
	H1993	AD	Sensitive	7.7 ± 0.2	*MET* amplification	
	H1395	AD	Resistant	860 ± 7.1	*B-raf* mutation	
	H2228	AD	Sensitive	20.4 ± 6.5	*EML4-ALK* fusion gene variant E6a/b;A20	
	H838	AD	Sensitive	17.1 ± 0.6	None	
MPM	H211	Biphasic	Sensitive	22.2 ± 3.8		
	H290	Epithelial	Sensitive	27.3 ± 3.8		
	H28	Sarcomatoid	Resistant	89.2 ± 8.2		
	HP1	Biphasic	Resistant	1,070 ± 10		
	H2052	Epithelial	Resistant	24,100 ± 4,900		
BC	SKBR3		Sensitive	9.7 ± 3.5		

NSCLC, non-small cell lung cancer: MPM, malignant pleural mesothelioma; BC, breast cancer; AD, adenocarcinoma; LC, large cell carcinoma; SQ, squamous cell carcinoma. *Sensitivity: sensitive cell lines, IC_{50} value $\leq$ 50 nM; resistant cell lines, IC_{50} value > 50 nM; del, deletion; NVP-AUY922 exhibited strong effects to most NSCLC cell lines with *EGFR* and *K-ras* mutation or *HER2* and *MET* amplification.

FIGURE LEGENDS

Fig. 1. IC_{50} values of non-small cell lung cancer and malignant pleural mesothelioma cell lines.

Fig. 2. The profiles of protein expression under the treatment of different AUY922 concentration for 24 hours.

Fig. 3. The profiles of protein expression according to exposure time with AUY922. Each NSCLC cell line (H1975, A549, and H838) was treated with AUY922 of which concentration was five times as high as each IC_{50}. H1395 was exposed to 100 nM of AUY922.

Fig. 4. The impact of AUY922 on cell cycle distribution and induction of apoptosis. Using two sensitive cell lines, cell cycle distribution was analyzed using flow cytometry and cleaved PARP expression was examined using Western blotting. After treatment of AUY922, sub-G_0-G_1 DNA content increased in a time-dependent manner and cleaved PARP also increased with AUY922 treatment.

Supplementary Fig. 1. The profiles of phospho-protein expression under the treatment of different AUY922 concentration for 24 hours.

Supplementary Fig. 2. The profiles of phospho-protein expression according to exposure time with AUY922. Each NSCLC cell line (H1975, A549, and H838) was treated with AUY922 of which concentration was five times as high as each IC_{50}. H1395 was exposed to 100 nM of AUY922.

Fig. 1

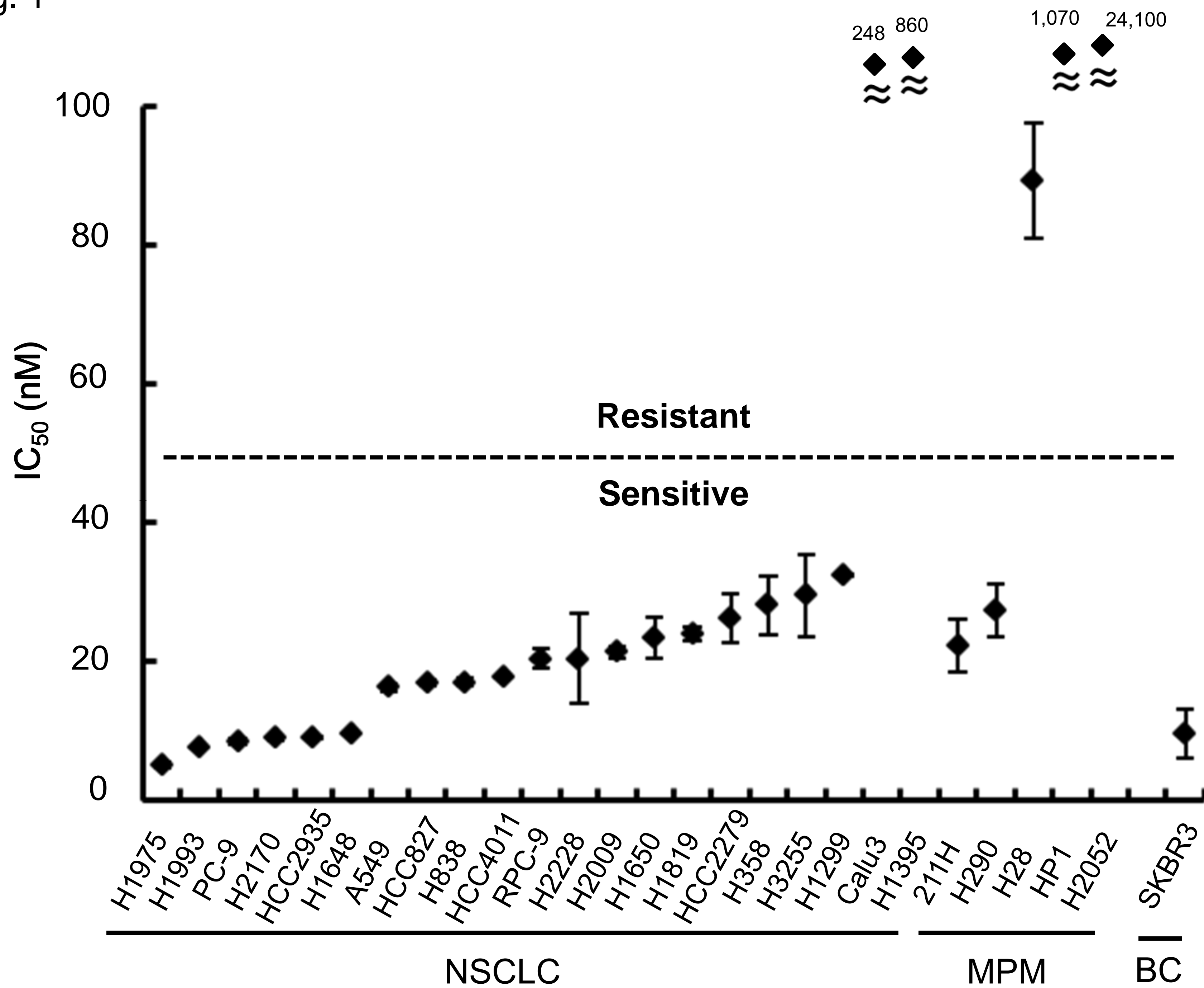


Fig. 2

H1975 A549 H838 H1395 Calu3

AUY922(nM) 0 10 50 100 500 0 10 50 100 500 0 10 50 100 500 0 10 50 100 500 0 10 50 100 500

EGFR

MET

HER2

AKT

CCND1

MAPK

Hsp90

Hsp70

Actin

Fig. 3

H1975 A549 H838 H1395

AUY922(hr) 0 12 24 48 72 0 12 24 48 72 0 12 24 48 72 0 12 24 48 72

EGFR

MET

HER2

AKT

CCND1

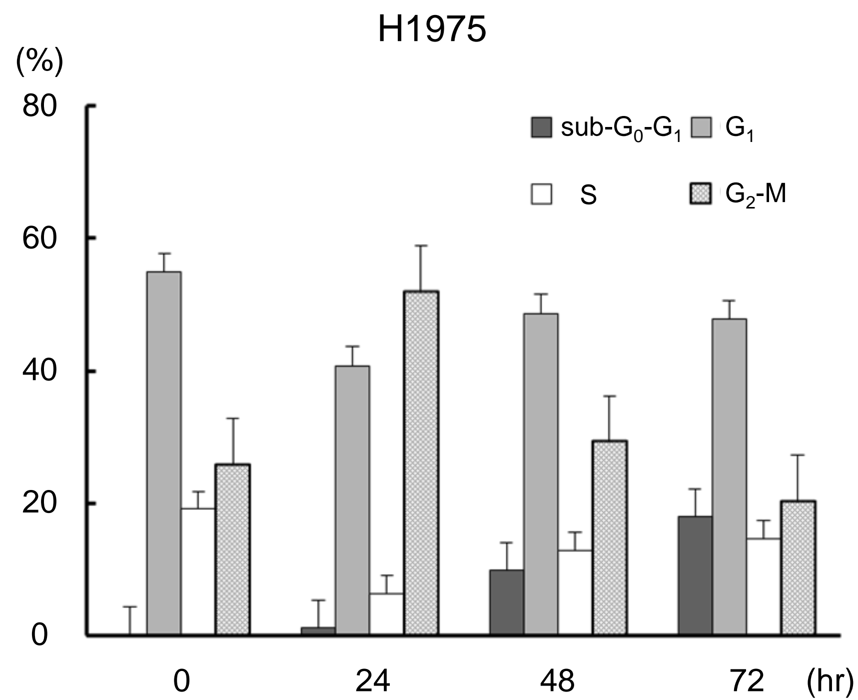
MAPK

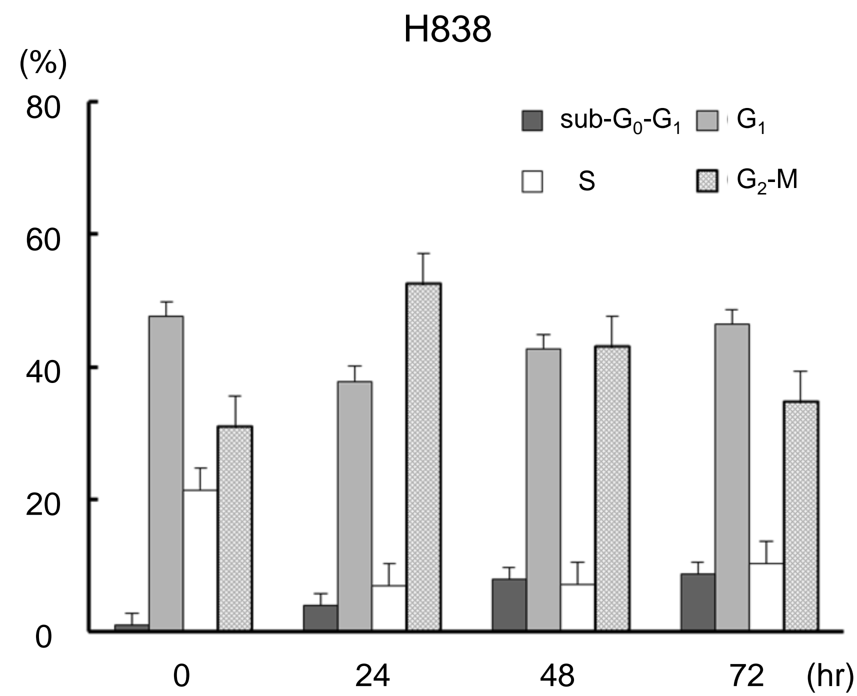
Hsp90

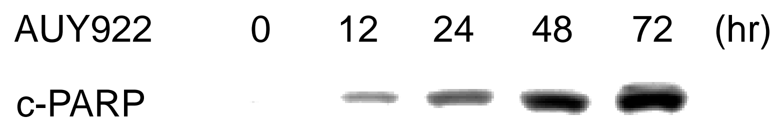
Hsp70

Actin

Fig. 4







Supplementary Fig.1

	H1975	A549	H838	H1395	Calu3
AUY922(nM)	0 10 50 100 500	0 10 50 100 500	0 10 50 100 500	0 10 50 100 500	0 10 50 100 500
p-EGFR					
p-MET					
p-HER2					
p-AKT					
p-MAPK					

Supplementary Fig.2