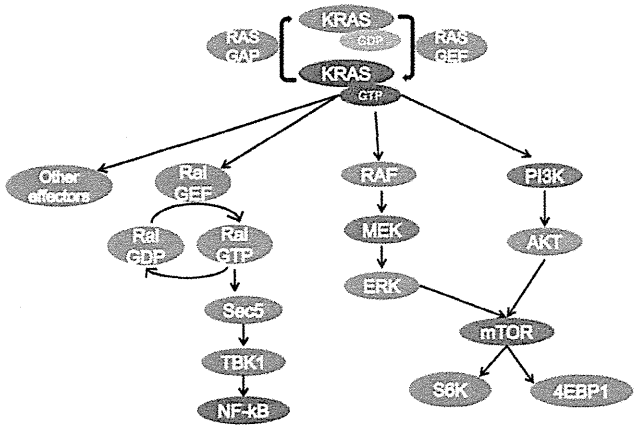

Fig. 1. Effector pathways of Kirsten rat-sarcoma (KRAS). Proteins highlighted green are pharmacologically targetable.

Zeneca, Macclesfield, UK) failed to show clinical activity in an unselected pretreated patient population with a high-rate of *KRAS* mutations.[10–12]

PI3K pathway. The precise role of KRAS in regulating PI3K has been difficult to elucidate because PI3K can be activated by multiple upstream signals, not all of which integrate KRAS to promote downstream signaling. Several lines of evidence suggest PI3K associates with, and is activated by KRAS, thus serving as a principal mechanism of PI3K regulation. The binding of KRAS to p110α induces a conformational change in p110α, which opens and orients the active site of KRAS toward its substrate. Although RBD mutants of p110α fail to bind KRAS, they still maintain enzymatic activity. Interestingly, mice engineered to express RBD-mutant p110α cannot develop mutant *Kras*-driven lung tumors.[13] Furthermore, by using an inducible mouse model of mutant *Kras*-driven lung cancer, Downward and colleagues showed that loss of Kras-p110α binding leads to long-term tumor stasis and partial regression.[14] These elegant studies showed that the interaction between mutant KRAS and p110α is not only required for tumorigenesis but also for tumor maintenance.

In addition to direct activation by KRAS, PI3K can also be activated by receptor tyrosine kinases (RTKs) in *KRAS* mutant cancers. We have reported in colorectal cancers that insulin-like growth factor 1 receptor (IGF-IR) exerts dominant control over PI3K signaling through binding to insulin receptor substrate (IRS) adaptor proteins even in the presence of mutant *KRAS*.[15] PI3K activity is also dependent on basal IGF-IR activity in *KRAS* mutant lung cancer, although in this context mutant KRAS is still thought to be involved in PI3K activation. It has been shown that IGF-IR activation causes IRS-1:p85 complex formation, which in turn relieves an inhibitory effect of p85 on PI3K signaling.[16] Additionally, a recent study showed the *KRAS* mutant NCI-H358 non-small cell lung cancer (NSCLC) cell line still remains dependent on ERBB3 for PI3K signaling.[17] Altogether, these studies suggest numerous contributors, including mutant KRAS and RTKs, activate PI3K signaling in *KRAS* mutant cancers. Another confounding issue is that the role of mutant KRAS may further differ depending on other mutations that may be more or less prevalent among the different tissue types of origin. For example, oncogenic mutations in *KRAS* and *PIK3CA* often coexist in colorectal cancer but less often in pancreatic cancer.[18] The coexistence of *KRAS* and *PIK3CA* mutations in colorectal cancers suggests that mutant KRAS is not sufficient for robust PI3K activity. Similar to MEK inhibitors, single agent PI3K inhibitors are also ineffective for treatment of *KRAS* mutant cancers; murine lung cancers driven by oncogenic *Kras* do not respond to the PI3K/mammalian target of rapamycin (mTOR) inhibitor, NVP-BEZ235.[19] Furthermore, *KRAS* mutations predict resistance to PI3K inhibitors in cell culture experiments.[20,21]

Ral-NF-κB pathway. While the RAF-MEK-ERK and PI3K pathways have been established as key KRAS-effector pathways, KRAS has a number of additional effectors. Among them, the guanine exchange factors of the Ras-like (Ral) GTPases (RalGEFs) have emerged as important effectors of KRAS. Ras-like GTPases directly interact with RAS, and subsequently activates Ral small GTPases.[22,23] Two Ral small GTPases, RalA and RalB, appear to have distinct biological roles in *KRAS* mutant cancers. For instance, inhibition of RalA alone is enough to inhibit tumor initiation, while RalB is vital for tumor invasion and metastasis.[24–26] Similar to KRAS, activated Ral-GTP interacts with multiple downstream effector proteins including RalBP1, which promotes membrane ruffling and filopodia formation through Rac1 and CDC42, as well as receptor trafficking via endocytic regulation.[27] Additional effectors of Ral are the octometric exocyst subunits Sec5 and Exo84, important for secretory vesicle delivery to different membrane compartments.[28,29] Lastly, active RalB signaling causes the association of Sec5 complex with the atypical IkB-related protein kinase TBK1 to promote cell survival through activation of the oncogenic transcription factor NF-κB.[30]

Targeting PI3K-AKT and MEK-ERK Signaling by Combinatorial Approaches

The lack of efficacy seen following suppression of single effector pathway (e.g. use of MEK inhibitors or PI3K inhibitors) in *KRAS* mutant cancers suggests that a combinatorial approach targeting multiple effector pathways is needed. When cancer cells exhibit dependency on a single oncogene ("oncogene addiction"), inhibition of the oncogene leads to downregulation of both PI3K/AKT and MEK/ERK signaling in most instances. Importantly, combination of both a PI3K inhibitor and a MEK inhibitor is sufficient to recapitulate much of the apoptosis and suppression of tumor growth induced by EGFR inhibitors in *EGFR* mutant NSCLC.[31] Moreover, *HER2* amplified and/or *PIK3CA* mutant breast cancers are particularly sensitive to single agent PI3K inhibitors, which surprisingly downregulate both PI3K and MEK/ERK signaling in these cancers, resulting in apoptosis.[32] These results suggest that concomitant disruption of PI3K/AKT and MEK/ERK signaling may underlie much of the antitumor effects observed with targeted therapies in oncogene-addicted models. Consistent with this concept, pharmaceutical inhibition of both the MEK and PI3K pathways has shown durable responses in *KRAS* mutant cancers *in vivo*.[8,19]

Currently, a large number of clinical trials to assess the combination of PI3K inhibitors and MEK inhibitors are ongoing (Table 1). A recent dose-escalation trial tested the combination of the dual PI3K/mTOR inhibitor SAR245409 (Sanofi, Paris, France) with the MEK1/2 inhibitor pimasertib (Merck KGAA, Darmstadt, Germany) in 46 cancer patients. Among the patients, two partial responses were observed: one in a patient with *KRAS* mutant colorectal cancer whose tumor exhibited neuroendocrine features, and a low-grade ovarian cancer patient with simultaneous *KRAS* and *PI3KCA* muta-

Table 1. Currently ongoing trials combining phosphatidylinositol 3-kinase (PI3K) inhibitor and MEK inhibitor

NCT no.	Phase	Company	PI3K inhibitor	MEK inhibitor	Patient selection
01347866	I	Pfizer (New York, NY, USA)	PF-05212384 (PI3K/mTOR inhibitor)	PD-0325901	At the MTD dose, further assessment of these combinations will be done in patients with KRAS mutated colorectal cancer
01363232	Ib	Novartis	BKM120 (pan PI3K inhibitor)	MEK162	At the MTD dose, this combination is explored in patients with EGFR mutant NSCLC, whom have progressed on EGFR inhibitors and triple negative breast cancer, as well as other advanced solid tumors with KRAS, NRAS, and/or BRAF mutations
01390818	I	EMD Serono (Rockland, MA, USA)	SAR245409 (PI3K/mTOR inhibitor)	Pimasertib	Locally advanced or metastatic solid tumors
01155453	Ib	Novartis	BKM120 (pan PI3K inhibitor)	Trametinib	At the MTD dose, further assessment will be done in patients with KRAS or BRAF mutated NSCLC, ovarian, and pancreatic cancer
01859351	I	Wilex (München, Germany)	WX-037 (pan PI3K inhibitor)	WX-554	Solid tumor
01337765	Ib	Novartis	BEZ235 (PI3K/mTOR inhibitor)	MEK162	At the MTD dose, this combination was assessed in patients with EGFR mutant NSCLC, whom have progressed on EGFR inhibitors and triple negative breast cancer, as well as other advanced solid tumors with KRAS, NRAS, and/or BRAF mutations
01392521	Ib	Bayer (Leverkusen, Germany)	BAY80-6946 (pan class I PI3K inhibitor)	BAY86-9766	Advanced cancer
00996892	Ib	Genentech (San Francisco, CA, USA)	GDC-0941 (Pan PI3K inhibitor)	GDC-0973	Locally advanced or metastatic solid tumors
01449058	Ib	Novartis	BYL719 (PI3K alpha-specific inhibitor)	MEK162	Advanced solid tumors or AML or high risk and very high risk MDS, with documented RAS or BRAF mutations
01248858	I	GlaxoSmithKline	GSK2126458 (pan PI3K/mTOR inhibitor)	Trametinib	Advanced solid tumors

AML, acute myeloid leukemia; EGFR, epidermal growth factor receptor; MDS, myelodysplastic syndromes; MEK, mitogen-activated protein kinase kinase; MTD, Maximum Tolerated Dose; mTOR, mammalian target of rapamycin; NCT, national clinical trial that is given to each registered clinical trial; NSCLC, non–small-cell lung cancer; PI3K, phosphatidylinositol 3-kinase.

tions. Grade 3 and 4 toxicities were infrequent, with the most common grade 3 event being skin rash in 14% of patients.[33] In a separate trial combining the PI3K inhibitor BKM120 (Novartis, Basel, Switzerland) and the MEK inhibitor trametinib (GlaxoSmithKline, Brentford, UK), three patients with *KRAS* mutant ovarian cancer achieved partial responses among 66 patients in an unselected population.[34] Based on these three responses, this trial is expanding cohorts to specifically include patients with *KRAS* or *BRAF* mutant tumors. These results suggest that the combination of PI3K and MEK inhibitors has activity, but the activity appears relatively limited. This lack of robust activity seems to be attributed to the difficulty of sufficiently suppressing both pathways without toxicities in a given patient. For example, a trial combining MK-2206 (Merck), an AKT inhibitor, and selumetinib, four of eight patients demonstrated biologically significant inhibition in one marker; however, at the maximum tolerated dose no patient had ≥70% inhibition of both targets.[35]

Alternative therapeutic strategies targeting RTKs that indirectly suppress the PI3K pathway in combination with MEK inhibition may be more tolerable, and as a consequence more effective. As mentioned, the IGF-IR is largely responsible for PI3K activation in *KRAS* mutant colorectal and lung cancer cell lines, and the combination of IGF-IR and MEK inhibitors results in tumor regressions in these xenografts.[15,16] This approach is currently being evaluated in a phase I/II trial of IGF-IR antibody ganitumab (Amgen, Thousand Oaks, CA, USA) combined with the MEK inhibitor MEK162 (Novartis) in *KRAS* mutant colorectal and pancreatic cancer and *BRAF* mutant melanoma (ClinicalTrilas.gov registry number, NCT01562899).

Targeting the Apoptotic Machinery

As mentioned above, in cancers addicted to a single oncogene, effective target inhibition generally results in apoptosis. This process involves the downstream BCL-2 family of proteins, which act as guardians of mitochondria-mediated apoptosis. For example, in *EGFR* mutant NSCLCs, treatment with an EGFR inhibitor shifts the balance of pro- and anti-apoptotic BCL-2 family members, reducing the expression of anti-apoptotic MCL-1 as a result of PI3K/mTORC1 inhibition,[31] and increasing the expression of pro-apoptotic BIM as a result of MEK/ERK suppression, leading to apoptosis.[31,36] In addition,

a recent study using engineered mice deficient for the pro-apoptotic BCL-2 family members BIM or PUMA provided evidence that BIM and PUMA are both key apoptotic effectors of tyrosine kinase inhibitors in *EGFR* mutant NSCLC and *HER2* amplified breast cancer.[37]

The TBK1/BCL-XL pathway. In addition to the PI3K and MEK/ERK pathway, mutant KRAS maintains proliferation and evades apoptosis through other pathways. For instance, shRNA screening using *KRAS* mutant cancer cell lines identified TBK1 as a synthetic lethal partner of oncogenic KRAS. Interestingly, BCL-XL, a known NF-κB target, was identified as a TBK1-regulated gene. Overexpression of BCL-XL rescued apoptosis induced by KRAS or TBK1 knockdown in the NCI-H23 KRAS mutant cell line.[38]

Combination of MEK inhibitor with BCL-XL inhibitor. Pharmacological inhibition of the MEK/ERK pathway is relatively more achievable compared with the PI3K pathway.[39,40] Therefore, MEK inhibitor therapy could be a backbone for combinatorial approaches for *KRAS* mutant cancers. To this point, shRNA screening was performed to identify genes that, when inhibited, cooperate with MEK inhibitors to reduce cell survival in *KRAS* mutant cell lines.[41] BCL-XL emerged as a top hit through this approach. That is, BIM induction following MEK inhibition is not enough to cause apoptosis, but BCL-XL knockdown disrupts an inhibitory complex between BIM and BCL-XL, leading to apoptosis in the presence of MEK inhibitor. Induction of apoptosis is recapitulated by combining the BCL-2/BCL-XL inhibitor navitoclax (ABT-263) with a MEK inhibitor. Two additional studies have also shown the efficacy of this combination.[42,43]

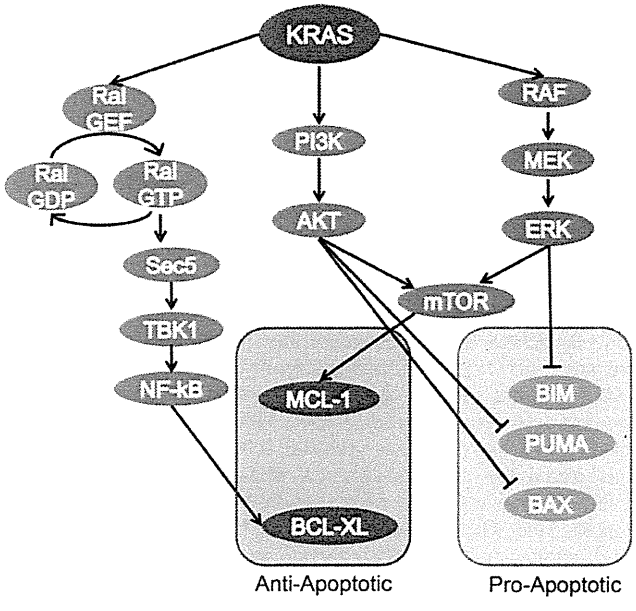

Fig. 2. Effector proteins of Kirsten rat-sarcoma (KRAS) and apoptosis. The BCL-2 family of proteins regulates mitochondrial-driven apoptosis in KRAS mutant cancers. The BCL-2 family consists of three subfamilies: the pro-survival members such as BCL-2 or MCL1, the pro-apoptotic BCL-2 homology domain 3 (BH3)-only proteins such as BIM and PUMA, and the pro-apoptotic BAX and BCL-2 antagonist/killer (BAK; not shown in this figure). The anti-apoptotic function of oncogenic KRAS is mediated by several effector pathways that converge on the BCL-2 family of proteins. The PI3K effector pathway suppresses pro-apoptotic protein PUMA and BAX, the RAS–RAF pathway downregulates the pro-apoptotic protein BIM, and the mTORC1 pathway regulates MCL-1. In addition, the Ral-NF-κB pathway has been implicated in the regulation of BCL-XL. Thus, KRAS suppresses cell death responses through regulation of both pro-apoptotic and anti-apoptotic BCL-2 family proteins.

Combination of mTORC1/2 inhibitor and BCL-2/BCL-XL inhibitor. We have recently showed *KRAS* mutant colorectal cancers are particularly vulnerable to simultaneous inhibition of the BCL-2 anti-apoptotic proteins BCL-2, BCL-XL and MCL-1.[44] Pure mTORC catalytic site inhibitors downregulated MCL-1 in *KRAS* mutant colorectal cancers, and targeting *KRAS* with shRNA similarly reduced mTORC1 signaling and MCL-1 levels, suggesting MCL-1 to be a vital KRAS-effector molecule in these cancers. When combined with the BCL-2/BCL-XL inhibitor navitoclax, the mTORC1/2 inhibitor AZD8055 induced tumor regressions in *KRAS* mutant human colorectal cancer xenografts and *Kras* mutant genetically engineered mouse models of colorectal cancers. In all, this study provides the rationale to use mTORC inhibitors in combination with BCL-2/BCL-XL inhibitors in *KRAS* mutant colorectal cancers. Altogether, these data mark the apoptotic machinery as an attractive target to treat *KRAS* mutant cancers (Fig. 2).

Combination of MEK inhibitor and docetaxel. Several studies have demonstrated that cytotoxic agents, including microtubule stabilizing drugs, stimulate MAPK signaling upon administration. Combining inhibitors of MAPK signaling with one such drug, docetaxel, results in an enhanced anti-tumorigenic phenotype.[45] One of the key mechanisms of this synergy is induction of pro-apoptotic proteins by inhibiting MAPK signaling, which reduces the threshold for apoptosis induction by cytotoxic agents. In fact, prolonged exposure to the MEK inhibitor selumetinib induced BIM expression in the *KRAS* mutant HCT-116 xenograft model. A prospective randomized phase II study assessing the impact of adding selumetinib to docetaxel in previously treated patients with advanced *KRAS* mutant NSCLC was conducted based on these pre-clinical results. Despite no differences in median overall survival, there was significant improvements in both progression-free survival and objective response rate in patients administered selumetinib.[46]

Concurrently with the clinical trials in human subjects, a *Kras* mutant transgenic mouse model was used to optimize treatment modalities, a so-called "co-clinical" trial.[47] This mouse study revealed that adding selumetinib was beneficial for mice with *Kras* or *Kras / p53* mutant lung cancer, but not with *Kras* and *Lkb1* mutations. Interestingly, *Kras/Lkb1* tumors show substantially less phosphorylation of ERK, suggesting that the ERK pathway is less active in these cancers. Furthermore, integrated genomic and proteomic profiles revealed SRC is activated in *Kras/Lkb1* tumors,[48] suggesting that *Kras/Lkb1* mutant tumors are a distinct subset of *KRAS* mutant cancers that may be less dependent on ERK signaling and more dependent on other pathways. Intriguingly, another recent report suggests that NSCLCs harboring mutations both in *KRAS* and *LKB1* are addicted to coatomer complex I (COPI)-dependent lysosome acidification, which participates in retrograde transport, is required for endosome maturation and is a CDC42 effector required for CDC42 transformation.[49]

Identifying Synthetic Lethal Interaction with KRAS

Recent high-throughput screening has provided an expanded list of targets for *KRAS* mutant tumors (Table 2). For example, siRNA screening in *KRAS* mutant NSCLC cell lines identified the transcription factor GATA2 as necessary for the survival

Table 2. Candidate genes showing synthetic lethal interaction with Kirsten rat-sarcoma (KRAS)

Synthetic lethal genes or pathways	Methodology	Pharmacological inhibition	References
TBK1	shRNA screening	Not assessed	38
Coatomer complex I (COPI)	Parallel screening of chemical and genetic perturbations	Saliphenylhalamide A	49
GATA2	siRNA screening	Bortezomib with Fasudil	50
CDC6	siRNA screening	Bortezomib and topotecan	51
STK33	shRNA screening	Specific inhibitor was subsequently developed, but failed to suppress growth of cells	52, 57
TAK1	Expression data based bioinfomatic analysis	5Z-7-oxozeaenol	53
Polo-like kinase (PLK) 1 and 2	shRNA screening and outlier kinase analysis	BI-2536	54, 58
CDK4	Mouse genetic studies	PD0332991	55
Reactive oxygen species	Chemical screening	Lanperisone	56

Fasudil is a Rho signaling inhibitor, approved for the treatment of cerebrovascular spasm in Japan.

of these cancers.[50] GATA2 maintains cell survival via the proteasome machinery, the IL-1/NF-κB signaling pathway, and the Rho-signaling cascade. Combined inhibition of the proteasome and Rho signaling recapitulates the effect of GATA2 loss on *KRAS*-driven tumorigenesis. CDC6, a critical regulator of DNA replication, has also been identified as a synthetic lethal protein with mutant *KRAS*.[51] Bioinformatic analysis suggests proteasome components functionally interact with CDC6, and knockdown of CDC6 showed additional synthetic lethal effects with proteasome inhibitor treatment. Other targets identified by synthetic lethal approaches include, as discussed above, TBK1,[38] as well as COPI,[49] STK33,[52] TAK1,[53] APC/C,[54] CDK4,[55] Polo-like kinase (PLK) 1,[54] and reactive oxygen species (ROS).[56] It should be cautioned that a major caveat associated with RNAi screening is potential off-target effects and the potential disconnect between reduction of total expression and inhibition of kinase function. For example, while STK33 knockdown was synthetic lethal for *KRAS* mutant cancers, inhibition of STK33 kinase activity does not appear to be effective therapy for *KRAS* mutant cancers.[57]

Other Means to Target KRAS

"Outlier kinase" approach. Using an innovative approach of identifying "outlier kinase" expression through analysis of transcriptome sequencing data from a large number of cancers, polo-like kinases (PLKs) were noted to be overexpressed in a subset of *KRAS* mutant pancreatic cancers, and these cancers had specific sensitivity to the PLK-pan inhibitor, BI-6727.[58]

HSP90 inhibitor combinations. Pharmaceutically targeting HSP90 has attracted significant interest. HSP90 inhibitors target HSP90 client proteins resulting in their rapid degradation. Although KRAS is not a client protein of HSP90, *KRAS* mutant NSCLCs are exquisitely sensitive to HSP90 inhibition,[59] most likely through the HSP90-inhibitor-mediated degradation of downstream signaling proteins such as C-RAF[60] as well as the production of ROS.[61] Interestingly, HSP90 inhibitors may have particular activity in combination with the mTOR inhibitor rapamycin in *KRAS/p53* mutant NSCLCs through rapamycin-mediated suppression of glutathione in the presence of HSP90-inhibitor induced ROS.[61]

Targeting posttranslational modification of KRAS. Lastly, targeting mutant *KRAS* by interfering with important KRAS post-translational modifications has recently been explored. The phosphorylation of KRAS on Serine 181, which is mediated by PKC,[62] is indispensable for full KRAS oncogenic activity.[63,64] As such, treatment of *KRAS* mutant cancers with PKC inhibitors has anti-proliferative and pro-apoptotic activity,[63,64] marking PKC as an intriguing therapeutic target.

Conclusion

Targeted therapies that directly disrupt oncogene function have changed the way cancers are treated. While one of the most obvious targets is oncogenic *KRAS*, mutated in roughly one-fourth of all cancers, direct targeting of KRAS has remained largely elusive. Instead, co-targeting pathways downstream of mutant KRAS has emerged in pre-clinical studies as a promising therapeutic strategy. However, validation of these pre-clinical studies has been hindered by unanticipated challenges, such as dose-limiting toxicity of combinatorial inhibition of PI3K and MEK/ERK signaling. Alternatively, blocking upstream activators of PI3K, such as IGF-IR, in combination with MEK inhibition, may be a less toxic and thus more successful strategy. More recently, targeting the apoptotic machinery in *KRAS* mutant cancers has garnered attention. For instance, mTORC inhibitors in combination with BCL-2/BCL-XL inhibitors showed dramatic pre-clinical efficacy in *KRAS* mutant colorectal cancers *in vivo*. Moreover, the identification of novel targets that offer synthetic lethality with mutant *KRAS* has paved the way toward new therapeutic strategies. However, whether effective drugs can be designed to disrupt these targets, and whether these drugs can be administered at doses high enough to inhibit their targets, remains to be seen. Lastly, the identification of already clinically available drugs that show efficacy in subsets of *KRAS* mutant cancers, such as the combination of docetaxel and selumetinib in *KRAS* mutant NSCLC with wild type *LKB1*, may speed up the implementation of much needed novel therapies.

Acknowledgments

We thank Drs Matt Niederst and Erin Coffee for critical reading of the manuscript.

Disclosure Statement

The authors have no conflict of interest.

References

1 Pylayeva-Gupta Y, Grabocka E, Bar-Sagi D. RAS oncogenes: weaving a tumorigenic web. *Nat Rev Cancer* 2011; **11**: 761–74.
2 Lawrence MS, Stojanov P, Mermel CH *et al.* Discovery and saturation analysis of cancer genes across 21 tumour types. *Nature* 2014; **505**: 495–501.
3 Kandoth C, McLellan MD, Vandin F *et al.* Mutational landscape and significance across 12 major cancer types. *Nature* 2013; **502**: 333–9.
4 Ostrem JM, Peters U, Sos ML, Wells JA, Shokat KM. K-Ras (G12C) inhibitors allosterically control GTP affinity and effector interactions. *Nature* 2013; **503**: 548–51.
5 Zimmermann G, Papke B, Ismail S *et al.* Small molecule inhibition of the KRAS-PDEdelta interaction impairs oncogenic KRAS signalling. *Nature* 2013; **497**: 638–42.
6 Buday L, Downward J. Many faces of Ras activation. *Biochim Biophys Acta* 2008; **1786**: 178–87.
7 Khosravi-Far R, White MA, Westwick JK *et al.* Oncogenic Ras activation of Raf/mitogen-activated protein kinase-independent pathways is sufficient to cause tumorigenic transformation. *Mol Cell Biol* 1996; **16**: 3923–33.
8 Sos ML, Fischer S, Ullrich R *et al.* Identifying genotype-dependent efficacy of single and combined PI3K- and MAPK-pathway inhibition in cancer. *Proc Natl Acad Sci USA* 2009; **106**: 18351–6.
9 Brognard J, Dennis PA. Variable apoptotic response of NSCLC cells to inhibition of the MEK/ERK pathway by small molecules or dominant negative mutants. *Cell Death Differ* 2002; **9**: 893–904.
10 Hainsworth JD, Cebotaru CL, Kanarev V *et al.* A phase II, open-label, randomized study to assess the efficacy and safety of AZD6244 (ARRY-142886) versus pemetrexed in patients with non-small cell lung cancer who have failed one or two prior chemotherapeutic regimens. *J Thorac Oncol* 2010; **5**: 1630–6.
11 Bennouna J, Lang I, Valladares-Ayerbes M *et al.* A Phase II, open-label, randomised study to assess the efficacy and safety of the MEK1/2 inhibitor AZD6244 (ARRY-142886) versus capecitabine monotherapy in patients with colorectal cancer who have failed one or two prior chemotherapeutic regimens. *Invest New Drugs* 2011; **29**: 1021–8.
12 Bodoky G, Timcheva C, Spigel DR *et al.* A phase II open-label randomized study to assess the efficacy and safety of selumetinib (AZD6244 [ARRY-142886]) versus capecitabine in patients with advanced or metastatic pancreatic cancer who have failed first-line gemcitabine therapy. *Invest New Drugs* 2012; **30**: 1216–23.
13 Gupta S, Ramjaun AR, Haiko P *et al.* Binding of ras to phosphoinositide 3-kinase p110alpha is required for ras-driven tumorigenesis in mice. *Cell* 2007; **129**: 957–68.
14 Castellano E, Sheridan C, Thin MZ *et al.* Requirement for interaction of PI3-kinase p110alpha with RAS in lung tumor maintenance. *Cancer Cell* 2013; **24**: 617–30.
15 Ebi H, Corcoran RB, Singh A *et al.* Receptor tyrosine kinases exert dominant control over PI3K signaling in human KRAS mutant colorectal cancers. *J Clin Invest* 2011; **121**: 4311–21.
16 Molina-Arcas M, Hancock DC, Sheridan C, Kumar MS, Downward J. Coordinate direct input of both KRAS and IGF1 receptor to activation of PI3 kinase in KRAS-mutant lung cancer. *Cancer Discov* 2013; **3**: 548–63.
17 Salt MB, Bandyopadhyay S, McCormick F. Epithelial to mesenchymal transition rewires the molecular path to PI3-Kinase-dependent proliferation. *Cancer Discov* 2014; **4**: 186–99.
18 COSMIC (Catalog of Somatic Mutations in Cancer) database. Wellcome Trust Sanger Institute. [Cited 14 Feb 2014.] Available from URL: http://www.sanger.ac.uk/cosmic.
19 Engelman JA, Chen L, Tan X *et al.* Effective use of PI3K and MEK inhibitors to treat mutant Kras G12D and PIK3CA H1047R murine lung cancers. *Nat Med* 2008; **14**: 1351–6.
20 Torbett NE, Luna-Moran A, Knight ZA *et al.* A chemical screen in diverse breast cancer cell lines reveals genetic enhancers and suppressors of sensitivity to PI3K isoform-selective inhibition. *Biochem J* 2008; **415**: 97–110.
21 Ihle NT, Lemos R Jr, Wipf P *et al.* Mutations in the phosphatidylinositol-3-kinase pathway predict for antitumor activity of the inhibitor PX-866 whereas oncogenic Ras is a dominant predictor for resistance. *Cancer Res* 2009; **69**: 143–50.
22 Neel NF, Martin TD, Stratford JK, Zand TP, Reiner DJ, Der CJ. The RalGEF-Ral effector signaling network: The road less traveled for anti-ras drug discovery. *Genes Cancer* 2011; **2**: 275–87.
23 Bodemann BO, White MA. Ral GTPases and cancer: linchpin support of the tumorigenic platform. *Nat Rev Cancer* 2008; **8**: 133–40.
24 Chien Y, White MA. RAL GTPases are linchpin modulators of human tumour-cell proliferation and survival. *EMBO Rep* 2003; **4**: 800–6.
25 Lim KH, Baines AT, Fiordalisi JJ *et al.* Activation of RalA is critical for Ras-induced tumorigenesis of human cells. *Cancer Cell* 2005; **7**: 533–45.
26 Lim KH, O'Hayer K, Adam SJ *et al.* Divergent roles for RalA and RalB in malignant growth of human pancreatic carcinoma cells. *Curr Biol* 2006; **16**: 2385–94.
27 Jullien-Flores V, Dorseuil O, Romero F *et al.* Bridging Ral GTPase to Rho pathways. RLIP76, a Ral effector with CDC42/Rac GTPase-activating protein activity. *J Biol Chem* 1995; **270**: 22473–7.
28 Moskalenko S, Henry DO, Rosse C, Mirey G, Camonis JH, White MA. The exocyst is a Ral effector complex. *Nat Cell Biol* 2002; **4**: 66–72.
29 Kashatus DF. Ral GTPases in tumorigenesis: emerging from the shadows. *Exp Cell Res* 2013; **319**: 2337–42.
30 Chien Y, Kim S, Bumeister R *et al.* RalB GTPase-mediated activation of the IkappaB family kinase TBK1 couples innate immune signaling to tumor cell survival. *Cell* 2006; **127**: 157–70.
31 Faber AC, Li D, Song Y *et al.* Differential induction of apoptosis in HER2 and EGFR addicted cancers following PI3K inhibition. *Proc Natl Acad Sci U S A* 2009; **106**: 19503–8.
32 Ebi H, Costa C, Faber AC *et al.* PI3K regulates MEK/ERK signaling in breast cancer via the Rac-GEF, P-Rex1. *Proc Natl Acad Sci USA* 2013; **110**: 21124–9.
33 Infante JR, Gandi L, Shapiro G *et al.* Combination of the MEK inhibitor, pimasertib (MSC1936369B), and the PI3K/mTOR inhibitor, SAR245409, in patients with advanced solid tumors: results of a phase Ib dose-escalation trial. *Cancer Res* 2013; **73**: LB–147.
34 Bedard P, Tabernero J, Kurzrock R *et al.* A phase Ib, open-label, multicenter, dose-escalation study of the oral pan-PI3K inhibitor BKM120 in combination with the oral MEK1/2 inhibitor GSK1120212 in patients (pts) with selected advanced solid tumors. *ASCO Meeting Abstracts* 2012; Abst 3003.
35 Speranza G, Kinders RJ, Khin S *et al.* Pharmacodynamic biomarker-driven trial of MK-2206, an AKT inhibitor, with AZD6244 (selumetinib), a MEK inhibitor, in patients with advanced colorectal carcinoma (CRC). *ASCO Meeting Abstracts* 2012; Abst 3529.
36 Gong Y, Somwar R, Politi K *et al.* Induction of BIM is essential for apoptosis triggered by EGFR kinase inhibitors in mutant EGFR-dependent lung adenocarcinomas. *PLoS Med* 2007; **4**: e294.
37 Bean GR, Ganesan YT, Dong Y *et al.* PUMA and BIM are required for oncogene inactivation-induced apoptosis. *Sci Signal* 2013; **6**: ra20.
38 Barbie DA, Tamayo P, Boehm JS *et al.* Systematic RNA interference reveals that oncogenic KRAS-driven cancers require TBK1. *Nature* 2009; **462**: 108–12.
39 Adjei AA, Cohen RB, Franklin W *et al.* Phase I pharmacokinetic and pharmacodynamic study of the oral, small-molecule mitogen-activated protein kinase kinase 1/2 inhibitor AZD6244 (ARRY-142886) in patients with advanced cancers. *J Clin Oncol* 2008; **26**: 2139–46.
40 Rodon J, Dienstmann R, Serra V, Tabernero J. Development of PI3K inhibitors: lessons learned from early clinical trials. *Nat Rev Clin Oncol* 2013; **10**: 143–53.
41 Corcoran RB, Cheng KA, Hata AN *et al.* Synthetic lethal interaction of combined BCL-XL and MEK inhibition promotes tumor regressions in KRAS mutant cancer models. *Cancer Cell* 2013; **23**: 121–8.
42 Tan N, Wong M, Nannini MA *et al.* Bcl-2/Bcl-xL inhibition increases the efficacy of MEK inhibition alone and in combination with PI3 kinase inhibition in lung and pancreatic tumor models. *Mol Cancer Ther* 2013; **12**: 853–64.
43 Sale MJ, Cook SJ. The BH3 mimetic ABT-263 synergizes with the MEK1/2 inhibitor selumetinib/AZD6244 to promote BIM-dependent tumour cell death and inhibit acquired resistance. *Biochem J* 2013; **450**: 285–94.
44 Faber AC, Coffee EM, Costa C *et al.* mTOR inhibition specifically sensitizes colorectal cancers with KRAS or BRAF mutations to BCL-2/BCL-XL inhibition by suppressing MCL-1. *Cancer Discov* 2014; **4**: 42–52.
45 Holt SV, Logie A, Odedra R *et al.* The MEK1/2 inhibitor, selumetinib (AZD6244; ARRY-142886), enhances anti-tumour efficacy when combined with conventional chemotherapeutic agents in human tumour xenograft models. *Br J Cancer* 2012; **106**: 858–66.
46 Janne PA, Shaw AT, Pereira JR *et al.* Selumetinib plus docetaxel for KRAS-mutant advanced non-small-cell lung cancer: a randomised, multicentre, placebo-controlled, phase 2 study. *Lancet Oncol* 2013; **14**: 38–47.
47 Chen Z, Cheng K, Walton Z *et al.* A murine lung cancer co-clinical trial identifies genetic modifiers of therapeutic response. *Nature* 2012; **483**: 613–7.
48 Carretero J, Shimamura T, Rikova K *et al.* Integrative genomic and proteomic analyses identify targets for Lkb1-deficient metastatic lung tumors. *Cancer Cell* 2010; **17**: 547–59.
49 Kim HS, Mendiratta S, Kim J *et al.* Systematic identification of molecular subtype-selective vulnerabilities in non-small-cell lung cancer. *Cell* 2013; **155**: 552–66.
50 Kumar MS, Hancock DC, Molina-Arcas M *et al.* The GATA2 transcriptional network is requisite for RAS oncogene-driven non-small cell lung cancer. *Cell* 2012; **149**: 642–55.
51 Steckel M, Molina-Arcas M, Weigelt B *et al.* Determination of synthetic lethal interactions in KRAS oncogene-dependent cancer cells reveals novel therapeutic targeting strategies. *Cell Res* 2012; **22**: 1227–45.

52 Scholl C, Frohling S, Dunn IF *et al.* Synthetic lethal interaction between oncogenic KRAS dependency and STK33 suppression in human cancer cells. *Cell* 2009; **137**: 821–34.
53 Singh A, Sweeney MF, Yu M *et al.* TAK1 inhibition promotes apoptosis in KRAS-dependent colon cancers. *Cell* 2012; **148**: 639–50.
54 Luo J, Emanuele MJ, Li D *et al.* A genome-wide RNAi screen identifies multiple synthetic lethal interactions with the Ras oncogene. *Cell* 2009; **137**: 835–48.
55 Puyol M, Martin A, Dubus P *et al.* A synthetic lethal interaction between K-Ras oncogenes and Cdk4 unveils a therapeutic strategy for non-small cell lung carcinoma. *Cancer Cell* 2010; **18**: 63–73.
56 Shaw AT, Winslow MM, Magendantz M *et al.* Selective killing of K-ras mutant cancer cells by small molecule inducers of oxidative stress. *Proc Natl Acad Sci USA* 2011; **108**: 8773–8.
57 Babij C, Zhang Y, Kurzeja RJ *et al.* STK33 kinase activity is nonessential in KRAS-dependent cancer cells. *Cancer Res* 2011; **71**: 5818–26.
58 Kothari V, Wei I, Shankar S *et al.* Outlier kinase expression by RNA sequencing as targets for precision therapy. *Cancer Discov* 2013; **3**: 280–93.
59 Sos ML, Michel K, Zander T *et al.* Predicting drug susceptibility of non-small cell lung cancers based on genetic lesions. *J Clin Invest* 2009; **119**: 1727–40.
60 Acquaviva J, Smith DL, Sang J *et al.* Targeting KRAS-mutant non-small cell lung cancer with the Hsp90 inhibitor ganetespib. *Mol Cancer Ther* 2012; **11**: 2633–43.
61 De Raedt T, Walton Z, Yecies JL *et al.* Exploiting cancer cell vulnerabilities to develop a combination therapy for ras-driven tumors. *Cancer Cell* 2011; **20**: 400–13.
62 Ballester R, Furth ME, Rosen OM. Phorbol ester- and protein kinase C-mediated phosphorylation of the cellular Kirsten ras gene product. *J Biol Chem* 1987; **262**: 2688–95.
63 Alvarez-Moya B, Lopez-Alcala C, Drosten M, Bachs O, Agell N. K-Ras4B phosphorylation at Ser181 is inhibited by calmodulin and modulates K-Ras activity and function. *Oncogene* 2010; **29**: 5911–22.
64 Barcelo C, Paco N, Morell M *et al.* Phosphorylation at Ser-181 of oncogenic KRAS is required for tumor growth. *Cancer Res* 2014; **74**: 1190–9.

JJCO Japanese Journal of Clinical Oncology

Jpn J Clin Oncol 2014;44(1)9–12
doi:10.1093/jjco/hyt188

Review Article: Strategy for Drug Discovery at Pharmaceutical Companies

The Current State of Molecularly Targeted Drugs Targeting HGF/Met

Seiji Yano[1,*] and Takayuki Nakagawa[1,2]

[1]Division of Medical Oncology, Cancer Research Institute, Kanazawa University, Kanazawa and [2]Tsukuba Research Laboratories, Eisai Co., Ltd., Ibaraki, Japan

*For reprints and all correspondence: Seiji Yano, Division of Medical Oncology, Cancer Research Institute, Kanazawa University, 13-1 Takara-machi, Kanazawa, Ishikawa 920-0934, Japan. E-mail: syano@staff.kanazawa-u.ac.jp

Received July 22, 2013; accepted October 27, 2013

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

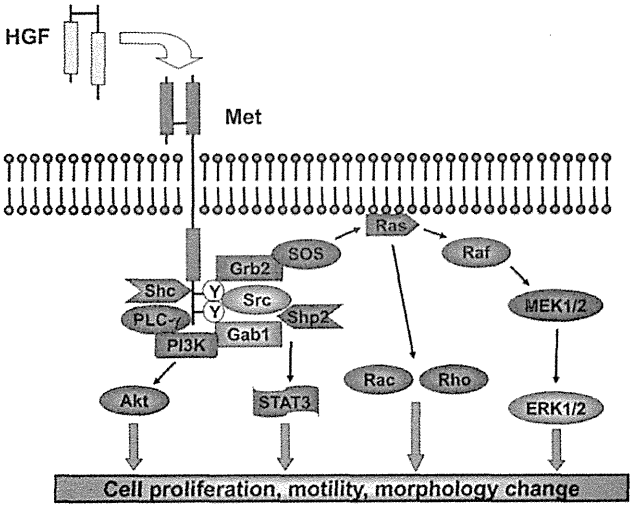

Figure 1. Hepatocyte growth factor/Met signaling pathway.

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.

Funding

This study was supported by Grants-in-Aid for Cancer Research (S.Y., 21390256), Scientific Research on Innovative Areas 'Integrative Research on Cancer Microenvironment Network' (S.Y., 22112010A01) and Grant-in-Aid for Project for Development of Innovative Research on Cancer Therapeutics (P-Direct) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan.

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.

References

1. Gherardi E, Birchmeier W, Birchmeier C, et al. Targeting MET in cancer: rationale and progress. *Nat Rev Cancer* 2012;12:89–103.
2. Kim SJ, Johnson M, Koterba K, et al. Reduced c-Met expression by an adenovirus expressing a c-Met ribozyme inhibits tumorigenic growth and lymph node metastases of PC3-LN4 prostate tumor cells in an orthotopic nude mouse model. *Clin Cancer Res* 2003;14:5161–70.
3. http://www.vai.org/Met/Index.aspx (29 November 2013, date last accessed).
4. Christensen JG, Burrows J, Salgia R. c-Met as a target for human cancer and characterization of inhibitors for therapeutic intervention. *Cancer Lett* 2005;225:1–26.
5. Shojaei F, Lee JH, Simmons BH, et al. HGF/c-Met acts as an alternative angiogenic pathway in sunitinib-resistant tumors. *Cancer Res* 2010;70:10090–100.
6. Chen CT, Kim H, Liska D, et al. MET activation mediates resistance to lapatinib inhibition of HER2-amplified gastric cancer cells. *Mol Cancer Ther* 2012;11:660–9.
7. Pao W, Chmielecki J. Rational, biologically based treatment of EGFR-mutant non-small-cell lung cancer. *Nat Rev Cancer* 2010;10:760–74.
8. Kobayashi S, Boggon TJ, Dayaram T, et al. EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. *N Engl J Med* 2005;352:786–92.
9. Yano S, Wang W, Li Q, et al. Hepatocyte growth factor induces gefitinib resistance of lung adenocarcinoma with epidermal growth factor receptor-activating mutations. *Cancer Res* 2008;68:9479–87.
10. Engelman JA, Zejnullahu K, Mitsudomi T, et al. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. *Science* 2007;316:1039–43.
11. Yano S, Yamada T, Takeuchi S, et al. Hepatocyte growth factor expression in EGFR mutant lung cancer with intrinsic and acquired resistance to tyrosine kinase inhibitors in a Japanese cohort. *J Thorac Oncol* 2011;6:2011–7.
12. Turke AB, Zeinullahu K, Wu YL, et al. Preexistence and clonal selection of MET amplification in EGFR mutant NSCLC. *Cancer Cell* 2010;17:77–88.
13. Yamada T, Matsumoto K, Wang W, et al. Hepatocyte growth factor reduces susceptibility to an irreversible epidermal growth factor receptor inhibitor in EGFR-T790M mutant lung cancer. *Clin Cancer Res* 2010;16:174–83.
14. Wang W, Li Q, Takeuchi S, et al. Met kinase inhibitor E7050 reverses three different mechanisms of hepatocyte growth factor-induced tyrosine kinase inhibitor resistance in EGFR mutant lung cancer. *Clin Cancer Res* 2012;18:1663–71.
15. Eathiraj S, Palma R, Volckova E, et al. Discovery of a novel mode of protein kinase inhibition characterized by the mechanism of inhibition of human mesenchymal-epithelial transition factor (c-Met) protein autophosphorylation by ARQ 197. *J Biol Chem* 2011;286:20666–76.
16. Katayama R, Aoyama A, Yamori T, et al. Cytotoxic activity of tivantinib (ARQ 197) is not due solely to c-MET inhibition. *Cancer Res* 2013;73:3087–96.
17. Catenacci DV, Henderson L, Xiao SY, et al. Durable complete response of metastatic gastric cancer with anti-Met therapy followed by resistance at recurrence. *Cancer Discov* 2011;1:573–9.
18. Ou SH, Kwak EL, Siwak-Tapp C, et al. Activity of crizotinib (PF02341066), a dual MET and anaplastic lymphoma kinase (ALK) inhibitor, in a non-small cell lung cancer patient with de novo MET amplification. *J Thorac Oncol* 2011;6:942–6.
19. Lennerz JK, Kwak EL, Ackerman A, et al. MET amplification identifies a small and aggressive subgroup of esophagogastric adenocarcinoma with evidence of responsiveness to crizotinib. *J Clin Oncol* 2011;29:4803–10.
20. Chi AS, Batchelor TT, Kwak EL, et al. Rapid radiographic and clinical improvement after treatment of a MET-amplified recurrent glioblastoma with a MET inhibitor. *J Clin Oncol* 2012;30:e30–3.

Cancer Science
Japanese Cancer Association JCA
Open Access

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

Qi Li,[1] Wei Wang,[1] Yusuke Machino,[2] Tadaaki Yamada,[1] Kenji Kita,[1] Masanobu Oshima,[3] Yoshitaka Sekido,[4] Mami Tsuchiya,[5] Yui Suzuki,[6] Ken-ichiro Nan-ya,[6] Shigeru Iida,[7] Kazuyasu Nakamura,[8] Shotaro Iwakiri,[9] Kazumi Itoi[9] and Seiji Yano[1]

[1]Division of Medical Oncology, Cancer Research Institute, Kanazawa University, Kanazawa; [2]Oncology Research Laboratories, R&D Division, Kyowa Hakko Kirin Co., Ltd., Tokyo; [3]Division of Genetics, Cancer Research Institute, Kanazawa University, Kanazawa; [4]Division of Molecular Oncology, Aichi Cancer Center Research Institute, Nagoya; [5]CNS Research Laboratories, R&D Division, Kyowa Hakko Kirin Co., Ltd., Shizuoka; [6]Translational Research Unit, R&D Division, Kyowa Hakko Kirin Co., Ltd., Shizuoka; [7]Research Core Function Laboratories, R&D Division, Kyowa Hakko Kirin Co., Ltd., Tokyo; [8]Tokyo Research Park, R&D Division, Kyowa Hakko Kirin Co., Ltd., Tokyo; [9]Department of Thoracic Surgery, Hyogo Prefectural Amagasaki Hospital, Amagasaki, Japan

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

Correspondence
Seiji Yano, Division of Medical Oncology, Cancer Research Institute, Kanazawa University, 13-1 Takara-machi, Kanazawa, Ishikawa 920-0934, Japan.
Tel.: +81-76-265-2780; Fax: +81-76-234-4524;
E-mail: syano@staff.kanazawa-u.ac.jp

Funding Information
Kyowa Hakko Kirin Co., Ltd.

Received September 14, 2014; Revised November 11, 2014; Accepted November 14, 2014

Cancer Sci **106** (2015) 102–107

doi: 10.1111/cas.12575

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

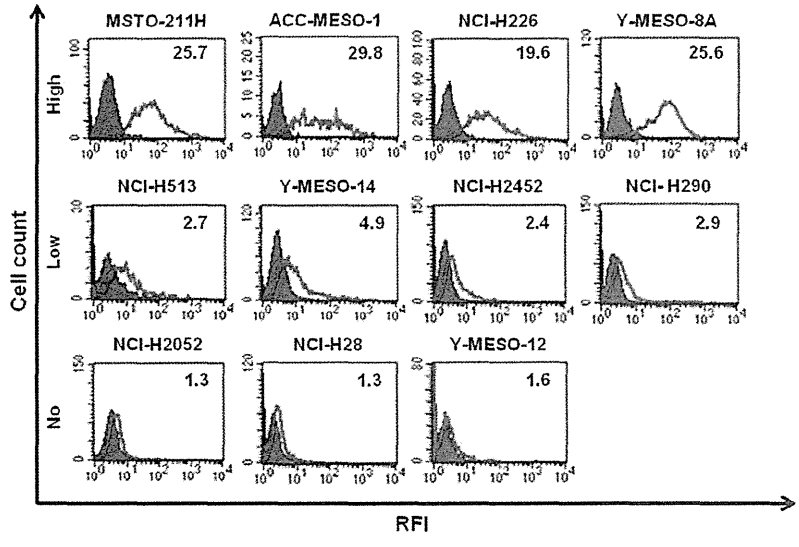

Fig. 1. Expression levels of ganglioside GM2 were evaluated in malignant pleural mesothelioma cell lines. Cells were detached and incubated with BIW-8962 or anti-dinitrophenol (DNP) antibodies on ice for 30 min. Bound Abs were detected with FITC-conjugated goat anti-human IgG Abs. The fluorescence intensity 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.

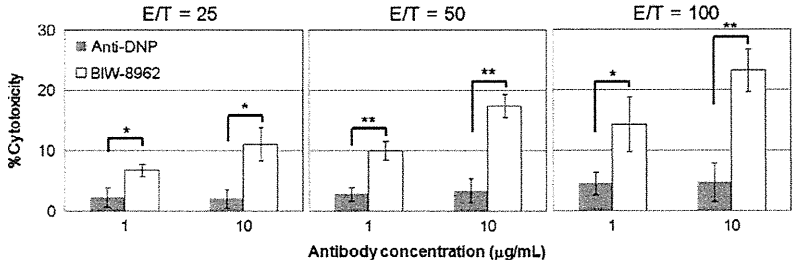

Fig. 2. Anti-GM2 antibody BIW-8962 exerted antibody-dependent cellular cytotoxicity activity against MSTO-211H malignant pleural mesothelioma cells. Human peripheral blood mononuclear cells were purified from healthy donors and used as effector cells. MSTO-211H cells (target cells) were incubated with 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.

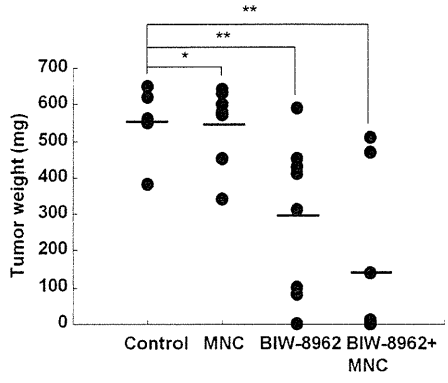

Fig. 3. Anti-GM2 antibody BIW-8962 showed therapeutic activity in an MSTO-211H orthotropic mouse model. MSTO-211H malignant pleural mesothelioma cells were inoculated into the thoracic cavity in SCID mice, and the animals were treated with BIW-8962 and/or human peripheral blood mononuclear cells (MNC). 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

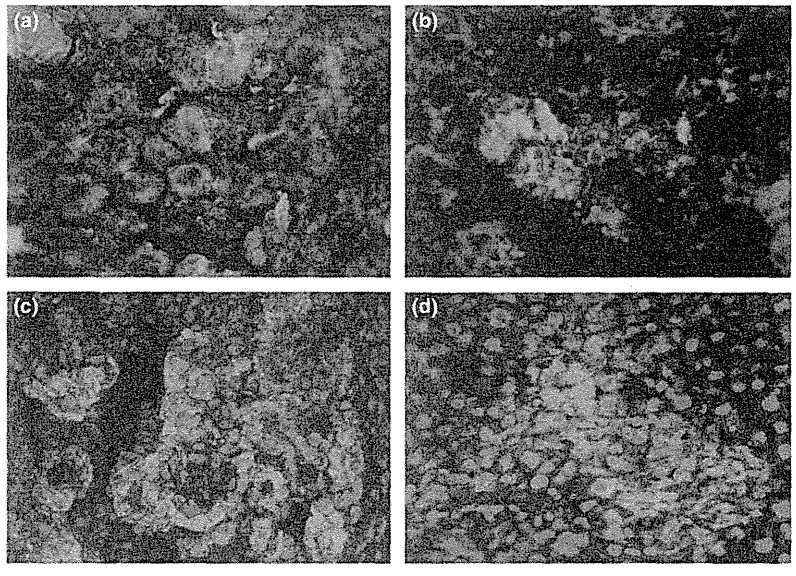

Fig. 4. Ganglioside GM2 expression in clinical malignant pleural mesothelioma specimens. Frozen sections obtained from 26 mesothelioma patients were incubated in DAPI at room temperature for 20 min. After washing, the sections were incubated in 5% FBS–PBS for 10 min for blocking and subsequently incubated 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).

Acknowledgments

We thank Dr. Hideki Murakami, Aichi Cancer Center Research Institute, for providing assistance with the immunofluorescent GM2 staining.

Disclosure Statement

Yusuke Machino, Mami Tsuchiya, Yui Suzuki, Ken-ichiro Nan-ya, Shigeru Iida, and Kazuyasu Nakamura are employees of Kyowa Hakko Kirin Co., Ltd. Seiji Yano received a research grant from Kyowa Hakko Kirin Co., Ltd.

References

1 Connelly RR, Spirtas R, Myers MH, Percy CL, Fraumeni JF Jr. Demographic patterns for mesothelioma in the United States. *J Natl Cancer Inst* 1987; **78**: 1053–60.
2 Ismail-Khan R, Robinson LA, Williams CC Jr, Garrett CR, Bepler G, Simon GR. Malignant pleural mesothelioma: a comprehensive review. *Cancer Control* 2006; **13**: 255–63.
3 Ruffie P, Feld R, Minkin S *et al.* Diffuse malignant mesothelioma of the pleura in Ontario and Quebec: a retrospective study of 332 patients. *J Clin Oncol* 1989; **7**: 1157–68.
4 Pass HI, Kranda K, Temeck BK, Feuerstein I, Steinberg SM. Surgically debulked malignant pleural mesothelioma: results and prognostic factors. *Ann Surg Oncol* 1997; **4**: 215–22.
5 Rusch VW, Piantadosi S, Holmes EC. The role of extrapleural pneumonectomy in malignant pleural mesothelioma. A Lung Cancer Study Group trial. *J Thorac Cardiovasc Surg* 1991; **102**: 1–9.
6 Vogelzang NJ, Rusthoven JJ, Symanowski J *et al.* Phase III study of pemetrexed in combination with cisplatin versus cisplatin alone in patients with malignant pleural mesothelioma. *J Clin Oncol* 2003; **21**: 2636–44.
7 Kurai J, Chikumi H, Hashimoto K *et al.* Therapeutic antitumor efficacy of anti-epidermal growth factor receptor antibody, cetuximab, against malignant pleural mesothelioma. *Int J Oncol* 2012; **41**: 1610–8.
8 Kubota T, Niwa R, Satoh M, Akinaga S, Shitara K, Hanai N. Engineered therapeutic antibodies with improved effector functions. *Cancer Sci* 2009; **100**: 1566–72.
9 Musolino A, Naldi N, Bortesi B *et al.* Immunoglobulin G fragment C receptor polymorphisms and clinical efficacy of trastuzumab-based therapy in patients with HER-2/neu-positive metastatic breast cancer. *J Clin Oncol* 2008; **26**: 1789–96.
10 Tamura K, Shimizu C, Hojo T *et al.* FcgammaR2A and 3A polymorphisms predict clinical outcome of trastuzumab in both neoadjuvant and metastatic settings in patients with HER2-positive breast cancer. *Ann Oncol* 2011; **22**: 1302–7.
11 Usuki S, Ren J, Utsunomiya I, Cashman NR, Inokuchi J, Miyatake T. GM2 ganglioside regulates the function of ciliary neurotrophic factor receptor in murine immortalized motor neuron-like cells (NSC-34). *Neurochem Res* 2001; **26**: 375–82.
12 Jennemann R, Rodden A, Bauer BL, Mennel HD, Wiegandt H. Glycosphingolipids of human gliomas. *Cancer Res* 1990; **50**: 7444–9.
13 Hakomori S. Aberrant glycosylation in cancer cell membranes as focused on glycolipids: overview and perspectives. *Cancer Res* 1985; **45**: 2405–14.
14 Jones PC, Sze LL, Liu PY, Morton DL, Irie RF. Prolonged survival for melanoma patients with elevated IgM antibody to oncofetal antigen. *J Natl Cancer Inst* 1981; **66**: 249–54.
15 Eggermont AM, Suciu S, Rutkowski P *et al.* Adjuvant ganglioside GM2-KLH/QS-21 vaccination versus observation after resection of primary tumor >1.5 mm in patients with stage II melanoma: results of the EORTC 18961 randomized phase III trial. *J Clin Oncol* 2013; **31**: 3831–7.
16 Nakamura K, Tanaka Y, Shitara K, Hanai N. Construction of humanized anti-ganglioside monoclonal antibodies with potent immune effector functions. *Cancer Immunol Immunother* 2001; **50**: 275–84.
17 Yamada T, Bando H, Takeuchi S *et al.* Genetically engineered humanized anti-ganglioside GM2 antibody against multiple organ metastasis produced by GM2-expressing small-cell lung cancer cells. *Cancer Sci* 2011; **102**: 2157–63.
18 Kawaguchi K, Murakami H, Taniguchi T *et al.* Combined inhibition of MET and EGFR suppresses proliferation of malignant mesothelioma cells. *Carcinogenesis* 2009; **30**: 1097–105.
19 Nishioka Y, Yano S, Fujiki F *et al.* Combined therapy of multidrug-resistant human lung cancer with anti-P-glycoprotein antibody and monocyte chemoattractant protein-1 gene transduction: the possibility of immunological overcoming of multidrug resistance. *Int J Cancer* 1997; **71**: 170–7.
20 Tanaka T, Tsudo M, Karasuyama H *et al.* A novel monoclonal antibody against murine IL-2 receptor beta-chain. Characterization of receptor expression in normal lymphoid cells and EL-4 cells. *J Immunol* 1991; **147**: 2222–8.
21 Nakataki E, Yano S, Matsumori Y *et al.* Novel orthotopic implantation model of human malignant pleural mesothelioma (EHMES-10 cells) highly expressing vascular endothelial growth factor and its receptor. *Cancer Sci* 2006; **97**: 183–91.
22 Nakamura K, Koike M, Shitara K *et al.* Chimeric anti-ganglioside GM2 antibody with antitumor activity. *Cancer Res* 1994; **54**: 1511–6.
23 Yamada N, Oizumi S, Kikuchi E *et al.* CD8+ tumor-infiltrating lymphocytes predict favorable prognosis in malignant pleural mesothelioma after resection. *Cancer Immunol Immunother* 2010; **59**: 1543–9.
24 Inami K, Abe M, Takeda K *et al.* Antitumor activity of anti-C-ERC/mesothelin monoclonal antibody *in vivo*. *Cancer Sci* 2010; **101**: 969–74.
25 Abe S, Morita Y, Kaneko MK *et al.* A novel targeting therapy of malignant mesothelioma using anti-podoplanin antibody. *J Immunol* 2013; **190**: 6239–49.
26 Nayak TK, Garmestani K, Milenic DE *et al.* HER1-targeted 86Y- panitumumab possesses superior targeting characteristics than 86Y-cetuximab for PET imaging of human malignant mesothelioma tumors xenografts. *PLoS ONE* 2011; **6**: e18198.
27 Gallinger S, Reilly RM, Kirsh JC *et al.* Comparative dual label study of first and second generation antitumor-associated glycoprotein-72 monoclonal antibodies in colorectal cancer patients. *Cancer Res* 1993; **53**: 271–8.
28 Musk AW, Olsen N, Alfonso H *et al.* Predicting survival in malignant mesothelioma. *Eur Respir J* 2011; **38**: 1420–4.
29 Flores RM, Pass HI, Seshan VE *et al.* Extrapleural pneumonectomy versus pleurectomy/decortication in the surgical management of malignant pleural mesothelioma: results in 663 patients. *J Thorac Cardiovasc Surg* 2008; **135**: 620–6.
30 Vacca P, Martini S, Garelli V, Passalacqua G, Moretta L, Mingari MC. NK cells from malignant pleural effusions are not anergic but produce cytokines and display strong antitumor activity on short-term IL-2 activation. *Eur J Immunol* 2013; **43**: 550–61.
31 Robinson BW, Lake RA. Advances in malignant mesothelioma. *N Engl J Med* 2005; **353**: 1591–603.

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

Azusa Tanimoto[1], Tadaaki Yamada[1], Shigeki Nanjo[1], Shinji Takeuchi[1], Hiromichi Ebi[1], Kenji Kita[1], Kunio Matsumoto[2] and Seiji Yano[1]

[1] Divisions of Medical Oncology, Cancer Research Institute, Kanazawa University, Kanazawa, Japan

[2] Tumor Dynamics and Regulation, Cancer Research Institute, Kanazawa University, Kanazawa, Japan

Correspondence to: Seiji Yano, email: syano@staff.kanazawa-u.ac.jp

Keywords: Hsp90 inhibitor, EML4-ALK, drug resistance, receptor ligands, New generation ALK inhibitor.

Received: April 1, 2014 *Accepted*: June 1, 2014 *Published*: June 3, 2014

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

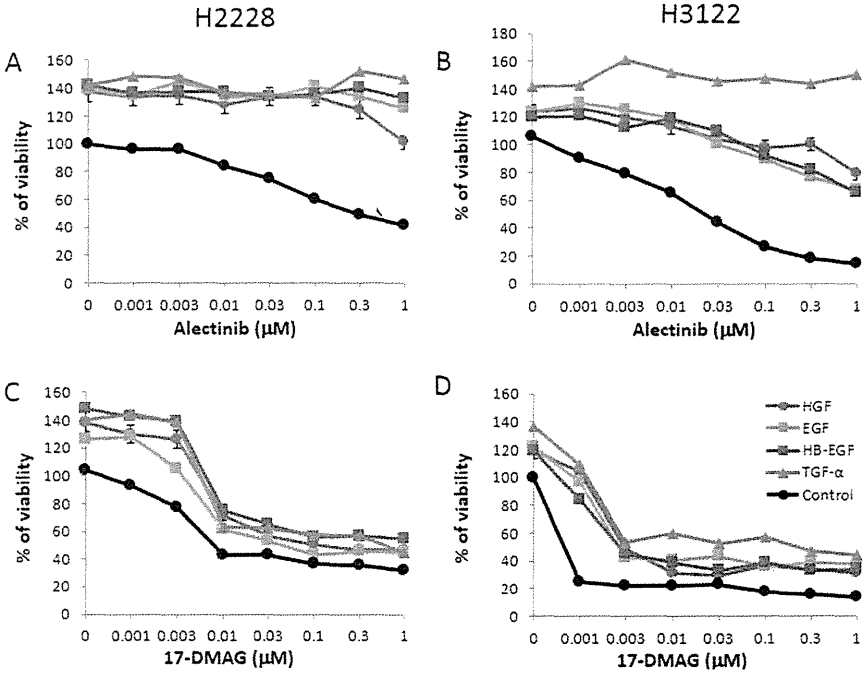

Figure 1: 17-DMAG suppresses the growth of EML4-ALK NSCLC cells in the presence of HGF and EGFR ligands. The EML4-ALK lung cancer cell lines human H2228 and human H3122 were treated with increasing concentrations of alectinib or 17-DMAG, with or without HGF (50 ng/mL), EGF (100 ng/mL), HB-EGF (10 ng/mL), 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.

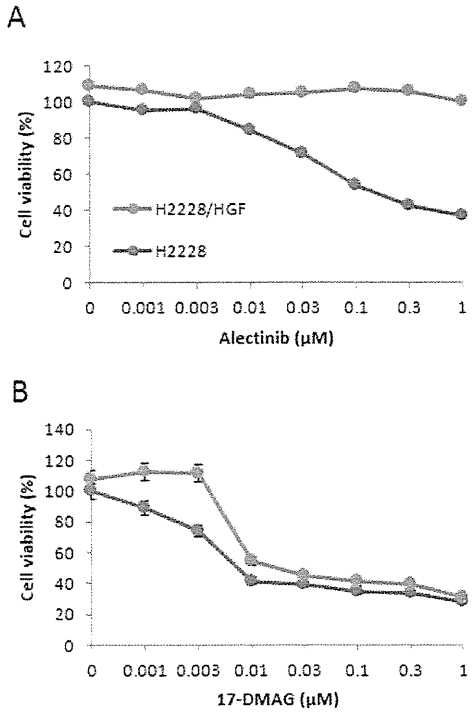

Figure 2: HGF-gene transfection resulted in reducing susceptibility of EML4-ALK NSCLC cells to alectinib but not 17-DMAG. H2228/Vec (A) or H2228/HGF (B) cells were treated with increasing concentrations of alectinib or 17-DMAG, and cell viability was determined after 72 h by MTT assay. Data shown are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

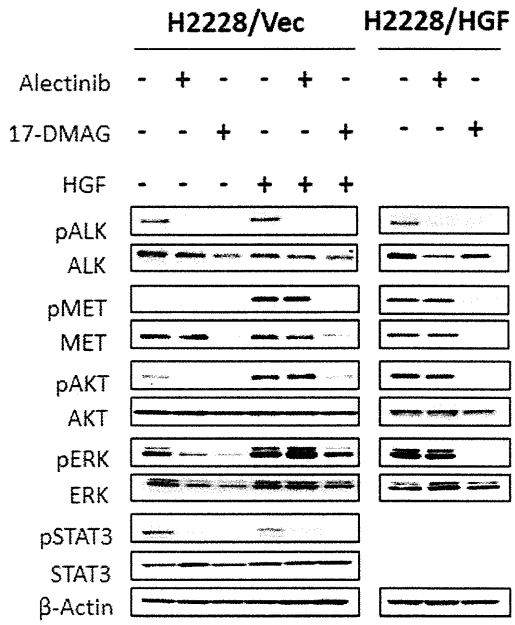

Figure 3: 17-DMAG reduced MET protein expression and inhibited downstream pathways, even in the presence of HGF. H2228/Vec or H2228/HGF cells were treated with or without alectinib (0.3 μmol/L) for 2 h or 17-DMAG (0.3 μmol/L) for 24 h and then stimulated with or without HGF (50 ng/mL) for 10 minutes. 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

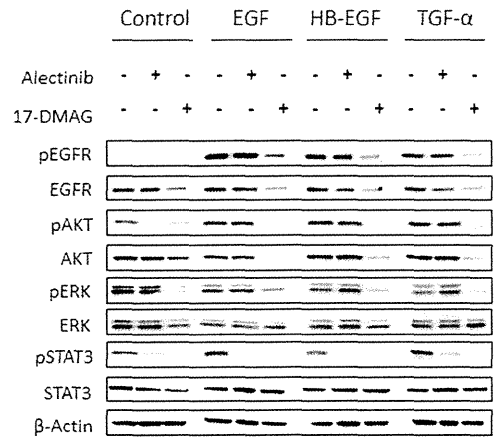

Figure 4: 17-DMAG reduced MET protein expression and inhibited downstream pathways, even in the presence of EGFR ligands. H2228 cells were treated with or without alectinib (0.3 μmol/L) for 2 h or 17-DMAG (0.3 μmol/L) for 24 h, and then stimulated with or without EGF (100 ng/mL), HB-EGF (10 ng/mL), and 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.

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.

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