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Review Article: Strategy for Drug Discovery at Pharmaceutical Companies

The Current State of Molecularly Targeted Drugs Targeting HGF/Met

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Met is a tyrosine kinase that has hepatocyte growth factor as its ligand. Met plays a major role in cell growth, migration and morphological changes. Overexpression of hepatocyte growth factor and Met and mutations and amplification of *MET* have been noted in many forms of cancer and are reportedly correlated with cancer progression and a poor prognosis. Over the past few years, these molecules have attracted attention as targets of molecularly targeted therapies. This article describes the association relationship between hepatocyte growth factor/Met and cancer and it describes the latest findings regarding inhibitors to target hepatocyte growth factor/Met that are currently being developed.

Key words: HGF – Met – EGFR-TKI resistance – tyrosine kinase

ABNORMALITIES IN HGF/MET SIGNALING PATHWAYS IN CANCER

A transmembrane tyrosine kinase receptor, Met is a heterodimer consisting of a 45 kDa extracellular α-subunit and 145 kDa transmembrane β-subunit. Hepatocyte growth factor (HGF) is the only known ligand of the tyrosine kinase receptor Met. When the ligand HGF binds to Met's Sema domain, Met dimerizes. In accordance with changes in its three-dimensional structure, tyrosine residues 1230, 1234 and 1235 in the tyrosine kinase domain are phosphorylated. Tyrosine residues 1349 and 1356 in the C-terminal region are also phosphorylated, and adapter proteins bind to these residues, activating Met. When Met is activated and adapter proteins bind to the tyrosine residues in the C-terminal region, activation of downstream signaling pathways such as PI3K/Akt, Ras/Rac/Rho and Ras/MAPK is facilitated. This signaling is known to induce cell growth, survival, and migration and angiogenesis (Fig. 1) (1,2).

Enhancement of abnormal HGF/Met signaling as a result of overexpression of HGF and Met and mutations and amplification of *MET* is associated with the progression of various forms of cancer. Overexpression of HGF has been noted in numerous forms of cancer, such as lung cancer (50%), breast cancer (91%), stomach cancer (87%), colon cancer (95%), cancer of the head and neck (45%) and liver cancer (33%) (3). Elevated levels of HGF in the blood are reportedly a factor for a poor prognosis for several forms of cancer (4). Moreover, overexpression of HGF in lung cancer is also known to be a factor for resistance to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (EGFR-TKIs). Overexpression of Met in tumor tissue has been noted in a range of cancers, such as lung cancer, stomach cancer, breast cancer, kidney cancer and colon cancer. Overexpression of=Met is reportedly a factor for a poor prognosis. *MET*-activating mutations in Met's tyrosine kinase domain have been noted in hereditary and sporadic renal cell carcinomas, pediatric liver cancer and squamous cell carcinoma of the head and neck. Other mutations in Met's juxtamembrane regain or in its Sema domain have been noted in cancers such as stomach cancer, breast cancer, pleural mesothelioma and small-cell lung cancer. *MET* amplification has been noted primarily in gastrointestinal cancers such as stomach cancer, esophageal cancer and colon cancer. In addition, Met inhibitors have become a key therapy to treat lung cancer over the past few years. *MET* amplification is reportedly involved in acquisition of resistance to EGFR-TKIs. An association between HGF/Met genetic abnormalities and cancer has been noted for

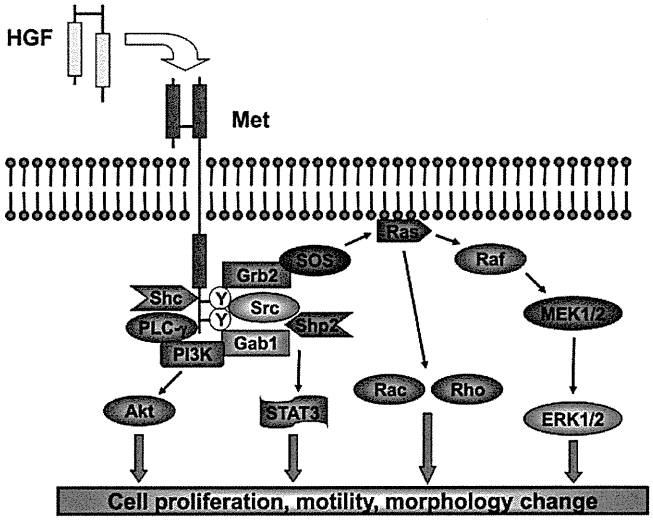

Figure 1. Hepatocyte growth factor/Met signaling pathway.

numerous forms of cancer. HGF/Met signaling is also reportedly involved in resistance to TKIs such as sunitinib and lapatinib as well as to EGFR-TKIs (5,6). HGF and Met are therapeutic targets that have attracted a great deal of attention over the past few years.

EGFR-TKI RESISTANCE AND HGF/MET SIGNALING

EGFR-activating mutations (exon 19 deletion or a point mutation in L858R in exon 21) are detected in 10–30% of non-small-cell lung cancers, and non-small-cell lung cancers with *EGFR*-activating mutations respond well to the EGFR-TKIs gefitinib and erlotinib (7). Nevertheless, many patients acquire drug resistance after 6 months to a year. Over the past few years, the mechanisms of resistance to EGFR-TKIs have been clarified. The first reported mechanism was a gatekeeper mutation (T790M) in exon 20 in *EGFR* (8). *MET* amplification is reportedly a primary mechanism for EGFR-TKI resistance; another is high levels of HGF expression (which the current authors noted) (9,10). As part of a joint study at 10 facilities in Japan, the current authors analyzed tumor specimens from patients with EGFR-TKI resistance. Analysis of specimens of 23 tumors from 23 patients with acquired resistance revealed the T790M mutation in 12/23 tumors (52%), *MET* amplification in 2/23 (9%) and high levels of HGF expression in 14/23 (61%). The increased incidence of activation of HGF/Met signaling may contribute to acquisition of resistance to EGFR-TKIs (11). High levels of HGF expression play three roles in the acquisition of EGFR-TKI resistance. The first is where Met is activated by HGF, and Met in turn activates Gab1/PI3K/Akt signaling; survival signaling occurs via these alternate pathways, inducing EGFR-TKI resistance. The second role that high HGF levels play is via stimulating growth of subpopulations of cells with *MET* amplification. A preclinical study cultured HCC827 lung cancer cells with *EGFR*-activating mutations in the presence of HGF and EGFR-TKIs. Growth of subpopulations of cells with *MET* amplification was stimulated, and these cells accounted for most of the cell growth (12). The third role that high HGF levels play is as a factor for resistance of T790M-mutant tumors to treatment with next-generation EGFR-TKIs such as irreversible EGFR-TKIs (CL-387, CL-785 and BIBW2992) or a mutant EGFR-selective TKI (WZ4002) (13,14). In a study involving lung cancer cell lines, the current authors found that HGF induced resistance to gefitinib and erlotinib as well as to next-generation EGFR-TKIs. EGFR-TKI resistance induced by HGF should be overcome by HGF/Met inhibitors. The current authors used E7050, a Met inhibitor, to determine whether EGFR-TKI resistance due to high levels of HGF expression could be overcome. E7050 is an ATP-competitive small molecular compound with an IC_{50} of 23 nM in relation to Met in a cell-free system. Clinical trials involving several forms of cancer are currently underway. Gefitinib powerfully inhibits the growth of PC-9 and HCC827 lung cancer cells with *EGFR*-activating mutations, but addition of HGF causes those cells to become resistant. However, gefitinib resistance as a result of added HGF is overcome by combined use of E7050. Gefitinib-resistant clones were created by long-term exposure to HGF and gefitinib, and the growth was found to be inhibited by combined use of E7050. One model of resistance due to HGF produced by tumor stroma is a mouse model in which HGF-producing human MRC-5 fibroblasts were subcutaneously implanted along with PC-9 cells. Combined therapy with E7050 was found to overcome gefitinib resistance due to HGF produced by MRC-5 cells, and this therapy was found to have tumor-shrinking action (14). In the future, the effectiveness with which combination therapy with EGFR-TKIs and E7050 overcomes resistance due to HGF/Met should be verified in clinical trials while carefully assessing the safety of that therapy.

CLINICAL DEVELOPMENT OF HGF/MET INHIBITORS

Numerous HGF/Met inhibitors besides E7050 are being developed, and clinical trials are being conducted with a wide range of cancers (Table 1). Ficlatuzumab is an anti-HGF antibody. A Phase II trial involving Asians with little history of smoking and untreated Stage IIIB/IV lung adenocarcinoma (188 patients) has been conducted. The trial compared gefitinib alone (94 patients) and gefitinib + ficlatuzumab (94 patients) (15). Differences in the response rate (the trial's primary endpoint) were not noted, but the two treatments did not result in significant differences in progression-free survival (4.7 vs. 5.6 months). However, stratified analysis of biomarkers in patients with stroma that expressed high levels of HGF indicated that patients administered gefitinib + ficlatuzumab had a significantly longer overall survival compared with patients administered gefitinib alone (94 patients).

Table 1. HGF/Met inhibitors in clinical trials

	Inhibitor		Target	Tumor types in clinical trials
Antibody	Rilotumumab	AMG102	HGF	Lung, colon, brain, stomach, ovary, renal
	Ficlatuzumab	AV-299	HGF	Lung
	Onartuzumab	MetMAb	Met	Lung, colon, breast
Small molecule	Crizotinib (Xalkori®)	PF-2341066	Met, ALK, ROS1	Lung, lymphoma
	Tivantinib	ARQ197		Lung, colon, breast, liver, prostate, myeloma
	Cabozantinib	XL184	Met, VEGFR2, Ret, Flt-3, Kit, Tie2	Lung, breast, prostate, thyroid, brain, pancreatic neuroendocrine tumor
	Foretinib	XL880	Met, VEGFR2, PDGFR, Ron, Flt-1, Flt-4, Tie2	Lung, breast, liver, renal, stomach, head and neck
	Golvatinib	E7050	Met, VEGFR2	Liver, head and neck, stomach
	MGCD265		Met, VEGFR2, Ron, Tie2	Lung
	BMS-777607		Met, Ron	Solid tumors
	AMG208		Met	Solid tumors

HGF, hepatocyte growth factor.

However, the trial had a small sample, and so further studies are needed to verify the effectiveness of anti-HGF antibodies. HGF's involvement in lung cancer treated with EGFR-TKIs was ascertained only with regard to the resistance it induced in EGFR-mutant lung cancer cells. This is directly related to the trial design. In other words, a trial should be designed so that only patients with EGFR-mutant lung cancer are selected and so that anti-HGF antibodies are added to therapy with EGFR-TKIs. Otherwise, the trial cannot verify the effectiveness of those antibodies.

MetMAb (onartuzumab) is a human monovalent anti-Met monoclonal antibody. Many anti-Met antibodies have a drawback in that they bind with Met, facilitating dimerization. As a result, they act agonistically. Thus, MetMAb was created to inhibit the activation of Met by HGF; MetMAb is monovalent, and so it avoids dimerizing Met even if it binds to Met. Since MetMAb has action to inhibit ligand-induced Met activation, it may not have action to inhibit amplified Met. A Phase I trial was conducted with a patient who had metastatic gastric cancer that was refractory to chemotherapy and expression of both HGF and Met. A complete response as a result of treatment with MetMAb was noted for 2 years, suggesting that MetMAb is effective in treating cancer with abnormal HGF/Met (16). A Phase II trial was conducted with 137 patients with non-small-cell lung cancer that was refractory to chemotherapy that did not include EGFR-TKIs. Patients were assigned to one of two groups, a group receiving MetMAb + erlotinib or a group receiving a placebo + erlotinib. The trial verified the efficacy of adding MetMAb to erlotinib. There were no significant differences in the progression-free survival of the two groups of patients; patients who were also administered MetMAb had a progression-free survival of 2.2 months, while patients given a placebo had a progression-free survival of 2.6 months (hazard ratio: 1.09, $P = 0.687$). However, levels of Met expression according to immunostaining were classified as high and low levels and then analyzed. Results revealed that the progression-free survival for patients with high levels of Met expression was 2.9 months for those who were also administered MetMAb and 1.5 months for those who were given a placebo. Patients who were also administered MetMAb had a progression-free survival that was about two times longer, and so significant improvement was noted (hazard ratio: 0.53, $P = 0.04$). In addition, the overall survival for patients with high levels of Met expression was 12.6 months for those who were also administered MetMAb and 3.8 months for those who were given a placebo. Patients who were also administered MetMAb had an overall survival that was about three times longer (hazard ratio: 0.37, $P = 0.002$). In the future, indicators of HGF/Met inhibitors must be verified further for combined therapy with EGFR-TKIs.

Tivantinib (ARQ197) is a small molecular compound that is being developed as a selective non-adenosine triphosphate (ATP)-competitive Met inhibitor (17). Recently, tivantinib was found to have microtubule-disrupting activity similar to that of vincristine (16). A Phase III clinical trial (MARQUEE Trial) was conducted to verify the effectiveness of adding tivantinib to erlotinib in patients with advanced non-squamous non-small-cell lung cancer that was resistant to platinum-based agents, but the trial was halted.

Crizotinib (PF-2341066, brand name: Xalkori®) is an anaplastic lymphoma kinase (ALK) inhibitor. Crizotinib was approved for non-small-cell lung cancer that tested positive for the *EML4-ALK* fusion gene in the USA in 2011, and the drug was similarly approved in Japan in March 2012. In addition to its inhibition of ALK, crizotinib has ROS1- and Met-inhibiting activity. The drug is currently being developed with a focus on its Met-inhibiting activity. Patients with non-small-cell lung cancer with *MET* amplification that tested negative for the *EML4-ALK* fusion gene reportedly responded to crizotinib (18). In addition, improvement in clinical

symptoms and tumor-shrinking action has been noted as a result of crizotinib administration in esophagogastric adenocarcinomas and glioblastoma multiforme with *MET* amplification (19,20). Thus, *MET* amplification may be a predictive biomarker for efficacy of Met inhibitors such as crizotinib.

CONCLUSION

Over 20 years have passed since Met and HGF were first discovered. Numerous studies have reported that abnormal HGF/Met signaling in cancer is related to disease progression, and Met and HGF have attracted attention as therapeutic targets. The efficacy and safety of numerous HGF/Met inhibitors are now being verified in clinical trials. Biomarkers and new molecularly targeted drugs are likely to be developed based on preclinical and clinical evidence regarding which inhibitors are efficacious in treating certain cancers with abnormal HGF/Met signaling.

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Conflict of interest statement

Takayuki Nakagawa is an employee of Eisai Co., Ltd for oncology research. Seiji Yano received honoraria from Chugai Pharmaceutical Co., Ltd and AstraZeneca. Seiji Yano received research funding from Chugai Pharmaceutical Co., Ltd, Kyowa Hakko Kirin Co., Ltd and Eisai Co., Ltd.

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Therapeutic activity of glycoengineered anti-GM2 antibodies against malignant pleural mesothelioma

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Key words
Antibodies, antibody-dependent cell cytotoxicity, ganglioside GM2, mesothelioma, therapeutics

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Malignant pleural mesothelioma (MPM) is a rare and highly aggressive neoplasm that arises from the pleural, pericardial, or peritoneal lining. Although surgery, chemotherapy, radiotherapy, and combinations of these therapies are used to treat MPM, the median survival of such patients is dismal. Therefore, there is a compelling need to develop novel therapeutics with different modes of action. Ganglioside GM2 is a glycolipid that has been shown to be overexpressed in various types of cancer. However, there are no published reports regarding the use of GM2 as a potential therapeutic target in cases of MPM. In this study, we evaluated the efficacy of the anti-GM2 antibody BIW-8962 as an anti-MPM therapeutic using *in vitro* and *in vivo* assays. Consequently, the GM2 expression in the MPM cell lines was confirmed using flow cytometry. In addition, eight of 11 cell lines were GM2-positive (73%), although the GM2 expression was variable. BIW-8962 showed a significant antibody-dependent cellular cytotoxicity activity against the GM2-expressing MPM cell line MSTO-211H, the effect of which depended on the antibody concentration and effector/target ratio. In an *in vivo* orthotropic mouse model using MSTO-211H cells, BIW-8962 significantly decreased the incidence and size of tumors. Additionally, the GM2 expression was confirmed in the MPM clinical specimens. Fifty-eight percent of the MPM tumors were positive for GM2, with individual variation in the intensity and frequency of staining. These data suggest that anti-GM2 antibodies may become a therapeutic option for MPM patients.

Malignant pleural mesothelioma (MPM) is a rare and highly aggressive neoplasm that arises from the pleural, pericardial, or peritoneal lining. Malignant pleural mesothelioma was previously considered to be very rare; however, the worldwide incidence is expected to increase substantially in the next decades, as MPM is usually associated with chronic asbestos exposure and there is a long latency period between the time of exposure and tumor development.[1,2] Although surgery, chemotherapy, radiotherapy, and combinations of these therapies are used to treat MPM, the median survival of such patients is dismal, at only 6–18 months.[3–5] Despite the use of a novel systemic chemotherapy regimen using the combination of pemetrexed and cisplatin, the long-term survival of patients with MPM remains limited.[6] Therefore, further specific, effective, and less toxic therapies are needed.[7]

The importance of antibody therapeutics is increasing due to the high efficacy and low toxicity of these agents. The mode of action of antibody therapeutics can be divided into two main types: antigen neutralization, and the killing of antigen-expressing cells due to antibody effector functions. The major effector functions of therapeutic antibodies include antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), both of which are mediated by the recruitment of immune cells, including natural killer (NK) cells and complement, respectively.[8] The ADCC activity, in particular, is considered to play an important role in the efficacy of antibody therapeutics for hematological and solid tumors. For example, trastuzumab has been shown to have clinical benefits in the setting of breast cancer. The beneficial effects of this drug depend on the patient's gene polymorphism of FcγRIIIA, the receptor for IgG expressed in NK cells.[9,10] These data strongly suggest that ADCC activity is involved in the therapeutic activity of trastuzumab.

Ganglioside GM2, a glycolipid consisting of ceramide and oligosaccharide, is a component of the cell membrane. The activity of GM2 is suggested to be associated with neuronal cell survival.[11] In addition, GM2 has been reported to be overexpressed in tissues of lung cancer, neuroblastoma, and glioma, although it is rarely expressed in normal cells.[12,13] Therefore, GM2 is considered to be an attractive target for therapeutic antibodies against cancer. Jones *et al.*[14] reported that melanoma patients with an elevated anti-GM2 antibody titer show prolonged survival. This finding indicates the potential importance of antibodies that recognize GM2 in the

immunological response against cancer. Although vaccination using GM2-conjugated proteins has been attempted clinically, sufficient efficacy has not yet been achieved.[15] Against this background, humanized anti-GM2 antibodies with potent ADCC and CDC activities have been generated.[16] Recently, the ADCC-enhancing modification of fucose removal from core Fc-linked oligosaccharides was applied to this antibody, and a non-fucosylated humanized anti-GM2 antibody, BIW-8962, was successfully developed. BIW-8962 was subsequently shown to have an *in vivo* therapeutic activity in a SCID mouse model of multiple organ metastasis induced by GM2-positive small-cell lung cancer (SCLC) cell lines, and overexpression of GM2 was detected in SCLC clinical specimens.[17] In order to further investigate the therapeutic potential of the non-fucosylated, humanized anti-GM2 antibody BIW-8962 as a novel anti-MPM agent, we evaluated the efficacy of BIW-8962 against MPM cell lines using *in vitro* ADCC and *in vivo* orthotropic mouse models. In addition, we analyzed GM2 expression levels in clinical samples of MPM.

Materials and Methods

Cell lines. Eleven human MPM cell lines were used in this study. ACC-MESO-1, Y-MESO-8A, Y-MESO-12, and Y-MESO-14 were established at the Aichi Cancer Research Center Institute (Nagoya, Japan).[18] NCI-H290 and NCI-H513 were provided by Dr. Adi F. Gazdar (University of Texas Southwestern Medical Center, Dallas, TX, USA). MSTO-211H, NCI-H28, NCI-H226, NCI-H2052, and NCI-H2452 were purchased from ATCC (Rockville, MD, USA). These cells were cultured in RPMI-1640 medium supplemented with 10% FBS (Life Technologies, Grand Island, NY, USA), 100 U/mL penicillin, and 100 mg/mL streptomycin (Meiji Seika Kaisha, Tokyo, Japan).

Animals. Male SCID mice, 5–6 weeks of age, were obtained from CLEA Japan (Osaka, Japan) and maintained under specific pathogen-free conditions throughout this study. All animals were acclimatized for at least 1 week before the experiments. All animal experiments complied with the Guidelines for the Institute for Experimental Animals, Kanazawa University Advanced Science Research Center (Kanazawa, Japan).

Reagents. The anti-GM2 antibody BIW-8962 and isotype control anti-dinitrophenol (DNP) antibody (fucose-removed human IgG1) were prepared by Kyowa Hakko Kirin Co., Ltd.

Flow cytometry. The GM2 expression in the MPM cells was examined using flow cytometry.[19] Briefly, cells (5×10^5) were resuspended in PBS, supplemented with 10% pooled AB serum to prevent non-specific binding to the Fc receptor, washed with cold PBS, and incubated on ice for 30 min with BIW-8962 or the isotype control. The cells were washed with cold PBS and incubated on ice for an additional 30 min with FITC-conjugated anti-human IgG antibodies (Beckman Coulter, Fullerton, CA, USA) then washed and resuspended in cold PBS. The cells were subsequently analyzed on a FACSCalibur flow cytometer using the CellQuest software program (Becton Dickinson, San Jose, CA, USA). The relative fluorescence intensity was calculated as the ratio of the mean fluorescence intensity of BIW-8962 to that of the isotype control.

Antibody-dependent cellular cytotoxicity activity. The *in vitro* ADCC activity was measured using the lactate dehydrogenase (LDH) release assay method. Human peripheral blood mononuclear cells (MNCs) prepared from healthy donors using Lymphoprep (Axis Shield, Dundee, UK) were used as effector cells, and the human MPM cell line MSTO-211H was used for the target cells. Detached MSTO-211H cells were plated at a density of 1×10^4 cells/well into round-bottom 96-well microplates, and freshly isolated MNCs were added to the same plates in order to achieve an appropriate effector/target (E/T) ratio (E/T = 25/1, 50/1, and 100/1). Serial dilutions of BIW-8962 were then added to the plates to start the reaction. Following incubation at 37°C for 4 h, the supernatants from each well were recovered by centrifugation at 50 *g* for 5 min. The LDH activity in each supernatant was measured using a non-radioactive cytotoxicity assay kit (Promega, Madison, WI, USA). The absorbance at 490 nm was determined using an ELISA reader. The specific cytotoxicity level was calculated according to the following formula:

$$\%\,\text{Cytotoxicity} = 100\times(\text{Exp} - \text{Espo} - \text{Tspo})/(\text{Total} - \text{Espo})$$

where Exp is the amount of LDH experimentally released from the target cells incubated with the effector cells and antibodies, Espo is the amount of LDH spontaneously released from the effector cells, Tspo is the amount of LDH spontaneously

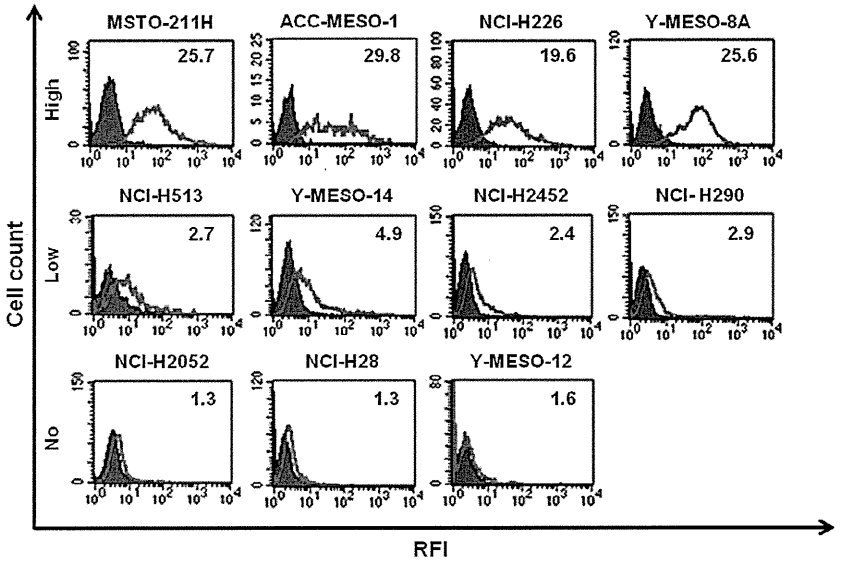

Fig. 1. Expression levels of ganglioside GM2 were evaluated in malignant pleural mesothelioma cell lines. Cells were detached and incubated with BIW-8962 or anti-dinitrophenol (DNP) antibodies on ice for 30 min. Bound Abs were detected with FITC-conjugated goat anti-human IgG Abs. The fluorescence intensity of the stained cells was measured using flow cytometry, and the mean fluorescence intensity was calculated. The open red histograms represent BIW-8962-stained samples and the filled blue histograms represent anti-DNP antibody-stained samples. The relative fluorescent intensity (RFI) versus anti-DNP is indicated.

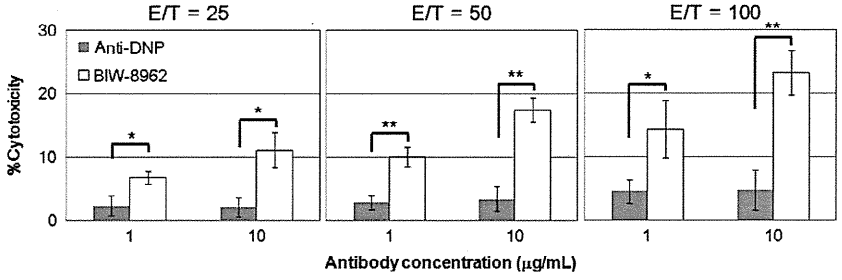

Fig. 2. Anti-GM2 antibody BIW-8962 exerted antibody-dependent cellular cytotoxicity activity against MSTO-211H malignant pleural mesothelioma cells. Human peripheral blood mononuclear cells were purified from healthy donors and used as effector cells. MSTO-211H cells (target cells) were incubated with effector cells (effector/target = 25/1, 50/1, and 100/1) and antibodies (BIW-8962 or anti-dinitrophenol antibodies) at 37°C for 4 h. The released lactate dehydrogenase activity was measured and the % cytotoxicity was calculated. The experiments were carried out in triplicate, and the values are expressed as the mean of the values for four donors ± SD. $*P < 0.01$, $**P < 0.001$ BIW-8962 treatment versus anti-dinitrophenol antibody treatment.

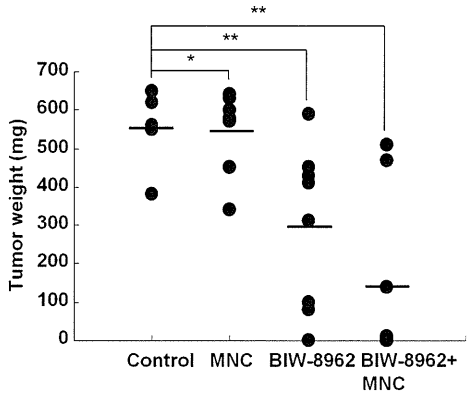

Fig. 3. Anti-GM2 antibody BIW-8962 showed therapeutic activity in an MSTO-211H orthotropic mouse model. MSTO-211H malignant pleural mesothelioma cells were inoculated into the thoracic cavity in SCID mice, and the animals were treated with BIW-8962 and/or human peripheral blood mononuclear cells (MNC). The mice were then i.v. administered BIW-8962 and/or MNC on days 7 and 14. At 3 weeks after tumor cell inoculation, the mice were sacrificed and their tumor weights were measured. The bars represent the mean of the group data. $*P < 0.05$ and $**P < 0.01$ between each treatment and the control groups.

released from the target cells, and Total is the maximum amount of LDH released from the target cells incubated with 9% Triton X.

Orthotropic *in vivo* assay. Cultured MSTO-211H cells were harvested using trypsin, washed twice, and resuspended in PBS, and 1×10^6 cells in 100 μL PBS were subsequently injected into the thoracic cavity of each SCID mouse, which was pretreated with TM-β1 antibodies (previously established mouse IL-2Rβ antibodies)[20] 2 days before cancer cell inoculation.[21] The mice were then i.v. given BIW-8962 (10 μg /animal) and/or 1×10^6 cells/animal MNC prepared according to the above method on days 7 and 14. At 3 weeks after tumor cell inoculation, the mice were sacrificed, their thoracic tumors were carefully removed and weighed, and the volume of pleural effusion was measured.

Immunofluorescent staining. Twenty-six MPM clinical tumor specimens were purchased from Origene Technologies (Rockville, MD, USA). Tumor specimens obtained by orthotopic inoculation of H290 cells in SCID mice were also used as a positive control. Frozen sections of these samples were fixed with acetone, washed with PBS, and incubated with 1 μg/mL of DAPI (Vector Laboratories, Burlingame, CA, USA) at room temperature for 20 min. The sections were then washed with PBS, blocked with 5% FBS in PBS for 10 min and incubated with 30 μg/mL of Alexa Fluor488 (Invitrogen, Carlsbad, CA, USA) conjugated BIW-8962 or isotype control at 4°C for 6 h. After being washed with PBS, the sections were analyzed using fluorescence microscopy. The percentages of cells with positive cytoplasmic and/or membrane GM2 immunoreactivity were evaluated as 0–100% and the modal intensity of the positively staining cells were determined on a scale from 0 to 3+: 0, complete absence of staining; 1+, weaker staining than H290 cells; 2+, similar staining to H290 cells; 3+, clearly more intense staining than H290 cells (Fig. S1).

Statistical analysis. Differences in the results of the *in vitro* experiments were evaluated using Student's two-tailed *t*-test, and differences in the results of the *in vivo* experiments were analyzed according to Dunnett's multiple comparison test. In all analyses, differences were considered to be significant at a *P*-value of <0.05.

Results

GM2 expression in MPM cell lines. First, we carried out a flow cytometric analysis to determine the GM2 expression levels in the MPM cell lines. In this assay, 11 histologically different MPM cell lines were used, as follows: ACC-MESO-1, Y-MESO-12, NCI-H290, NCI-H513, NCI-H226, and NCI-H2452 as epithelioid type cells; NCI-H28 and NCI-H2052 as sarcomatoid type cells; and Y-meso-8A, Y-meso-14, and MSTO-211H as biphasic type cells. Membrane-bound GM2 antigens were detected using the anti-GM2 antibody BIW-8962. The GM2 expression levels in these cell lines were categorized into three groups based on the relative fluorescence intensity: high (>10) in four cell lines (36%); low (2–10) in four cell lines (36%); and negative (<2) in three cell lines (28%) (Fig. 1). We found no cell type-dependent high expression of GM2, and no GM2 expression was detected in the sarcomatoid MPM cell lines.

Antibody-dependent cellular cytotoxicity activity against MPM cell line. The *in vitro* ADCC activity of BIW-8962 against the MPM cell line was determined using MNCs obtained from four healthy volunteers as effector cells and MSTO-211H, as target cells, which highly express GM2. Consequently, BIW-8962 showed significant ADCC activity at antibody concentrations of 1 and 10 μg/mL (Fig. 2), the efficacy of which increased in correlation with the E/T ratio (25, 50, and 100). The potent ADCC activity of BIW-8962

Table 1. Therapeutic evaluation of anti-GM2 antibody BIW-8962 in an *in vivo* orthotropic malignant pleural mesothelioma model

Treatment	Dose	Thoracic tumor			Pleural effusion		
		Incidence	Weight, mg		Incidence	Volume, μL	
			Median	Range		Median	Range
Control	DW	8/8	550	380–650	3/8	0	0–300
MNC	1×10^6 cells	8/8	590	340–640	2/8	0	0–200
BIW-8962	10 μg	7/8	360	0–590	1/8	0	0–250
BIW-8962 + MNC	10 μg, 1×10^6 cells	4/8	<10	0–510	1/8	0	0–150

DW, distilled water; MNC, human peripheral blood mononuclear cells.

was consistently observed in MNCs obtained from the four donors.

***In vivo* therapeutic activity of BIW-8962 in orthotropic mouse model.** The therapeutic efficacy of BIW-8962 was evaluated using an *in vivo* SCID mouse orthotropic model in which the animals were inoculated in the thoracic cavity with GM2-positive MSTO-211H cells and treated with BIW-8962 and/or human MNCs. BIW-8962 significantly decreased both the incidence and size of tumors (Fig. 3, Table 1). Notably, the concomitant administration of BIW-8962 and MNC had a greater effect on tumor incidence and size, whereas MNC injection alone showed weak antitumor activity. In addition, the incidence and volume of pleural effusion tended to be decreased by the co-administration of BIW-8962 and MNC.

Expression of GM2 in MPM clinical specimens. In order to determine GM2 expression in clinical MPM specimens, immunofluorescent staining of frozen MPM tissue samples obtained from 26 patients was carried out. The GM2 expression was subsequently confirmed in 58% of the donors, although the intensity and frequency of staining varied greatly from donor to donor (Fig. 4, Table 2). In contrast, we found no correlation between GM2 expression pattern and MPM tissue type or stage. GM2 positivity was detected in four of eight biphasic type samples (50%), three of five desmoplastic type samples (60%), and one of two epithelial type samples (50%). Similarly, GM2-positive cells were detected in two of three stage I tumors (67%), three of four stage II tumors (75%), five of seven stage III tumors (71%), and two of five stage IV tumors (40%).

Discussion

Ganglioside GM2 is recognized to be a cancer-associated antigen. A previous histological analysis showed that GM2 is widely expressed in human lung cancer cells, including SCLC and Non-small-cell Lung cancer (NSCLC).[22] However, GM2 expression in MPM has not yet been characterized. To the best of our knowledge, this study is the first experimental report to show GM2 expression in MPM cell lines and clinical MPM tumors. Furthermore, in this study, the non-fucosylated humanized anti-GM2 antibody, BIW-8962, showed an antitumor effect and a trend toward decreasing pleural effusion in the *in vivo* mouse orthotropic model. These results suggest the possibility that the anti-GM2 antibody BIW-8962 may be applied therapeutically in MPM patients.

Expression of GM2 was confirmed in eight of 11 MPM cell lines, four of which showed high levels of GM2 expression. A previous study showed that the main mode of actions of BIW-8962 are ADCC and CDC.[17] Actually, BIW-8962 was shown to exert ADCC activity against the highly GM2-positive MPM cell line MSTO-211H in a dose-dependent and E/T

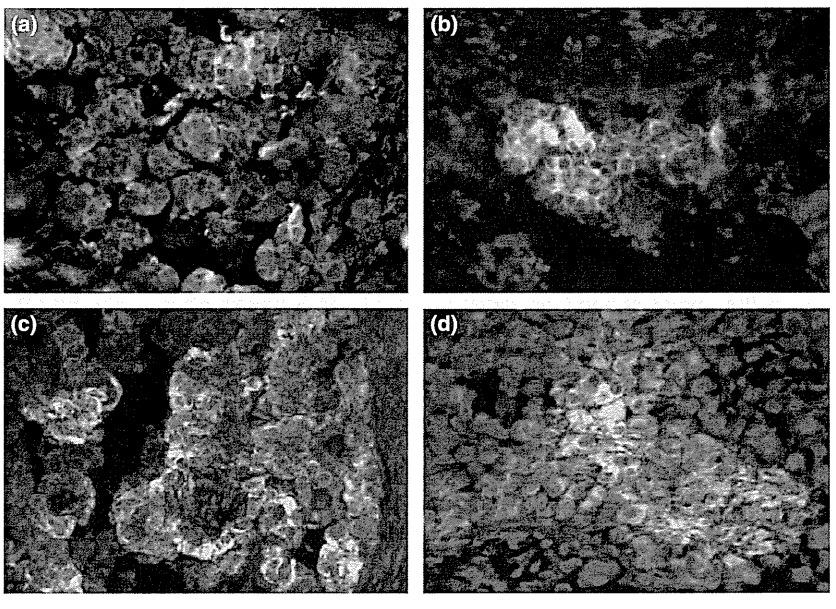

Fig. 4. Ganglioside GM2 expression in clinical malignant pleural mesothelioma specimens. Frozen sections obtained from 26 mesothelioma patients were incubated in DAPI at room temperature for 20 min. After washing, the sections were incubated in 5% FBS–PBS for 10 min for blocking and subsequently incubated in 30 μg/mL BIW-8962 or anti-dinitrophenol antibodies conjugated with Alexa Fluor488 at 4°C for 6 h. Representative images are shown. Samples of patients #12 (a), 19 (b), 20 (c), and 23 (d) are shown.

Table 2. Expression of ganglioside GM2 in mesothelioma patient samples

Patient ID	Staining frequency, %	Staining intensity	Tissue type	Age, years	Sex	Stage
#1	0	0	Biphasic	58	M	II
#2	30	2	Biphasic	69	M	I
#3	0	0	Meso	53	M	IV
#4	0	0	Meso	57	F	III
#5	30	3	Meso	73	M	NR
#6	0	0	Biphasic	52	M	III
#7	0	0	Biphasic	71	M	IV
#8	80	2	Biphasic	50	M	IV
#9	30	1	Meso	65	M	III
#10	0	0	Meso	71	M	IIA
#11	0	0	Desmoplastic	81	M	NR
#12	50	3	Meso	66	M	II
#13	0	0	Meso	86	M	NR
#14	100	2	Desmoplastic	55	M	I
#15	0	0	Desmoplastic	63	NR	I
#16	90	1	Epithelial	61	M	IV
#17	10	1	Meso	68	M	III
#18	40	3	Biphasic	62	M	III
#19	80	3	Desmoplastic	57	M	NR
#20	100	3	Meso	69	M	II
#21	100	1	Biphasic + desmoplastic	68	M	III
#22	100	3	NR	65	M	NR
#23	50	3	Meso	69	M	II
#24	20	2	Meso	57	F	III
#25	0	0	Biphasic	50	M	IV
#26	0	0	Epithelial	81	M	NR

Criteria for staining intensity: 0, negative; 1, faint; 2, moderate; 3, strong. F, female; M, male; Meso, unclassified mesothelioma; NR, not reported.

ratio-dependent manner. In contrast, CDC activity against MPM cell lines was not observed when BIW-8962 was used at the high concentration of 100 μg/mL (data not shown). In addition, BIW-8962 treatment resulted in a significant reduction in the incidence and size of tumors in the *in vivo* orthotropic mouse model. As the concomitant administration of human MNC augmented the therapeutic activity of BIW-8962 in this study, the main mechanism of therapeutic action is thought to involve ADCC. One of the primary subsets of lymphocytes exerting an ADCC activity is NK cells, which are believed to be present in the tumors of MPM patients.[23] These data suggest that the ADCC activity is stimulated in MPM tumors by treatment with BIW-8962, thus eliciting an antitumor effect. The therapeutic effect of BIW-8962 was observed at a dose of 10 μg/animal (roughly equal to 0.5 mg /kg) given i.v., and the efficacy of the therapy appeared to be higher than that of other antibody therapeutics reported previously.[24,25] The antibody distribution in human MPM has not been defined. In an MPM mouse xenograft model, it was reported that biodistribution of ^{86}Y labeled cetuximab and panitumumab in tumor was approximately 30% ID/g.[26] Extrapolating from this report, antibody concentration in tumor when 0.5 mg/kg dose of antibody is given i.v. is calculated to be approximately 3 μg/mL. This is the sufficient concentration for BIW-8962 to exert ADCC activity, based on the in vitro experiment result. However, a previous study reported that antibody distribution in tumor was approximately 0.001% ID/g in colorectal cancer patients, lower than the mouse xenograft model.[27] If we extrapolate the biodistribution ratio to MPM, a dose of 10 mg/kg can achieve an antibody concentration of more than 5 μg/mL in tumor, which is required for ADCC exertion.

In this study, GM2 expression was also confirmed in the clinical specimens of MPM patients, with individual differences in the expression levels and a rate of GM2 positivity of 58%. Although no correlations were observed between the GM2 expression profile and the histological findings or stage, all of the tissue types were GM2-positive. In the setting of MPM, differences in the histological type are known to affect the patient's prognosis, with cases of biphasic and sarcomatoid MPM having an especially poor prognosis.[28,29] In this study, the antitumor activities of BIW-8962 were observed in the *in vitro* and *in vivo* models using the biphasic MPM cell line MSTO-211H. If this antibody possesses therapeutic activity in biphasic and sarcomatoid MPM tissues in the clinical setting, it would be a valuable treatment option for MPM patients.

In the *in vivo* orthotropic mouse model using the human MPM cell line, the mice given BIW-8962 had a tendency to develop a smaller amount of pleural effusion. As NK cells have been shown to be present in the fluid of pleural effusion,[30] it is possible that ADCC reactions may occur in the pleural effusion of MPM patients. More than 60% of MPM patients present with pleural effusion associated with breathlessness, often accompanied by chest wall pain, which subsequently compromises their quality of life.[31] BIW-8962 treatment may help to improve the prognosis of these patients as well as increase their quality of life by inhibiting the accumulation of pleural effusion.

Cytotoxic agents such as pemetrexed and cisplatin are primarily used in MPM therapy. Hence, the development of a novel therapeutic agent with a different mode of action is eagerly anticipated. In this study, we showed that GM2 is overexpressed in MPM clinical specimens and that the non-fucosylated anti-GM2 antibody BIW-8962 has therapeutic activity in an *in vivo* orthotropic MPM model. As the ADCC activity of antibody therapeutics plays an important role in the efficacy of oncologic treatment, anti-GM2 antibodies may become an effective therapy for MPM. A clinical study of BIW-8962 as monotherapy in subjects with NSCLC, SCLC, and mesothelioma is currently being carried out (NCT01898156).

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Disclosure Statement

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Representative staining of ganglioside GM2 in H290 cells in SCID mice.

Receptor ligand-triggered resistance to alectinib and its circumvention by Hsp90 inhibition in EML4-ALK lung cancer cells

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ABSTRACT

Alectinib is a new generation ALK inhibitor with activity against the gatekeeper L1196M mutation that showed remarkable activity in a phase I/II study with *echinoderm microtubule associated protein-like 4* (*EML4*) - *anaplastic lymphoma kinase* (*ALK*) non-small cell lung cancer (NSCLC) patients. However, alectinib resistance may eventually develop. Here, we found that EGFR ligands and HGF, a ligand of the MET receptor, activate EGFR and MET, respectively, as alternative pathways, and thereby induce resistance to alectinib. Additionally, the heat shock protein 90 (Hsp90) inhibitor suppressed protein expression of ALK, MET, EGFR, and AKT, and thereby induced apoptosis in EML4-ALK NSCLC cells, even in the presence of EGFR ligands or HGF. These results suggest that Hsp90 inhibitors may overcome ligand-triggered resistance to new generation ALK inhibitors and may result in more successful treatment of NSCLC patients with EML4-ALK.

INTRODUCTION

Non-small cell lung carcinoma (NSCLC) can be classified into distinct molecular subsets based on specific genomic alterations that drive tumorigenesis [1]. *ALK* rearrangement, most commonly *EML4 -ALK*, is detected in approximately 3–7% of unselected NSCLCs [2, 3]. *EML4-ALK* NSCLC is more frequently observed in patients with adenocarcinoma than with other diseases , in young adults than in older patients, and in non-smokers or light smokers (<15 packs/year) than in heavier smokers [4]. Crizotinib, a multiple tyrosine kinase inhibitor (TKI) of ALK, MET, and ROS1, is the only agent that has been approved for *ALK*-rearranged NSCLC. It shows dramatic clinical efficacy, with a response rate of about 60–80% and a progression free survival (PFS) of approximately 9–10 months in *ALK*-rearranged NSCLC patients [5]. However, almost all patients who strongly responded to crizotinib acquired resistance to these agents after varying periods of time [6].

Known mechanisms for resistance to crizotinib include the gatekeeper L1196M mutation [6], other secondary *ALK* gene mutations (F1174L, C1156Y, G1202R, S1206Y, 1151-T-ins, and G1269A) [7, 8, 9, 10], *ALK* amplification [7], and activation of bypass signals via activation of other receptors (*KIT* amplification and epidermal growth factor receptor (EGFR) autophosphorylation) [8]. We recently reported that receptor ligands, such as epidermal growth factor (EGF), heparin binding-epidermal growth factor (HB-EGF), and transforming growth factor-α (TGF-α) , also activate EGFR as a bypass signal and induce crizotinib resistance in EML4-ALK NSCLC cells [11].

Alectinib is a highly selective, new generation ALK-TKI that also has inhibitory activity against EML4-ALK NSCLC cells with the gatekeeper L1196M mutation [12]. In a clinical trial for crizotinib-treatment naïve NSCLC patients with *ALK* rearrangement, there was a response rate of 93.5% to alectinib [13]. Moreover, alectinib demonstrated promising effects, even in the crizotinib-treated NSCLC patients with *ALK* rearrangement [14]. While it is clear that resistance may also develop against this class of inhibitor, the mechanisms of resistance to alectinib are largely unknown.

Heat shock protein 90 (Hsp90) is a molecular chaperone that plays a central role in regulating the

correct folding, stability, and function of numerous "client proteins," including human epidermal growth factor receptor 2 (HER2), BRAF , mutant EGFR, and EML4-ALK, Bcr-Abl, Raf-1, which are required for cancer cell survival [15, 16, 17, 18]. Hsp90 inhibition is therefore thought to be a promising strategy for controlling tumors, including those of EML4-ALK NSCLC. A natural product, geldanamycin, was found to directly bind to the ATP-binding pocket in the N-terminal domain of Hsp90 and block the binding of nucleotides to Hsp90; hence, geldanamycin was found to inhibit Hsp90 function. The first water-soluble, semi-synthetic derivative of geldanamycin is 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG) , which has shown excellent bioavailability and is quantitatively metabolized much less than other geldanamycin derivatives, such as 17-Allylamino 17-demethoxygeldanamycin (17-AAG) [19, 20].

In the present study, we examined whether receptor ligands would trigger resistance to a highly selective ALK-TKI, alectinib. Additionally, since we previously demonstrated that the Hsp90 inhibitor overcame EGFR-TKI resistance triggered by HGF, a ligand of MET, in *EGFR*-mutant lung cancer cells [21], we determined whether Hsp90 inhibition by 17-DMAG would overcome ligand-triggered alectinib resistance in *ALK*-rearranged NSCLC cells.

RESULTS

Exogenously added HGF and EGFR ligands induce resistance to alectinib in EML4-ALK NSCLC cells

Two EML4-ALK NSCLC cell lines, H2228 and H3122, were sensitive to crizotinib (IC50 0.3 μmol/L and 0.06 μmol/L, respectively). These cell lines were also sensitive to alectinib (IC50 0.24 μmol/L and 0.03 μmol/L, respectively). Exogenously added HGF and EGFR ligands (EGF, HB-EGF, and TGF-α) slightly stimulated cell growth, as determined by cell counting (Supplementary Fig. 1), and increased cell viability was determined by MTT assay (Fig. 1A-B). Under these experimental conditions, HGF and EGFR ligands remarkably reduced susceptibility of H2228 and H3122 cells to alectinib.

17-DMAG inhibits the viability of EML4-ALK NSCLC cells, irrespective of the presence of exogenously added HGF or EGFR ligands

Only the Hsp90 inhibitor 17-DMAG inhibited the viability of H2228 (Fig. 1C) and H3122 (Fig. 1D) cells

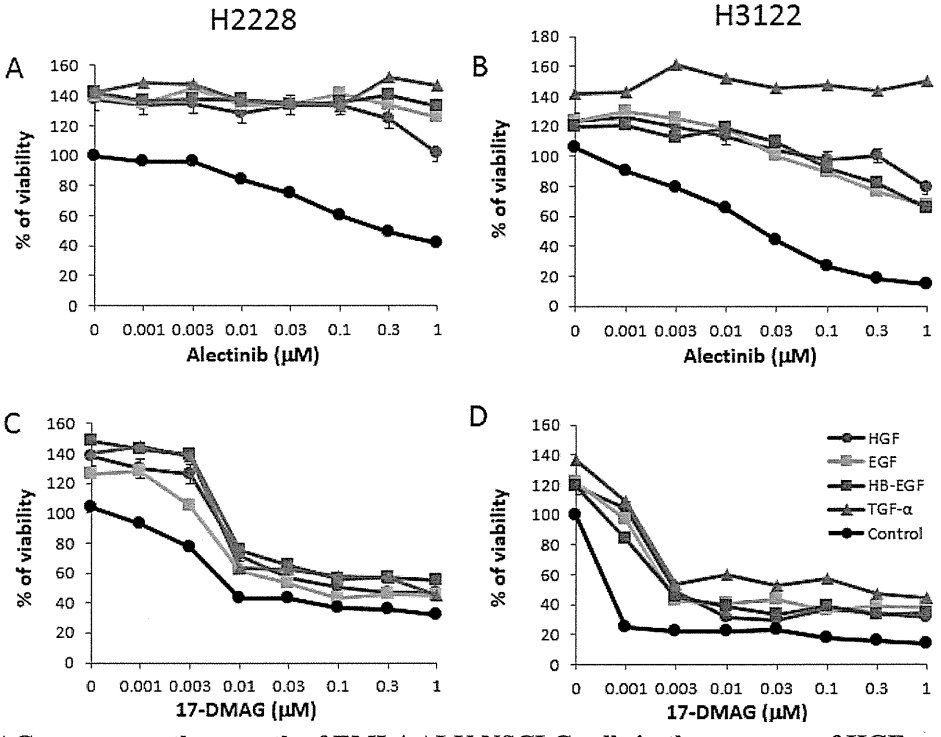

Figure 1: 17-DMAG suppresses the growth of EML4-ALK NSCLC cells in the presence of HGF and EGFR ligands. The EML4-ALK lung cancer cell lines human H2228 and human H3122 were treated with increasing concentrations of alectinib or 17-DMAG, with or without HGF (50 ng/mL), EGF (100 ng/mL), HB-EGF (10 ng/mL), and TGF-α (100 ng/mL), and cell viability was determined after 72 h by MTT assay. Data shown are representative of at least 3 independent experiments. Error bars indicate standard deviation (SD) of triplicate cultures.

in a dose-dependent manner. Importantly, 17-DMAG inhibited the viability of H2228 and H3122 cells, even in the presence of HGF or EGFR ligands. These results suggest that 17-DMAG may overcome alectinib resistance triggered by HGF or EGFR ligands, such as EGF, HB-EGF, and TGF-α.

17-DMAG inhibits the viability of EML4-ALK NSCLC cells in the presence of endogenous HGF

Recently, HGF was reported to induce resistance to various molecular-targeted drugs in various types of cancers with oncogene drivers [22, 23]. Moreover, our previous study reported that HGF was overexpressed in the *EGFR* mutant cancer cells that acquired resistance to EGFR-TKIs, indicating endogenous HGF production by cancer cells [24]. These findings suggest that HGF can be overexpressed in EML4-ALK NSCLC cells that acquire resistance to ALK inhibitors.

Therefore, we next examined whether endogenously expressed HGF induced alectinib resistance in EML4-ALK NSCLC cells. To assess this question, we generated stable HGF-gene transfectants in H2228 cells (H2228/HGF); as a control, we generated H2228/Vec cells transfected with vector alone . H2228/HGF cells secreted high concentrations of HGF (16.0 ± 0.4 ng/mL), whereas the HGF concentrations secreted by H2228 and H2228/Vec cells were under the detection limit. Consistent with the results of exogenously added HGF, HGF-transfected H2228 (H2228/HGF) cells became insensitive to alectinib (Fig. 2A), indicating that endogenously-expressed HGF also induced resistance to alectinib in EML4-ALK NSCLC cells.

We further found that 17-DMAG inhibited the growth of both H2228/Vec and H2228/HGF cells, because each had an IC50 of 0.01 μmol/L (Fig. 2B). These findings indicate that 17-DMAG may overcome alectinib resistance triggered by endogenously-produced HGF.

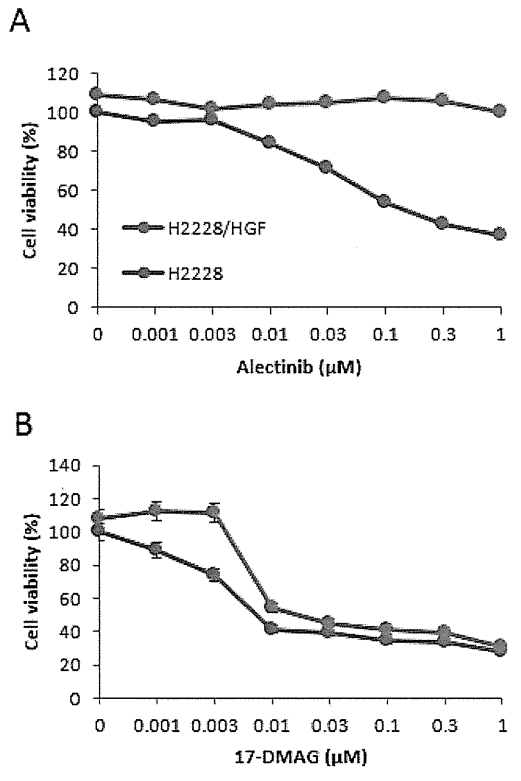

Figure 2: HGF-gene transfection resulted in reducing susceptibility of EML4-ALK NSCLC cells to alectinib but not 17-DMAG. H2228/Vec (A) or H2228/HGF (B) cells were treated with increasing concentrations of alectinib or 17-DMAG, and cell viability was determined after 72 h by MTT assay. Data shown are representative of at least 3 independent experiments. Error bars indicate SD of triplicate cultures.

HGF reduces alectinib susceptibility via MET phosphorylation, and 17-DMAG reduces expression of ALK and MET

To explore the molecular mechanism by which HGF reduced susceptibility to alectinib and 17-DMAG inhibited cell growth, even in the presence of HGF, we examined the protein expression and phosphorylation status of MET, ALK, and their downstream molecules (PI3K/AKT, ERK1/2, and STAT3) by Western blotting (Fig. 3). Since

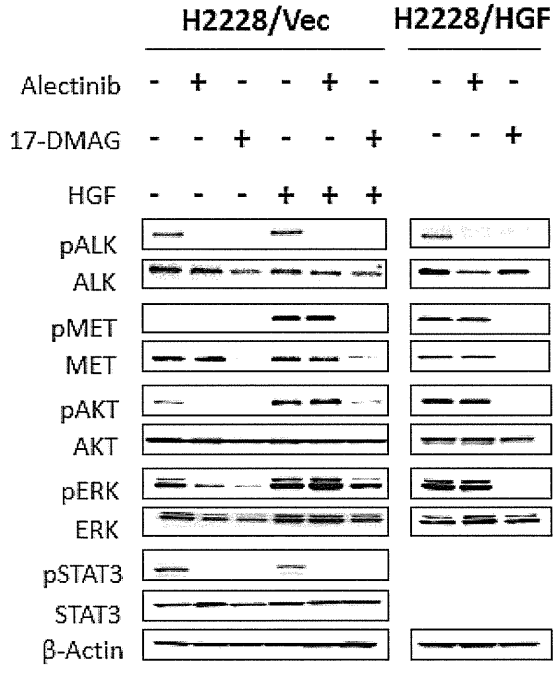

Figure 3: 17-DMAG reduced MET protein expression and inhibited downstream pathways, even in the presence of HGF. H2228/Vec or H2228/HGF cells were treated with or without alectinib (0.3 μmol/L) for 2 h or 17-DMAG (0.3 μmol/L) for 24 h and then stimulated with or without HGF (50 ng/mL) for 10 minutes. The resultant cells were lysed, and the indicated proteins were detected by immunoblotting. Data shown are representative of at least 3 independent experiments.

HGF reduced alectinib susceptibility more potently in H2228 compared with H3122 cells (Fig. 1A-B), we mainly used H2228 cells in the following experiments. H2228 (data not shown) and H2228/Vec (Fig. 3) cells expressed ALK and MET proteins (ALK were phosphorylated but MET were not), as well as the downstream molecules AKT, ERK1/2, and STAT3. In the absence of HGF, alectinib inhibited ALK phosphorylation, thereby inhibiting AKT, ERK1/2, and STAT3 phosphorylation.

In the presence of HGF, alectinib failed to inhibit MET, AKT, and ERK1/2 phosphorylation, although it inhibited ALK and STAT3 phosphorylation. These results suggest that HGF reduced susceptibility to alectinib by mainly restoring AKT and ERK1/2 pathways via MET activation.

In parallel experiments, 17-DMAG decreased the expression of ALK and MET proteins and inhibited their phosphorylation and AKT, ERK1/2, and STAT3 phosphorylation, irrespective of HGF presence. Similar results were observed in H2228/HGF (Fig. 3) and H3122 cells (Supplementary Fig. 2). These results indicate that 17-DMAG decreases protein expression of ALK and MET, thereby suppressing downstream signaling and overcoming alectinib resistance caused by HGF.

17-DMAG reduces EGFR and AKT protein expression and inhibits downstream pathways, even in the presence of EGFR ligands

We also examined the protein expression and phosphorylation status of EGFR and its downstream molecules in H2228 cells stimulated with EGFR ligands (Fig. 4). H2228 expressed EGFR, but EGFR was not constitutively phosphorylated in our experimental conditions. The EGFR ligands EGF, HB-EGF, and TGF-α remarkably induced EGFR phosphorylation. In these experimental conditions, alectinib failed to inhibit phosphorylation of EGFR or downstream AKT and ERK1/2, while it inhibited STAT3 phosphorylation. These results suggest that EGFR ligands reduced susceptibility to alectinib mainly by restoring AKT and ERK1/2 pathways via EGFR activation.

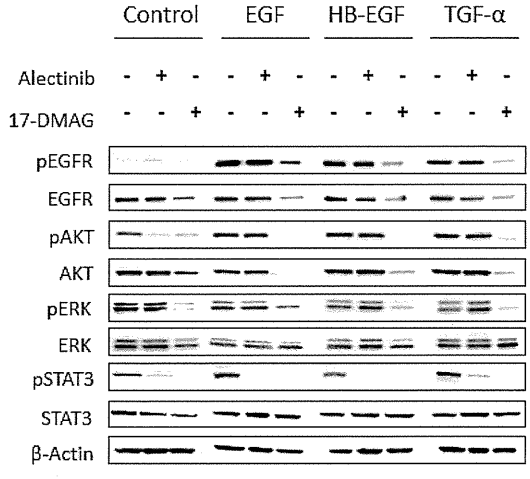

Figure 4: 17-DMAG reduced MET protein expression and inhibited downstream pathways, even in the presence of EGFR ligands. H2228 cells were treated with or without alectinib (0.3 μmol/L) for 2 h or 17-DMAG (0.3 μmol/L) for 24 h, and then stimulated with or without EGF (100 ng/mL), HB-EGF (10 ng/mL), and TGF-α (100 ng/mL) for 10 min. The resultant cells were lysed, and the indicated proteins were detected by immunoblotting. Data shown are representative of at least 3 independent experiments.

On the other hand, 17-DMAG decreased EGFR protein expression, resulting in inhibition of AKT, ERK1/2, and STAT3 phosphorylation, irrespective of the presence of EGFR ligands. These results suggest that 17-DMAG decreases EGFR protein expression, thereby suppressing downstream signaling and overcoming alectinib resistance triggered by EGFR ligands.

17-DMAG induces apoptosis of EML4-ALK lung cancer cells, even in the presence of HGF

We next assessed whether alectinib and 17-DMAG induced H2228/Vec cell apoptosis in the absence or presence of HGF. Alectinib induced apoptosis of H2228/Vec cells in the absence, but not presence, of HGF (Fig. 5). In contrast, 17-DMAG induced apoptosis in both the presence and absence of HGF. In a similar fashion, 17-DMAG, but not alectinib, induced H2228/HGF cell apoptosis.

17-DMAG inhibits H2228 cell viability, even in the presence of both of HGF and EGFR ligands

Since several growth factors can be simultaneously produced in cancer microenvironments [25, 26], it is possible that HGF and EGFR ligands are co-expressed in EML4-ALK NSCLC cells . Crizotinib inhibits MET, ALK, and ROS1, and it is supposed to overcome alectinib resistance caused by HGF alone. We therefore examined the effect of 17-DMAG compared with crizotinib in the presence of HGF plus EGFR ligands. H2228 and H3122 cells became insensitive to alectinib in the presence of HGF, TGF-α, and HGF with TGF-α (Fig. 6). These cells were sensitive to crizotinib in the presence of HGF, but they became much less sensitive to crizotinib in the presence of TGF-α with or without HGF. However, H2228 and H3122 were sensitive to 17-DMAG in the presence of HGF, TGF-α, or HGF with TGF-α. These results suggest that 17-DMAG may overcome alectinib resistance, even in the presence of ligands for two different receptors.

DISCUSSION

We demonstrated that ligands of MET (HGF) and

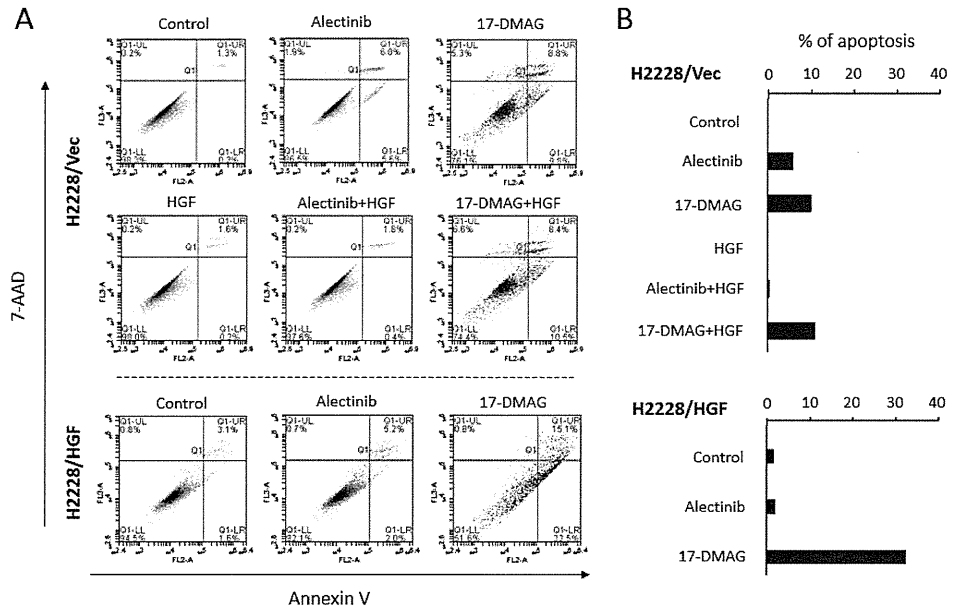

Figure 5: 17-DMAG induced apoptosis of EML4-ALK NSCLC cells, even in the presence of HGF. A. Apoptotic cells were evaluated by the 7-AAD cell viability assay , as described in the Materials and Methods. B. Quantification of apoptotic cells.

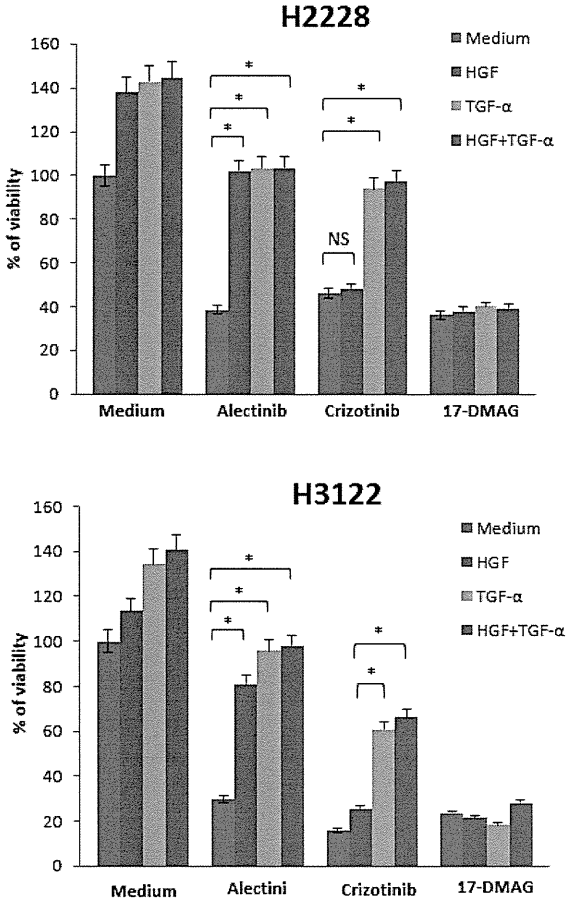

Figure 6: 17-DMAG reduced viability of EML4-ALK NSCLC cells, even in the presence of both HGF and TGF-α. H2228 and H3122 cells were incubated with or without alectinib (0.1 μmol/L), crizotinib (0.1 μmol/L), and/or HGF (50 ng/mL) and TGF-α (100 ng/mL), and cell viability was determined after 72 h by MTT assay. The percentage of cell viability is shown relative to controls without HGF or TGF-α treatment. * , $P < 0.001$ (one-way ANOVA). NS, not significant. Data shown are representative of at least 3 independent experiments. Error bars indicate SD of triplicate cultures.

EGFR (EGF, HB-EGF, and TGF-α) triggered resistance to alectinib in *ALK*-rearranged NSCLC cells, and that the Hsp90 inhibitor 17-DMAG overcame the resistance triggered by these receptor ligands. 17-DMAG inhibited protein levels of ALK, EGFR, and MET, even in the presence of ligand activation, and suppressed of AKT and ERK1/2 phosphorylation, thereby inducing apoptosis of *ALK*-rearranged NSCLC cells, irrespective of the presence of HGF or EGFR ligands. Since the Hsp90 inhibitor by itself could inhibit both driver (from rearranged *ALK*) and resistance signals (from activated receptors; MET and EGFR), it may be an ideal agent for overcoming ligand-triggered alectinib resistance in *ALK*-rearranged NSCLC.

Activation of bypass signals is a common resistance mechanism for targeted drugs. For example, EGFR-TKI resistance could be caused by *MET* amplification [27], HGF-triggered MET activation [23], Gas6-triggered AXL activation [28], and *HER2* amplification [29] in *EGFR* mutant lung cancer. BRAF inhibitor resistance could be caused by HGF-triggered MET activation [30], and IGF-1 triggered its receptor activation [31] in *BRAF* mutant melanoma. Crizotinib resistance could be caused by EGFR ligand-triggered EGFR activation [11], and stem cell factor (SCF)-triggered amplified cKIT activation [8] in EML4-ALK NSCLC. Therefore, HGF and EGFR ligands may be common resistance triggers that activate bypass survival signal via their receptor activation. The results in the present study are consistent with previous research indicating that alectinib resistance was induced by HGF and EGFR ligands.

Previous studies reported that several signaling pathways, including PI3K/AKT, MEK/ERK, and STAT3, are essential for survival and/or resistance to ALK inhibitors in *ALK*-rearranged NSCLC cells [12, 32]. Accordingly, we found that alectinib inhibited STAT3 and ALK phosphorylation. In the presence of alectinib, HGF or EGFR ligands restored AKT and ERK1/2, but not STAT3, phosphorylation and thereby made EML4-ALK cells insensitive to alectinib. These observations indicate that, when activated by their ligands, AKT and ERK signals from MET or EGFR play pivotal roles in alectinib resistance of EML4-ALK NSCLC cells.

It is of interest in the present study that HGF and EGFR ligands induced not only ALK-TKI resistance but also increased cell growth of EML4-ALK NSCLC cells. HGF and EGFR ligands also induced morphological change of H2228 cells (Supplementary Fig. 3). Therefore, these receptor ligands may modulate various cancer phenotypes of EML4-ALK NSCLC cells. HGF-MET and EGFR-ligands-EGFR axises play pivotal roles in progression of various types of tumors (33, 34). We are planning further studies to explore the molecular mechanisms of this morphological change and co-relation between the expression of receptor ligands in patient specimens and clinical characteristics in *ALK*-rearranged NSCLC.

Inter- and/or intra-tumor heterogeneity is a critical obstacle in cancer therapy with targeted drugs [35]. This is also the case in ALK-TKI resistance. Intra-tumor heterogeneity caused by crizotinib resistance results from the L1196M gatekeeper *ALK* mutation, and other *ALK* secondary C1156Y mutations co-existed in malignant pleural effusion of a patient who acquired crizotinib resistance [6]. Moreover, activation of different two receptors, EGFR and amplified *KIT* (both of which could induce crizotinib resistance), also co-existed in one crizotinib-resistant tumor [8].

Hsp90 inhibitors have been reported to overcome crizotinib resistance caused by several mechanisms, including *ALK* amplification, L1196M gatekeeper *ALK* mutation, other secondary *ALK* mutations (including F1174L), and epithelial to mesenchymal transition. Furthermore, we demonstrated that the Hsp90 inhibitor may overcome alectinib resistance, even when ligands of MET and EGFR co-exist . A new generation of Hsp90 inhibitors, including ganetespib, has recently been developed, and remarkable efficacy has been reported in a co-clinical model and early phase clinical studies [36]. Therefore, Hsp90 inhibition using new generation inhibitors may be a promising strategy to treat *ALK*-rearranged NSCLCs that acquire resistance to alectinib.

MATERIALS AND METHODS

Cell culture

The H2228 human lung adenocarcinoma cell line, with EML4-ALK fusion protein variant3 (E6;A20), was purchased from the American Type Culture Collection (Manassas, VA). The H3122 human lung adenocarcinoma cell line, with EML4-ALK fusion protein variant1 (E13;A20), was kindly provided by Dr. Jeffrey A. Engelman of the Massachusetts General Hospital Cancer Center (Boston, MA) [37]. H2228 and H3122 cells were cultured in RPMI-1640 medium supplemented with 5% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (50 μg/mL) in a humidified CO_2 incubator at 37°C. All cells were passaged for less than 3 months before renewal from frozen, early-passage stocks obtained from the indicated sources. Cells were regularly screened for *Mycoplasma* using a MycoAlert *Mycoplasma* Detection Kit (Lonza , Basel, Swiss).

Reagents

Alectinib, crizotinib, and 17-DMAG were purchased from Seleck Chemicals (Houston, TX). Recombinant EGF, TGF-α, and HB-EGF were purchased from R&D Systems (Minneapolis, MN). Recombinant HGF was prepared as described in a previous study [38].

Cell growth assay

Cell proliferation was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl terazolium bromide (MTT) dye reduction method [39]. Tumor cells were harvested at 80% confluence, seeded at 2 × 10^3 cells per well in 96-well plates, and incubated in appropriate medium for 24 h. Several concentrations of alectinib, crizotinib, 17-DMAG, and/or EGF, TGF-α, HB-EGF, and HGF were added to each well, and incubation continued for another 72 h. Fifty μL MTT (2 mg/mL; Sigma, St.Louis, MO) was added to each well, followed by incubation for 2 h at 37°C. The media were removed and the dark blue crystals in each well were dissolved in 100 μL of dimethyl sulfoxide (DMSO). Absorbance was measured with an MTP-120 Microplate reader (Corona Electric , Hitachinaka, Ibaraki, Japan) at test and reference wavelengths of 550 and 630 nm, respectively. The percentage growth was calculated relative to untreated controls. Each assay was carried out at least in triplicate, with results based on 3 independent experiments.

HGF-gene transfection

One day before transfection, aliquots of 1 × 10^5 H2228 cells in 1 mL of antibiotic-free medium were plated on 6-well plates. The full-length *HGF* cDNA cloned into the BCMGSneo expression vector [40] was transfected using Lipofectamine 2000 according to the manufacturer's instructions. After incubation for 24 h, the cells were washed with phosphate buffered saline (PBS) and incubated for an additional 72 h in antibiotic-containing medium. Then, the cells were selected in G418 sulfate (Calbiochem, Jolla, CA). After limiting dilution, the HGF-producing cells, H2228/HGF, were established. HGF production by H2228/HGF was confirmed by enzyme linked immunosolvent assay (ELISA) .

HGF production

Cells (2 × 10^5) were cultured in RPMI-1640 medium with 10% FBS for 24 h. The cells were washed with PBS and incubated for 48 h in 2 mL of RPMI-1640 medium with 10% FBS. Then, culture medium was harvested and centrifuged, and the supernatant was stored at –70°C until analysis. HGF concentrations were determined by IMMUNIS HGF EIA (Institute of Immunology, Tokyo) according to the manufacturer's protocols. All samples were run in duplicate. Color intensity was measured at 450 nm using a spectrophotometric plate reader. Growth factor concentrations were determined by comparison with standard curves, and the HGF detection limit was 100 pg/mL.

Apoptosis assay

Cell apoptosis induced by alectinib and 17-DMAG was measured by the PE Annexin V Apoptosis Detection Kit I (BD Biosciences, San Jose, CA) which detects and quantifies apoptotic cells with phycoerythrin (PE) Annexin V and 7-amino-actinomycin (7-AAD) staining. Cells were analyzed on a FACSCalibur flow cytometer with CellQuest software (Becton Dickinson, Franklin Lakes, NJ).

Western blotting

Sodium dodesyl sulfate (SDS) polyacrylamide gels (Bio-Rad , Hercules, CA) were loaded with 40 μg total protein per lane; following electrophoresis, the proteins were transferred onto polyvinylidene difluoride membranes (Bio-Rad), which were incubated with Blocking One (Nacalai Tesque, Kyoto, Japan) for 1 h at room temperature, followed by overnight incubation at 4°C with anti-ALK (C26G7), anti-phospho-ALK (Tyr1604), anti-phospho-EGFR (Tyr1068), anti-STAT3 (79D7), anti-phospho-STAT3 (Y705), anti-AKT, anti-phospho-AKT (Ser473), anti-ErbB4 (111B2), anti-phospho-ErbB4 (Tyr1284), anti-MET (25H2), anti-phospho-MET (Y1234/Y1235) (3D7), or anti-β-actin (13E5) antibodies (1:1,000 dilution each; Cell Signaling Technology , Danvers, MA), or with anti-human EGFR (1 μg/mL), anti-human/mouse/rat extracellular signal-regulated kinase (Erk)1/Erk2 (0.2 μg/mL), or anti-phospho-Erk1/Erk2 (T202/Y204) (0.1 μg/mL) antibodies (R&D Systems). After washing 3 times, the membranes were incubated for 1 h at room temperature with secondary antibodies (horseradish peroxidase-conjugated species-specific antibodies).

Immunoreactive bands were visualized with SuperSignal West Dura Extended Duration Substrate Enhanced Chemiluminescent Substrate (Pierce, Osaka, Japan). Each experiment was independently carried out at least 3 times.

Statistical analysis

Differences were analyzed by one-way ANOVA. All statistical analyses were carried out using GraphPad StatMate 4 (GraphPad Software, Inc ., San Diego, CA). $P < 0.05$ was considered significant.

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