UICC
global cancer control

IJC
International Journal of Cancer

Antitumor activity of HM781-36B, a highly effective pan-HER inhibitor in erlotinib-resistant NSCLC and other EGFR-dependent cancer models

Mi Young Cha[1,2], Kwang-Ok Lee[1], Mira Kim[1], Ji Yeon Song[1], Kyu Hang Lee[1], Jongmin Park[2], Yun Jung Chae[1], Young Hoon Kim[1], Kwee Hyun Suh[1], Gwan Sun Lee[1], Seung Bum Park[2,3] and Maeng Sup Kim[1]

[1] Department of Drug Discovery, Hanmi Research Center, Gyeonggi-do 445-813, Korea
[2] Department of Chemistry, Seoul National University, Seoul 151-747, Korea
[3] Department of Biophysics and Chemical Biology, Seoul National University, Seoul 151-747, Korea

The epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases has been implicated in a variety of cancers. In particular, activating mutations such as the L858R point mutation in exon 21 and the small in-frame deletions in exon 19 of the EGFR tyrosine kinase domain are correlated with sensitivity to EGFR tyrosine kinase inhibitors in non-small cell lung cancer (NSCLC) patients. Clinical treatment of patients is limited by the development of drug resistance resulting mainly from a gatekeeper mutation (T790M). In this study, we evaluated the therapeutic potential of a novel, irreversible pan-HER inhibitor, HM781-36B. The results from this study show that HM781-36B is a potent inhibitor of EGFR *in vitro*, including the *EGFR*-acquired resistance mutation (T790M), as well as HER-2 and HER-4, compared with other EGFR tyrosine kinases inhibitors (erlotinib, lapatinib and BIBW2992). HM781-36B treatment of *EGFR* DelE746_A750-harboring erlotinib-sensitive HCC827 and *EGFR* L858R/T790M-harboring erlotinib-resistant NCI-H1975 NSCLC cells results in the inhibition of EGFR phosphorylation and the subsequent deactivation of downstream signaling proteins. Additionally, HM781-36B shows an excellent efficacy in a variety of EGFR- and HER-2-dependent tumor xenograft models, including erlotinib-sensitive HCC827 NSCLC cells, erlotinib-resistant NCI-H1975 NSCLC cells, HER-2 overexpressing Calu-3 NSCLC cells, NCI-N87 gastric cancer cells, SK-Ov3 ovarian cancer cells and EGFR-overexpressing A431 epidermoid carcinoma cancer cells. On the basis of these preclinical results, HM781-36B is the most potent pan-HER inhibitor, which will be advantageous for the treatment of patients with NSCLC including clinical limitation caused by acquired mutation (*EGFR* T790M), breast cancer and gastric cancer.

Members of the EGFR tyrosine kinase family, including epidermal growth factor receptor (EGFR; also called HER-1, ErbB-1), HER-2 (neu, ErbB-2), HER-3 (ErbB-3) and HER-4 (ErbB-4), play a crucial role in regulating cell proliferation and differentiation in many tissue types.[1–3] The dysregulation of the EGFR signaling pathway caused by genetic alterations in the kinase domain of EGFR is associated with malignant transformation in non-small cell lung cancer (NSCLC).[4] The hyperactivation of the EGFR family, in particular, HER-1 and HER-2, frequently observed in several solid tumors can be caused by overexpression, mutations resulting in constitutive activation or autocrine expression of endogenous ligands.[5] Therefore, the EGFR family remains a major target for the development of anticancer agents.[6]

Small-molecule inhibitors of the EGFR tyrosine kinases have been developed and approved for the treatment of patients with NSCLC.[7,8] The first generation of EGFR-targeting therapeutic agents includes the HER-1-specific reversible and quinazoline-based adenosine triphosphate (ATP) competitive inhibitors, such as gefitinib and erlotinib.[9,10] These are effective for the treatment of patients with NSCLC who have activating mutations in the HER-1 kinase domain.[11,12] The most common activating mutations are L858R in exon 21 and DelE746_A750 in exon 19.[13] Despite an initial good response to these drugs, however, patients almost invariably develop resistance to HER-1-specific inhibitors, which has been clinically observed after treatment for 1 year. Resistance is often associated with the T790M mutation within HER-1

Key words: HM781-36B, pan-HER inhibitor, erlotinib-resistance, EGFR T790M, NSCLC, breast cancer, gastric cancer
Additional Supporting Information may be found in the online version of this article.
This work was presented at 102nd AACR Annual Meeting in Orlando, FL, in 2011. (Proceedings supplement, p 115, no. LB-389)
Grant sponsor: Korea Healthcare Technology R&D Project, Ministry of Health & Welfare, Republic of Korea; **Grant numbers:** A092074, A060203; **Grant sponsors:** National Research Foundation of Korea (NRF), Korean Ministry of Education, Science, and Technology (MEST), BK21 Program
DOI: 10.1002/ijc.26276
History: Received 8 Mar 2011; Accepted 6 Jun 2011; Online 5 Jul 2011
Correspondence to: Seung Bum Park, Ph. D., Department of Chemistry, Seoul National University, Seoul 151-747, Korea, Tel: +82-2-8809090, Fax: +82-2-8844025, E-mail: sbpark@snu.ac.kr; or Maeng Sup Kim, Ph. D., Hanmi Research Center, 377-1 Yeongcheon-ri, Dongtan-myeon, Hwaseong-si, Gyeonggi-do, 445-813, Korea, Tel: +82-31-3715000, Fax: +82-31-3715006, E-mail: kims@hanmi.co.kr

tyrosine kinase.[14,15] The resistance mechanisms caused by T790M, the gatekeeper mutation, have been explained in several aspects, such as steric hindrance, increased ATP affinity, decreased affinity by disruption of favorable interactions and decreased binding velocity to the mutant kinase.[16–19]

The second generation of EGFR-targeting therapeutic agents includes the HER-1/HER-2 reversible dual inhibitor such as lapatinib, for the treatment of HER-2-positive breast cancer.[20] Lapatinib is also a promising treatment option for gastric cancer with HER-2 amplification.[21] HER-1/HER-2 dual inhibition is more effective than just HER-1 inhibition, because the HER-1 receptor tyrosine kinase can homo- or heterodimerize with HER-1 or HER-2 receptor tyrosine kinases for signal transduction in the EGFR signaling cascade.[22] The third generation of EGFR-targeting therapeutic agents includes the EGFR irreversible inhibitors, such as HKI-272, CI-1033, PF00299804 and BIBW2992, all of which covalently modify reactive cysteine residues (Cys773 of HER-1 and Cys805 of HER-2) at their respective active sites.[23–29] Several advantages of irreversible inhibition of EGFR tyrosine kinases have been demonstrated. For example, irreversible EGFR inhibitors are capable of overcoming the acquired resistance toward HER-1-specific reversible inhibitors, gefitinib and erlotinib, that are associated with the *EGFR* T790M mutation in NSCLC.[30–32] The irreversible EGFR inhibitors also show a high potency with a broad spectrum because of their pan-HER inhibitory profiles. However, some irreversible EGFR inhibitors with clinically insufficient exposure level in plasma suffer remarkably decreased efficacy against patients with advanced lung tumor harboring the T790M-acquired mutation in the EGFR kinase domain, although they were effective in animal models.[19] In this study, we describe the identification of a novel, small-molecular and irreversible pan-HER inhibitor, HM781-36B, with excellent potency and sufficient exposure level in human. We evaluate its anticancer activity *in vitro* and *in vivo* to provide a "best-in-class" agent for the treatment of various EGFR-dependent cancer cells, including the erlotinib-resistant NCI-H1975 cells, which harbor the *EGFR* L858R/T790M double mutation.

Material and Methods

Reagents

The novel pan-HER inhibitor, HM781-36B,[33,34] and other EGFR tyrosine kinase inhibitors including erlotinib,[35] lapatinib[36] and BIBW2992,[37] were synthesized as described in patents.

In vitro kinase assay

$EGFR^{WT}$ (Upstate, Billerica/MA), $EGFR^{T790M}$ (Upstate, Billerica/MA), $EGFR^{T790M/L858R}$ (Upstate, Billerica/MA), HER-2 (Invitrogen, Carlsbad/CA) and HER-4 (Invitrogen, Carlsbad/CA) were expressed by baculovirus in Sf21 insect cells and used for kinase assays and IC_{50} determinations, which were performed as previously described.[38] To determine the selectivity of HM781-36B against various kinases, the SelectScreen™ Kinase Profiling service was used (Invitrogen, Carlsbad/CA).

Cell growth inhibition assay

Calu-3, NCI-H1975, NCI-H358, NCI-H1781, HCC827, A549, A431, SK-Br3, BT-474, MDA-175, NCI-N87, Hs-27 and Balb/c3t3 (clone A31) cells were purchased from the American Type Culture Collection (ATCC, Manassas/VA). Calu-3 and A549 cells were maintained in Minimum Essential Medium (MEM) and F-12K culture medium, respectively. NCI-H1975, NCI-H358, NCI-H1781, HCC827, SK-Br3 and NCI-N87 cell lines were cultured in RPMI medium containing 1 mM sodium pyruvate. A431 and Hs-27 cell lines were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM), and Balb/c3t3 cell line was maintained in low-glucose DMEM. BT-474 and MDA-175 cells were incubated in Hybri-Care medium and L-15 medium, respectively. All culture media were supplemented with 1% penicillin–streptomycin and 10% fetal bovine serum (FBS) except for Balb/c3t3 cells (10% fetal calf serum and 1% penicillin–streptomycin). Cells were incubated in a humidified atmosphere under 5% CO_2 except for MDA-175 cells (CO_2 free) at 37°C. Cell growth inhibition assays and GI_{50} and GI_{90} determinations were performed as previously described.[38]

Western blot analysis

Cells were treated with erlotinib or HM781-36B in the presence of media supplemented with 10% FBS for 24 hr. After harvesting the cells, total cell lysate was immunoblotted. Primary antibodies against p-EGFR (pY1086 in H1975 and pY1148 in HCC827), p-HER-2 (pY877) and p-AKT (pS473) were purchased from Cell Signaling (Danvers/MA) and EGFR, HER-2, AKT, p-ERK1 and ERK1/2 were purchased from Santa Cruz Biotechnology (Santa Cruz/CA).

Prolongation of phosphorylation inhibition

Cells were plated at a density of 5×10^5/well in 6-well plates under normal culture conditions (10% FBS and 1% penicillin-streptomycin). After 24 hr, the media were changed to 0.1% FBS media and cells were incubated for 16 hr. Cells were then treated with 1 μM erlotinib, BIBW2992 or HM781-36B for 4 hr. Each set was washed four times with warmed compound-free medium and incubated for 0, 8 and 24 hr. Each set was stimulated with EGF (100 ng/ml) for 5 min. The phosphorylation rates of EGFR or HER-2 were measured by ELISA using Human EGFR (pY1173, Biosource, Victoria/Australia: Cat No. KHR9071) and HER-2 (Cell Signaling, Danvers/MA: Cat No. 7148) immunoassay kit.

Irreversible binding studies with Cy3-HM781-36B

Enzymes (1 μg of $EGFR^{WT}$, $EGFR^{T790M}$ or PDGFRα, Upstate) were incubated with 0.1% DMSO or 1 μM of Cy3-HM781-36B in the presence or absence of 5 μM of unlabeled HM781-36B for 15 min at 0°C. Subsequent to incubation, samples were boiled for 5 min in SDS buffer, and proteins were separated by SDS-PAGE (10% acrylamide). The proteins were visualized by Coomassie staining and the fluorescence

Table 1. Inhibition of EGFR family kinases by HM781–36B, Erlotinib, Lapatinib and BIBW-2992

Enzyme (IC_{50}, nM)[1]	$EGFR^{WT}$	$EGFR^{T790M}$	$EGFR^{T790M/L858R}$	HER-2	HER-4
HM781–36B	3.2	4.2	2.2	5.3	23.5
Erlotinib	>1,000	>1,000	>1,000	>1,000	>1,000
Lapatinib	52.2	>1,000	>1,000	36.3	>1,000
BIBW2992	8.0	9.8	24.0	24.9	26.6

[1]All biological data are mean values for three independent experiments performed in duplicate. The IC_{50} was calculated from data at six different concentrations.
Abbreviation: WT, wild-type.

Table 2. Inhibition of kinases by HM781–36B

Kinase	IC_{50} (nM)[1]
FGFR 1, 2, 3, 4	>1,000
GSK3 beta	>10,000
IGF1R	>10,000
IKK beta	>10,000
INSR	>10,000
BLK	10
BMX	<10
BTK	<10
TEK	>10,000
ITK	>10,000
JAK 1, 2	>10,000
JAK 3	1,000
KIT	>10,000
MAPK 1, 3, 8	>10,000
MET	>10,000
mTOR	>10,000
PDGFR alpha, beta	>1,000
CDK1/CyclinB	>10,000
AKT 1, 2, 3	>10,000

[1]This test was conducted by Invitrogen Profiling Service.
The IC_{50} was calculated from data at three different concentrations: 0.1, 1 and 10 μM ($n = 2$).

gel image was obtained using Typhoon Trio (GE Healthcare, Piscataway/NJ).

Xenograft mouse model

The SK-Ov3 human ovarian cancer cell line was obtained from ATCC (Manassas/VA). SK-Ov3 cells were maintained in McCoy's 5A containing 1% penicillin-streptomycin and 10% FBS in a humidified atmosphere containing 5% CO_2 at 37°C. In xenograft models, a total of 30 mg of mouse tumor fragments were implanted subcutaneously into the right flank of NU/NU Balb/C mice (female, body-weight range: 20 ± 5 g). Treatment was initiated approximately 7 days after implantation. Animals were randomized into treatment groups ($n = 8$) with similar mean tumor volumes. Distilled water containing 20% PEG400 and 3% Tween80 (Sigma Aldrich, St. Louis/MO) was used as vehicle in the *in vivo* studies. Different doses of HM781-36B, BIBW2992, erlotinib or vehicle alone were orally administered once daily for 10 days. Tumor volumes (mg) and body weights (g) were recorded twice a week from all groups using a Vernier caliper and balance.

For immunohistochemistry staining of HCC827 model, two tumor-bearing mice in the group of control and HM781-36B (0.3 mg/kg) group were killed 6 hr after the last dose and tumor sections were then stained with antibodies recognizing phospho-EGFR (Tyr1173), phospho-AKT (Ser473) and phospho-ERK1/2 (Tyr202/Tyr204) by HM781-36B (Cell Signaling, Danvers/MA). The samples were observed by using an optical microscope (Olympus, Tokyo/Japan).

Statistical analyses

The statistical analysis was performed with GraphPad Prism version 5.0 (GraphPad software, Inc., San Diego/CA). The significance of differences among multiple groups was assessed using an ANOVA. When the data sets failed the normality test, the Kruskal–Wallis one-way ANOVA on ranks was used. *Post hoc* pairwise comparison between groups was tested for significance using Dunnett's method.

Results

HM781-36B is a potent inhibitor of EGFR family kinases

HM781-36B, an irreversible and quinazoline-based pan-HER inhibitor, possesses a functional α,β-unsaturated carbonyl group as Michael acceptor moiety at the C_6 position that allows covalent modifications of the EGFR kinase domain active site in a manner similar to that with other irreversible EGFR inhibitors, such as EKB-569, HKI-272 and BIBW2992. We examined the specificity of HM781-36B by conducting *in vitro* assays against purified tyrosine kinases of the EGFR family (Table 1) and against a panel of other protein kinases (Table 2). HM781-36B effectively inhibited EGFR family kinases with IC_{50} values of 3.2, 5.3 and 23.5 nM against $EGFR^{wt}$, HER-2 and HER-4, respectively. In particular, HM781-36B showed excellent inhibitory activities against mutated EGFRs, including $EGFR^{T790M}$ and $EGFR^{L858R/T790M}$, both of which have been associated with acquired resistance to erlotinib and lapatinib. As shown in Table 1, the inhibitory efficacy of HM781-36B against $EGFR^{T790M}$ and $EGFR^{L858R/T790M}$, with IC_{50} values of 4.2 and 2.2 nM,

Table 3. Effect of HM781-36B, Erlotinib, Lapatinib and BIBW-2992 on cell proliferation

			GI_{50} (GI_{90}), nM[1]			
Tissue	Cell line	Characterization	HM781–36B	Erlotinib	Lapatinib	BIBW 2992
Lung	HCC827	EGFR (Del E746_A750)	1.2 (4.0)	2.4	271.4	1.2 (4.6)
	NCI-H358	EGFR (WT)	4.8 (38.7)	286.7	420.7	37.8 (286)
	NCI-H1975	EGFR (L858R T790M)	5.7 (12.5)	>1,000	>1,000	42.0 (261)
	Calu-3	HER-2 (WT, amplified)	2.1 (5.3)	377.5	27.8	7.3 (37.7)
	NCI-H1781	HER-2 (G776V, Cins)	4.0	3,246	3,402	60.0
	A549	*K-ras* mutation (G12S)	>1,000	>10,000	>10,000	>1,000
Epidermis	A431	EGFR (WT, amplified)	0.9 (8.4)	95.6	104.2	4.1 (41.8)
Breast	SK-Br3	HER-2 (WT, amplified)	1.0 (4.4)	>1,000	28.8	2.8 (8.2)
	BT-474	HER-2 (WT, amplified)	1.3 (4.6)	>1,000	30.6	7.5 (43.8)
	MDA-175	HER-2 (WT)	1.8 (5.0)	>1,000	852	15.3 (139)
Gastric	NCI-N87	HER-2 (WT, amplified)	0.6 (3.3)	>1,000	30.8	2.8 (7.2)
Normal cell	Hs-27	Human fibroblast	3,830	>10,000	>10,000	2,835
	Balb/c3T3	Mouse fibroblast	2,409	>10,000	>10,000	2,105

[1]All biological data are mean values for three independent experiments performed in duplicate. The GI_{50} (GI_{90}) was calculated from data at five different concentrations.
Abbreviation: WT, wild-type.

respectively, is far above that of other reported EGFR inhibitors, such as erlotinib, lapatinib and BIBW2992. HM781-36B also showed excellent selectivity in *in vitro* assays with other kinases with greater than 100- to 1,000-fold IC_{50} values compared with EGFR family members, except for TEC family kinases, including BLK, BMX and BTK. In fact, recent studies reported that TEC family kinases are also target proteins for anticancer therapy and inhibited by other irreversible EGFR inhibitors.[39,40] The beneficial effects of HM781-36B by the inhibition of TEC family kinases are currently under evaluation.

HM781-36B has potent growth inhibitory activity against EGFR-dependent cancer cell lines, including erlotinib-resistant NSCLC cell lines

The *in vitro* activity of HM781-36B was assessed by performing a side-by-side comparison of HM781-36B with other EGFR inhibitors in a variety of EGFR-dependent cancer cell lines, including erlotinib-sensitive (*EGFR* DelE746_A750), moderately sensitive (*EGFR* WT) and erlotinib-resistant (*EGFR* L858R/T790M) NSCLC cell lines (HCC827, NCI-H358 and NCI-H1975), HER-2 (G776V, Cins)[41] and *K-Ras* mutated (G12S) NSCLC cell lines (NCI-H1781 and A549), HER-2 dependent NSCLC (Calu-3), breast cancer (SK-Br3, BT-474 and MDA-175)[42] and gastric cancer (NCI-N87) cell lines and the *EGFR* WT-amplified epithelial cancer cell line (A-431). The results revealed that HM781-36B strongly suppressed the growth of cancer cells with GI_{50} values of 0.6–5.7 nM, which are far superior to other EGFR inhibitors except for *K-Ras* mutated A549 NSCLC cell line (Table 3). In particular, the GI_{50} values of HM781-36B for NCI-H1975, a NSCLC cell line that harbors the *EGFR* L858R/T790M double mutation, and NCI-H1781, a NSCLC cell line that harbors the HER-2 somatic mutation (in-frame insertion in exon 20), were 5.7 nM and 4.0 nM, which is 7.4- and 15-fold lower than the GI_{50} of BIBW2992. The cellular growth inhibitory activity in NCI-H1975 clearly differentiates HM781-36B from BIBW2992 in the aspect of GI_{90} value (21-fold higher potency: 12.5 nM *vs.* 261 nM), compared to its 7.4-fold higher potency over BIBW2992 in GI_{50} value. On the basis of these data, we are confident that HM781-36B can overcome the current limitation of irreversible EGFR inhibitors in clinical trials to tackle the acquired T790M mutation in NSCLC patients due to their insufficient levels of exposure in human plasma for effective blockage of cancer.[19] We also investigated the cellular effects of HM781-36B on normal cells, such as Hs-27 and Balb/c3T3, and observed that HM781-36B has excellent selectivity toward cancer cells over normal cells in its cell growth inhibitory activity with 1,000-fold higher GI_{50} values against normal cell lines.

HM781-36B inhibits EGFR phosphorylation and its downstream signaling proteins, which leads to apoptosis in cells with the EGFR DelE746_A750 or EGFR L858R/T790M mutations

We showed that HCC827 cells with the *EGFR* DelE746_A750 mutation are sensitive to erlotinib and NCI-H1975 cells harboring *EGFR* L858R/T790M mutations are resistant to erlotinib. Yet, HM781-36B effectively inhibits the proliferation of both clinically relevant cancer cell lines (Table 3). As shown in Figure 1, the growth inhibitory activity of HM781-36B against HCC827 and NCI-H1975 cells is correlated with the dose-dependent inhibition of EGFR phosphorylation as well as the phosporylation level of HER-2, AKT and ERK.

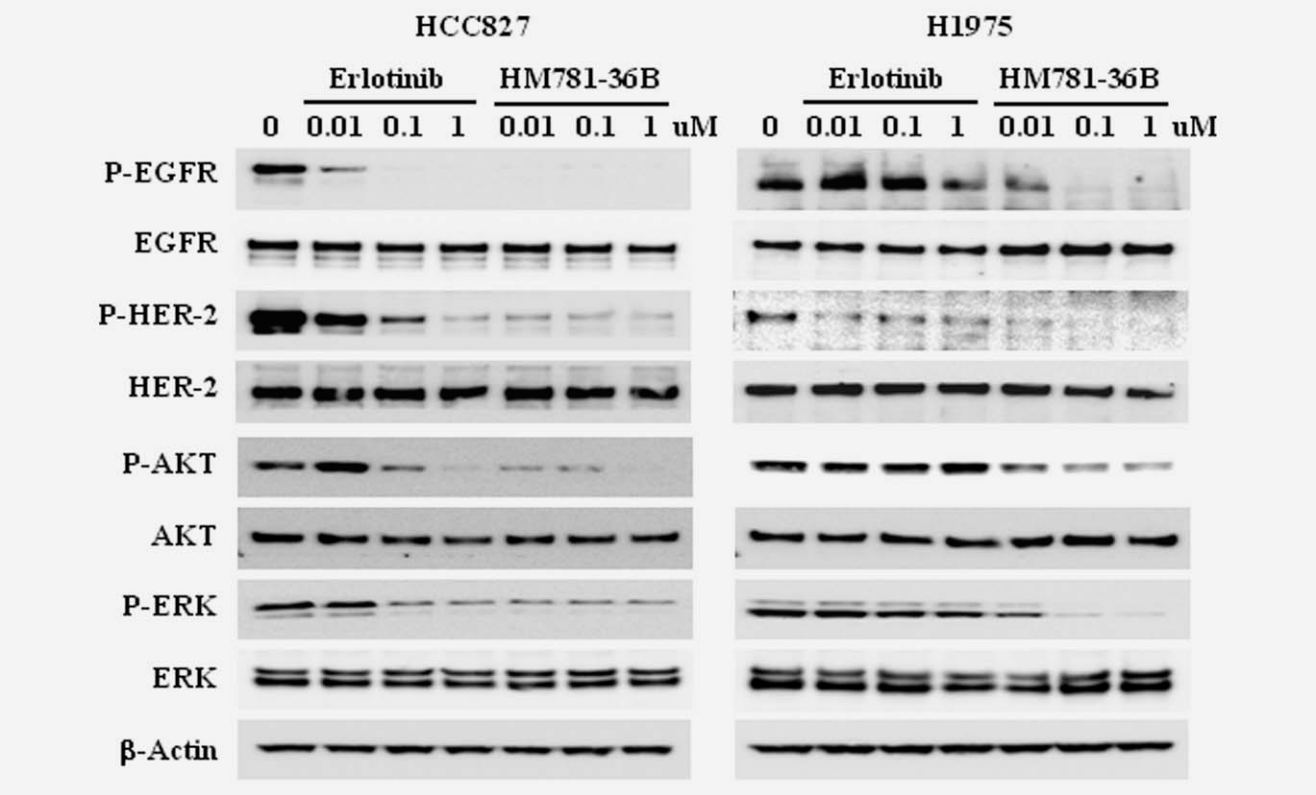

Figure 1. HM781-36B inhibits EGFR signaling in the *EGFR* Del E746_A750-containing HCC827 and *EGFR* L858R/T790M-containing NCI-H1975 cells. Cells were treated with increasing concentrations (0.01, 0.1, 1 μM) of erlotinib or HM781-36B for 24 hr. Cells were lysed and proteins were analyzed by Western Blotting with the indicated antibodies. Phosphorylations of EGFR/HER-2 and its down-stream AKT/ERK pathways were inhibited by both erlotinib and HM781-36B in erlotinib-sensitive HCC827 cells; however, in erlotinib-resistant NCI-H1975 cells, EGFR signaling was inhibited only by treatment of HM781-36B.

However, erlotinib, an EGFR-specific reversible inhibitor, showed no inhibition of EGFR phosphorylation as well as other related proteins at the highest treated concentration in NCI-H1975 cells with *EGFR* L858R/T790M mutations. In addition, we observed the drastic deterioration in the phosphorylation level of these proteins on the dose-dependent treatment of erlotinib in the case of HCC827 cells with the *EGFR* DelE746_A750 mutation (Fig. 1).

To investigate whether HM781-36B can induce the cell cycle arrest, the DNA contents of EGFR-dependent NSCLC cell lines (erlotinib-sensitive HCC827 and erlotinib-resistant NCI-H1975) were analyzed by staining cells with the DNA-intercalating dye, propidium iodide (PI), and analyzing them using FACS after treatment of either HM781-36B (0.01, 0.1 and 1 μM) or erlotinib (0.1, 1 and 5 μM) at different concentrations for 48 hr (Figs. S1*a* and S1*b*). Both NSCLC cell lines (HCC827 and NCI-H1975) showed a slight increase in the number of cells in G_0/G_1 phase and a slight decrease in the number of cells in the S phase upon the treatment of HM781-36B. The apoptotic cell death monitored by using FACS with annexin V confirmed that HM781-36B is effective against both HCC827 and NCI-H1975 cell lines in a dose-dependent fashion, which was not observed in the case of NCI-H1975 cells on treatment of erlotinib (Figs. S2*a* and S2*b*).

HM781-36B irreversibly binds to $EGFR^{WT}$, HER-2 and $EGFR^{T790M}$

Irreversible modification of HM781-36B to specific cysteine residues within the active sites of EGFR family tyrosine kinase domains (Cys773 of EGFR and Cys805 of HER-2) is expected *via* the covalent immobilization with the Michael acceptor moiety of HM781-36B. To validate this hypothesis, the phosphorylation levels of EGFR tyrosine kinases (EGFR and HER-2) in EGFR-overexpressing A431 and HER-2-overexpressing SK-Br3 cells was measured immediately after or 8 or 24 hr after removal of HM781-36B and two classes of other EGFR inhibitors (erlotinib as a reversible inhibitor and BIBW2992 as an irreversible inhibitor) from the medium. As shown in Figures 2*a* and 2*b*, we observed the complete disappearance of the erlotinib-induced inhibitory effect on the phosphorylation of EGFR and HER-2 in both A431 and SK-Br3 cells within 8 hr after media washing. However, HM781-36B and BIBW2992, both irreversible inhibitors, continued to block the phosphorylation of receptor kinases up to 24 hr after withdrawal of drugs (HM781-36B and

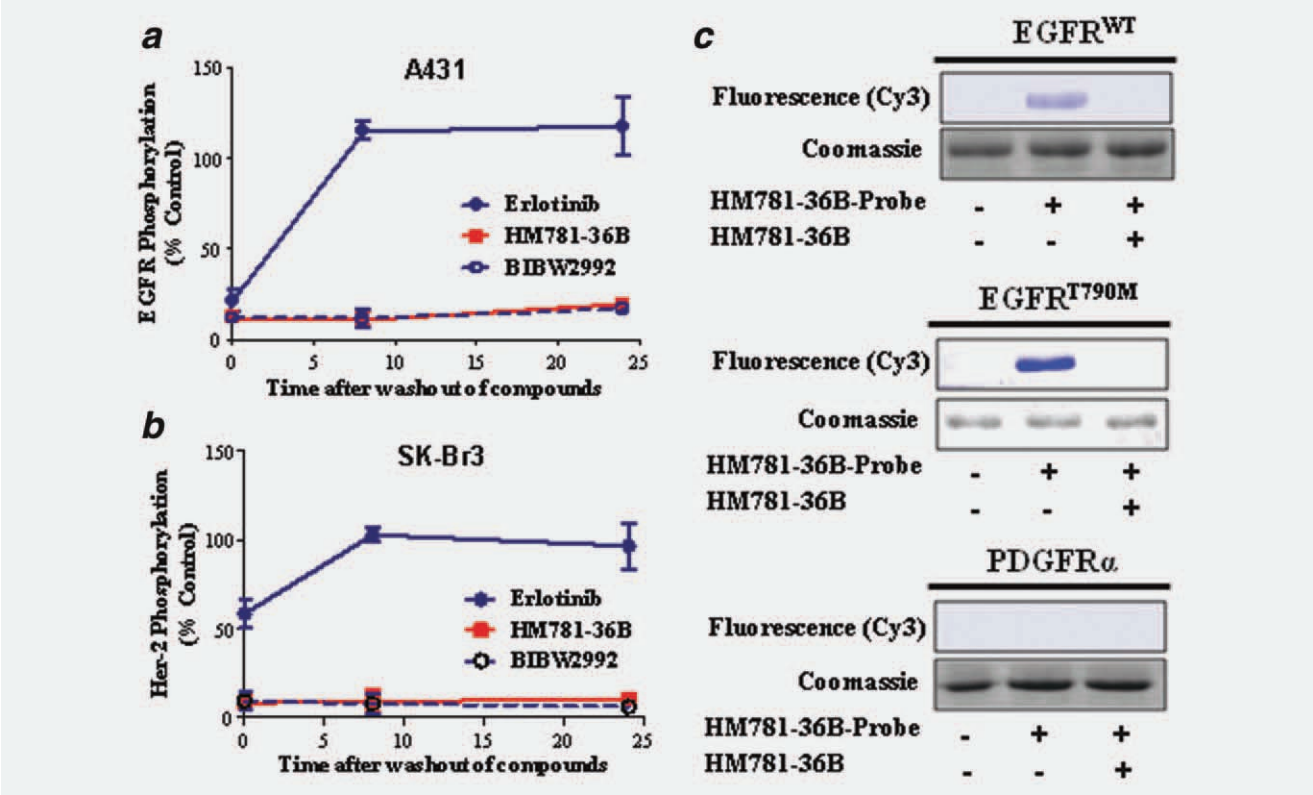

Figure 2. HM781-36B binds irreversibly to tyrosine kinases of $EGFR^{WT}$, $EGFR^{T790M}$ and HER-2. (*a*,*b*) Inhibition of EGFR or HER-2 phosphorylation was prolonged up to 24 hr in $EGFR^{WT}$-overexpressing A431 (*a*) and HER-2-overexpressing SK-Br3 (*b*) cells after removal of 1 μM of HM781-36B and BIBW2992, but not in the case of erlotinib. The phosphorylation inhibition levels of EGFR or HER-2 were measured by ELISA. (*c*) Covalent immobilization at the EGFR kinase domains by HM781-36B was confirmed by the analysis of fluorescence-based SDS-PAGE through the incubation of Cy3-labeled HM781-36B with $EGFR^{WT}$, $EGFR^{T790M}$ and PDGFRα.

BIBW2992) in both A431 and SK-Br3 cells. We also confirmed the covalent immobilization of HM781-36B at the active sites of EGFR tyrosine kinase domains with $EGFR^{WT}$ and $EGFR^{T790M}$ using a Cy3-labeled HM781-36B probe (Supporting Information Table S1). After incubation of a Cy3-labeled HM781-36B probe with purified $EGFR^{WT}$, $EGFR^{T790M}$ and PDGFRα, these kinases were denatured and analyzed by SDS-PAGE. As shown in Figure 2*c*, we clearly observed a fluorescent band associated with covalently immobilized Cy3-labeled HM781-36B probe within the kinase domain of $EGFR^{WT}$ and $EGFR^{T790M}$ (M_r ~87,000), which was effectively blocked in the presence of a fivefold excess of HM781-36B through the competition. However, we did not observe any immobilization of Cy3-labeled HM781-36B with PDGFRα used as a negative control, which confirms the specificity of the immobilization event of HM781-36B.

HM781-36B shows excellent anti-tumor activity in EGFR-dependent xenograft models

The *in vivo* activity of HM781-36B was assessed in xenograft mice models with various EGFR-dependent cancer cell lines through a direct comparison with BIBW2992, an irreversible EGFR/HER-2 inhibitor and erlotinib, an EGFR selective inhibitor.[29,43] To evaluate the *in vivo* activity of HM781-36B, we generated a standard xenograft model with NSCLC cell line HCC827 having the erlotinib-sensitive *EGFR* DelE746_A750 mutation. Daily oral treatments of HM781-36B at 0.3 mg/kg/day or 1 mg/kg/day for 10 days resulted in a dramatic reduction of tumor size with an 83% maximum inhibition rate (mIR, IR = [1 − (relative tumor growth in treated group/relative tumor growth in control group)] × 100) at 0.3 mg/kg/day without body-weight loss. As shown in Figure 3*a*, the antitumor efficacy upon treatment with HM781-36B (1 mg/kg/day, mIR 89%, $p < 0.01$; Kruskal–Wallis test) was comparable to that with 10 mg/kg/day of BIBW2992 (mIR 93%, $p < 0.001$; Kruskal–Wallis test) and 100 mg/kg/day of erlotinib (mIR 91%, $p < 0.001$; Kruskal–Wallis test). We also evaluated the effect of HM781-36B on HCC827 tumor endothelium by immunohistochemistry and observed the significant reduction in the expression level of pEGFR, pAKT and pERK upon treatment with HM781-36B (0.3 mg/kg/day) for 10 days (Fig. 3*b*). In this dose schedule with statistically significant inhibition of tumor growth, the anti-tumor efficacy of HM781-36B was studied in mice models xenografted with NCI-H1975 NSCLC cell line harboring erlotinib-resistant $EGFR^{L858R/T790M}$ and with Calu-3 NSCLC

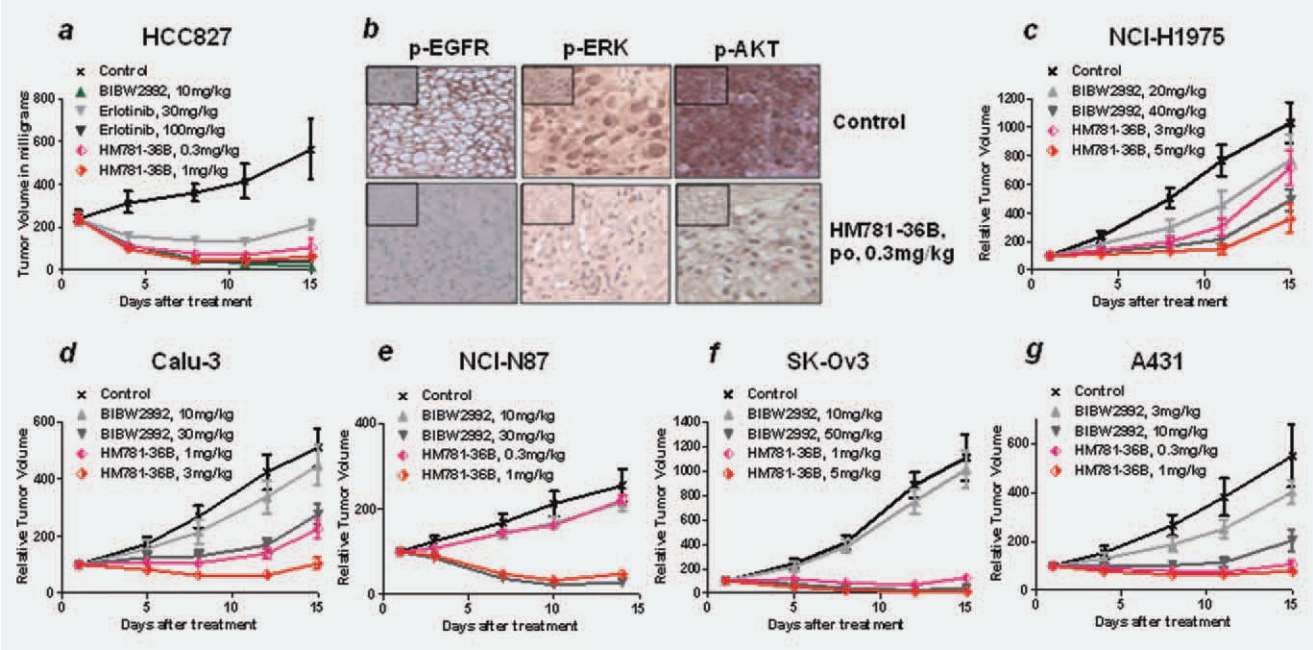

Figure 3. *In vivo* efficacy of HM781-36B in xenograft models. (*a*) Nude mice ($n = 8$) carrying tumors established from HCC827 cells were orally treated once-a-day for 10 days with HM781-36B, BIBW2992, and erlotinib at indicated doses. (*b*) Immunohistochemical analysis of HCC827 tumor sections obtained 6 hr after the last dose confirms the *in vivo* modulation on the phosphorylation levels of EGFR, AKT and ERK upon treatment with HM781-36B (0.3 mg/kg/day). (*c–g*) Nude mice ($n = 8$) carrying tumors established from NCI-H1975 cells (*c*), Calu-3 cells (*d*), NCI-N87 cells (*e*), SK-Ov3 cells (*f*) and A431 cells (*g*) were orally treated once-a-day for 10 days with HM781-36B and BIBW2992 at indicated doses.

cell line harboring a high level of HER-2 expression (Figs. 3*c* and 3*d*, respectively). HM781-36B showed the strong *in vivo* efficacy, with 80.8% mIR at 5 mg/kg/day for 10 days ($p < 0.001$; Kruskal–Wallis test) in the NCI-H1975 xenograft model and with 67.4% mIR at 1 mg/kg/day for 10 days ($p < 0.05$; Kruskal–Wallis test) in the Calu-3 xenograft model. In fact, the comparable *in vivo* efficacy was achieved with BIBW2992 at much higher doses; 40 mg/kg/day and 30 mg/kg/day in the NCI-H1975 and the Calu-3 xenograft model, respectively ($p < 0.05$; Kruskal–Wallis test).

HM781-36B was also effective in different kinds of cancers with a high level of HER-2 expression, including NCI-N87, a human gastric cancer cell line, and SK-Ov3, a human ovarian cancer cell line (Figs. 3*e* and 3*f*). HM781-36B effectively induced tumor regression with 84.4% mIR for 10 days at 1 mg/kg/day ($p < 0.001$; Kruskal–Wallis test) in the NCI-N87 model and 92.3% mIR at 1 mg/kg/day for 10 days ($p < 0.05$; Kruskal–Wallis test) in the SK-Ov3 model. Again, the comparable *in vivo* efficacy was achieved with BIBW2992 at much higher doses; 89.6% mIR at 30 mg/kg/day ($p < 0.01$; Kruskal–Wallis test) in the NCI-N87 model and 97.1% mIR at 50 mg/kg/day ($p < 0.05$; Kruskal–Wallis test) in the SK-Ov3 model. We also confirmed the excellent *in vivo* efficacy in the mice xenograft model of A431 epidermoid carcinoma cell line with a high level of wild-type EGFR expression through the observation of a dramatic reduction in tumor size with an 80.7% mIR upon treatment of HM781-36B at 0.3 mg/kg/day ($p < 0.001$; Kruskal–Wallis test) without body-weight loss (Fig. 3*g*).

Discussion

The overexpression of EGFR and HER-2 is observed in many human cancers, including lung, breast and gastric cancers, and a strong correlation has been found between solid tumors with high levels of EGFR/HER-2 and poor prognosis.[44–46] We developed HM781-36B as a very potent pan-HER inhibitor and demonstrated that HM781-36B is effective against the erlotinib-resistant *EGFR* T790M and *EGFR* L858R/T790M double mutant along with the inhibition of wild-type EGFR, HER-2 and HER-4 (Table 1). On the basis of a kinase panel assay, we also confirmed the excellent selectivity of HM781-36B toward EGFR family kinases with the exception of TEC family kinases (BLK, BMX and BTK, Table 2), which have been recognized as attractive targets of anticancer therapy and subjected for the study on additional effects upon their inhibition.

HM781-36B dramatically inhibits the cellular proliferation in a panel of cell lines with various expression levels of EGFR or HER-2 as well as disease-relevant mutations (Table 3). HM781-36B showed strong growth inhibitory activities in various cancer cells, including the erlotinib-sensitive small in-frame deletion in exon 19 (HCC827), erlotinib-resistant *EGFR* T790M/L858R double mutant (NCI-H1975), erlotinib-resistant *HER-2* G776insV_G/C mutant (NCI-H1781), *EGFR*

WT expressing cancer cells (NCI-H358 and A431), HER-2 overexpressing NSCLC cells (Calu-3), breast cancer cells (SK-Br3 and BT-474) and gastric cancer cells (NCI-N87). But HM781-36B did not induce growth inhibitory activity in *K-ras* mutated A549 NSCLC cells and normal cells (GI_{50} >1,000 nM). The *in vitro* cellular activities of HM781-36B were compared side-by-side with those of erotinib, lapatinib and BIBW2992. As shown in Table 3, HM781-36B has superior growth inhibitory activity to other leading pan-HER inhibitor, BIBW2992, considering GI_{50} and GI_{90} values.

Upon treatment with HM781-36B, the phosphorylation of EGFR and HER-2 was effectively blocked in HCC827 and NCI-H1975 cells along with the inhibition of ERK and AKT phosphorylation in the downstream signaling pathway (Fig. 1). The target enzyme is covalently modified with the Michael acceptor moiety of HM781-36B, which leads to the prolonged inhibition of EGFR and HER-2 phosphorylation after the removal of HM781-36B from media in EGFR-overexpressing A431 cells and HER-2-overexpressing SK-Br3 cells (Figs. 2*a* and 2*b*). This covalent immobilization at the kinase domains of EGFR was confirmed through direct fluorescence-based SDS-PAGE analysis by the incubation of Cy3-labeled HM781-36B probe with $EGFR^{WT}$ and $EGFR^{T790M}$ (Fig. 2*c*). This irreversible occupation at the kinase domain allows the enhanced efficacy of HM781-36B toward various EGFR-dependent cancers *via* the effective competition with high concentrations of cellular ATP and the extended inhibition of kinase activity for the enough time frames needed to affect the tumor growth.

Finally, we have shown that the treatment of HM781-36B induces excellent *in vivo* anti-tumor activity in xenograft models with various EGFR-dependent cancer cell lines. As shown in Figure 3, the daily oral treatment with HM781-36B resulted in a dramatic reduction of tumor size with much lower dose than a leading irreversible EGFR/HER-2 inhibitor, BIBW2992. The effective dose of HM781-36B for a significant inhibition of tumor growth is approximately 0.3–5 mg/kg/day and well tolerated by xenograft mice. The treatment of BIBW2992 or HM781-36B induced common side effects of EGFR inhibitors such as skin rash or diarrhea caused by the inhibition of *EGFR* WT in xenograft mice. But these symptoms have been recovered after the cessation of administration.

For 50% of clinically resistant patients to EGFR inhibitors mainly caused by an acquired mutation (T790M), some irreversible EGFR/HER-2 inhibitors were evaluated in clinic. Although induced good antitumor efficacy in animal models, they showed limited efficacy in humans caused by clinically unachievable plasma concentrations for the sufficient anticancer activity.[19] By this time, there are no approved therapies for the patients with *EGFR* T790M. Currently, phase I clinical trial of HM781-36B is underway in South Korea. In phase I study of HM781-36B, the MTD (maximum tolerated dose) was not reached, but HM781-36B already exceeded the clinically achievable plasma concentrations by single administration of BIBW2992 70mg (C_{max} = 76 ng/mL), which is the recommended dose for studies with BIBW2992 for 14 days followed by 14 days off medication (data not shown).[47,48] Therefore, we anticipate that HM781-36B will be effective for NSCLC patients with *EGFR* T790M, since HM781-36B showed excellent antitumor activity with much low dose in *in vitro* and *in vivo* models.

In conclusion, we showed that HM781-36B is a promising "best-in-class" anticancer agent that acts through the irreversible inhibition of EGFR family tyrosine kinases for the treatment of cancers such as NSCLC, breast cancer and gastric cancer. In particular, HM781-36B can address an urgent, unmet medical need for the treatment of NSCLC patients with an acquired erlotinib-resistant *EGFR* T790M mutation. Phase I clinical trial of HM781-36B is currently underway and the outcome of this study will be reported in due course.

Acknowledgements

J. Park is grateful for the fellowships awarded by the BK21 Program.

References

1. Schlessinger J. Cell signaling by receptor tyrosine kinases. *Cell* 2000;103:211–25.
2. Hynes NE, Lane HA. ERBB receptors and cancer: the complexity of targeted inhibitors. *Nat Rev Cancer* 2005;5:341–54.
3. Yarden Y, Sliwkowski MX. Untangling the ErbB signaling network. *Nat Rev Mol Cell Biol* 2001;2:127–37.
4. Tang X, Shigematsu H, Bekele BN, Roth JA, Minna JD, Hong WK, Gazdar AF, Wistuba II. EGFR tyrosine kinase domain mutations are detected in histologically normal respiratory epithelium in lung cancer patients. *Cancer Res* 2005;65:7568–72.
5. Herbst RS, Langer CJ. Epidermal growth factor receptors as a target for cancer treatment: the emerging role of IMC-C225 in the treatment of lung and head and neck cancers. *Semin Oncol* 2002;29:27–36.
6. Traxler P, Bold G, Buchdunger E, Caravatti G, Furet P, Manley P, O'Reilly T, Wood J, Zimmermann J. Tyrosine kinase inhibitors: from rational design to clinical trials. *Med Res Rev* 2001;21:499–512.
7. Adjei AA. Epidermal growth factor receptor tyrosine kinase inhibitors in cancer therapy. *Drugs Future* 2001;26:1087–92.
8. Renhowe PA. Inhibitors of growth factor receptor kinase-dependent signaling pathways in anticancer chemotherapy-clinical progress. *Curr Opin Drug Discov Dev* 2002;5:214–24.
9. Ranson M, Hammond LA, Ferry D, Kris M, Tullo A, Murray PI, Miller V, Averbuch S, Ochs J, Morris C, Feyereislova A, Swaisland H, et al. ZD1839, a selective oral epidermal growth factor receptor-tyrosine kinase inhibitor, is well tolerated and active in patients with solid, malignant tumors: results of a phase I trial. *J Clin Oncol* 2002;20:2240–50.
10. Ciardiello F, Tortora G. A novel approach in the treatment of cancer: targeting the epidermal growth factor receptor. *Clin Cancer Res* 2001;7:2958–70.
11. Mok TS, Wu YL, Thongprasert S, Yang CH, Chu DT, Saijo N, Sunpaweravong P, Han B, Margono B, Ichinose Y, Nishiwaki Y, Ohe Y, et al. Gefitinib or carboplatin-

paclitaxel in pulmonary adenocarcinoma. *N Engl J Med* 2009;361:947–57.
12. Rosell R, Moran T, Queralt C, Porta R, Cardenal F, Camps C, Majem M, Lopez-Vivanco G, Isla D, Provencio M, Insa A, Massuti B, et al. Screening for epidermal growth factor receptor mutations in lung cancer. *N Engl J Med* 2009;361:958–67.
13. Yun CH, Boggon TJ, Li Y, Woo MS, Greulich H, Meyerson M, Eck MJ. Structures of lung cancer-derived EGFR mutants and inhibitor complexes: mechanism of activation and insights into differential inhibitor sensitivity. *Cancer Cell* 2007;11:217–27.
14. Maheswaran S, Sequist LV, Nagrath S, Ulkus L, Brannigan B, Collura CV, Inserra E, Diederichs S, Iafrate AJ, Bell DW, Digumarthy S, Muzikansky A, et al. Detection of mutations in *EGFR* in circulating lung-cancer cells. *N Engl J Med* 2008;359:366–77.
15. Pao W, Miller VA, Politi KA, Riely GJ, Somwar R, Zakowski MF, Kris MG, Varmus H. Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. *PLoS Med* 2005;2:1–11.
16. Kobayashi S, Boggon TJ, Dayaram T, Jänne PA, Kocher O, Meyerson M, Johnson BE, Eck MJ, Tenen DG, Halmos B. *EGFR* mutation and resistance of non-small-cell lung cancer to gefitinib. *N Engl J Med* 2005;352:786–92.
17. Yun CH, Mengwasser KE, Toms AV, Woo MS, Greulich H, Wong KK, Meyerson M, Eck MJ. The T790M mutation in EGFR kinase causes drug resistance by increasing the affinity for ATP. *Proc Natl Acad Sci USA* 2008;105:2070–5.
18. Balius TE, Rizzo RC. Quantitative prediction of fold resistance for inhibitors of EGFR. *Biochemistry* 2009;48:8435–48.
19. Sos ML, Rode HB, Heynck S, Peifer M, Fischer F, Klüter S, Pawar VG, Reuter C, Heuckmann JM, Weiss J, Ruddigkeit L, Rabiller M, et al. Chemogenomic profiling provides insights into the limited activity of irreversible EGFR inhibitors in tumor cells expressing the T790M EGFR resistance mutation. *Cancer Res* 2010;70:868–74.
20. Xia W, Mullin RJ, Keith BR, Liu LH, Ma H, Rusnak DW, Owens G, Alligood KJ, Spector NL. Anti-tumor activity of GW572016: a dual tyrosine kinase inhibitor blocks EGF activation of EGFR/erbB2 and downstream Erk1/2 and AKT pathways. *Oncogene* 2002;21:6255–63.
21. Tanizaki J, Okamoto I, Takezawa K, Tsukioka S, Uchida J, Kiniwa M, Fukuoka M, Nakagawa K. Synergistic antitumor effect of S-1 and HER2-targeting agents in gastric cancer with HER2 amplification. *Mol Cancer Ther* 2010;9:1198–207.
22. Johnston SR, Leary A. Lapatinib: a novel EGFR/HER2 tyrosine kinase inhibitor for cancer. *Drugs Today* 2006;42:441–53.
23. Tsou HR, Overbeek-Klumpers EG, Hallett WA, Reich MF, Floyd MB, Johnson BD, Michalak RS, Nilakantan R, Discafani C, Golas J, Rabindran SK, Shen R, et al. Optimization of 6,7-disubstituted-4-(arylamino)quinoline-3-carbonitriles as orally active, irreversible inhibitors of human epidermal growth factor receptor-2 kinase activity. *J Med Chem* 2005;48:1107–31.
24. Rabindran SK, Discafani CM, Rosfjord EC, Baxter M, Floyd MB, Golas J, Hallett WA, Johnson BD, Nilakantan R, Overbeek E, Reich MF, Shen R, et al. Antitumor activity of HKI-272, an orally active, irreversible inhibitor of the HER-2 tyrosine kinase. *Cancer Res* 2004;64:3958–65.
25. Wissner A, Overbeek E, Reich MF, Floyd MB, Johnson BD, Mamuya N, Rosfjord EC, Discafani C, Davis R, Shi X, Rabindran SK, Gruber BC, et al. Synthesis and structure-activity relationships of 6,7-disubstituted 4-anilinoquinoline-3-carbonitriles: the design of an orally active, irreversible inhibitor of the tyrosine kinase activity of the epidermal growth factor receptor (EGFR) and the human epidermal growth factor receptor-2 (HER-2). *J Med Chem* 2003;46:49–63.
26. Smaill JB, Showalter HD, Zhou H, Bridges AJ, McNamara DJ, Fry DW, Nelson JM, Sherwood V, Vincent PW, Roberts BJ, Elliott WL, Denny WA. Tyrosine kinase inhibitors. 18. 6-Substituted 4-anilinoquinazolines and 4-anilinopyrido[3,4-*d*]pyrimidines as soluble, irreversible inhibitors of the epidermal growth factor receptor. *J Med Chem* 2001;44:429–40.
27. Campos S, Hamid O, Seiden MV, Oza A, Plante M, Potkul RK, Lenehan PF, Kaldjian EP, Varterasian ML, Jordan C, Charbonneau C, Hirte H. Multicenter, randomized phase II trial of oral CI-1033 for previously treated advanced ovarian cancer. *J Clin Oncol* 2005;23:5597–604.
28. Engelman JA, Zejnullahu K, Gale CM, Lifshits E, Gonzales AJ, Shimamura T, Zhao F, Vincent PW, Naumov GN, Bradner JE, Althaus IW, Gandhi L, et al. PF00299804, an irreversible pan-ERBB inhibitor, is effective in lung cancer models with EGFR and ERBB2 mutations that are resistant to gefitinib. *Cancer Res* 2007;67:11924–32.
29. Li D, Ambrogio L, Shimamura T, Kubo S, Takahashi M, Chirieac LR, Padera RF, Shapiro GI, Baum A, Himmelsbach F, Rettig WJ, Meyerson M, et al. BIBW2992, an irreversible EGFR/HER2 inhibitor highly effective in preclinical lung cancer models. *Oncogene* 2008;27:4702–11.
30. Paez JG, Jänne PA, Lee JC, Tracy S, Greulich H, Gabriel S, Herman P, Kaye FJ, Lindeman N, Boggon TJ, Naoki K, Sasaki H, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* 2004;304:1497–500.
31. Kobayashi S, Ji H, Yuza Y, Meyerson M, Wong KK, Tenen DG, Halmos B. An alternative inhibitor overcomes resistance caused by a mutation of the epidermal growth factor receptor. *Cancer Res* 2005;65:7096–101.
32. Kwak EL, Sordella R, Bell DW, Godin-Heymann N, Okimoto RA, Brannigan BW, Harris PL, Driscoll DR, Fidias P, Lynch TJ, Rabindran SK, McGinnis JP, et al. Irreversible inhibitors of the EGF receptor may circumvent acquired resistance to gefitinib. *Proc Natl Acad Sci USA* 2005;102:7665–70.
33. Nam HJ, Kim HP, Yoon YK, Hur HS, Song SH, Kim MS, Lee GS, Han SW, Im SA, Kim TY, Oh DY, Bang YJ. Antitumor activity of HM781-36B, an irreversible Pan-HER inhibitor, alone or in combination with cytotoxic chemotherapeutic agents in gastric cancer. *Cancer Lett* 2011;302:155–65.
34. Lee K-O, Cha MY, Kim M, Jung YH, Lee CG, Kim SY, Bang K, Park BW, Choi BI, Chae YJ, Ko MY, Kim HK, et al.; Hanmi Pharm. Co. Ltd., Novel amide derivative for inhibiting the growth of cancer cells. PCT patent WO 150,118, 2008.
35. Schnur SC, Arnold LD; Pfizer Inc. Quinazoline derivatives. PCT patent WO 9,630,347, 1996.
36. Carter MC, Cockerill GS, Guntrip SB, Lackey KE, Smith KJ; Glaxo Group Limited. Bicyclic heteroaromatic compounds as protein tyrosine kinase inhibitors. PCT patent WO 9,935,146, 1999.
37. Himmelsbach F, Langkopf E, Blech S, Jung B, Baum E, Solca F; Boehringer Ingelheim Pharma KG. Quinazoline derivatives, medicaments containing said compounds, their utilization and method for the production thereof. PCT patent WO 00,250,043, 2002.
38. Cha MY, Lee KO, Kim JW, Lee CG, Song JY, Kim YH, Lee GS, Park SB, Kim MS. Discovery of a novel Her-1/Her-2 dual tyrosine kinase inhibitor for the treatment of Her-1 selective inhibitor-resistant non-small cell lung cancer. *J Med Chem* 2009;52:6880–8.
39. Hur W, Velentza A, Kim S, Flatauer L, Jiang X, Valente D, Mason DE, Suzuki M, Larson B, Zhang J, Zagorska A, Didonato M, et al. Clinical stage EGFR inhibitors irreversibly alkylate Bmx kinase. *Bioorg Med Chem Lett* 2008;18:5916–19.

40. Zhou W, Ercan D, Chen L, Yun CH, Li D, Capelletti M, Cortot AB, Chirieac L, Iacob RE, Padera R, Engen JR, Wong KK, et al. Novel mutant-selective EGFR kinase inhibitors against EGFR T790M. *Nature* 2009;462:1070–4.
41. Shigematsu H, Takahashi T, Nomura M, Majmudar K, Suzuki M, Lee H, Wistuba II, Fong KM, Toyooka S, Shimizu N, Fujisawa T, Minna JD, et al. Somatic mutations of the HER2 kinase domain in lung adenocarcinomas. *Cancer Res* 2005;65: 1642–6.
42. Lewis GD, Figari I, Fendly B, Wong WL, Carter P, Gorman C, Shepard HM. Differential responses of human tumor cell lines to anti-p185HER2 monoclonal antibodies. *Cancer Immunol Immunother* 1993;37:255–63.
43. Naumov GN, Nilsson MB, Cascone T, Briggs A, Straume O, Akslen LA, Lifshits E, Byers LA, Xu L, Wu HK, Jänne P, Kobayashi S, et al. Combined vascular endothelial growth factor receptor and epidermal growth factor receptor (EGFR) blockade inhibits tumor growth in xenograft models of EGFR inhibitor resistance. *Clin Cancer Res* 2009;15: 3484–94.
44. Konecny GE, Pegram MD, Venkatesan N, Finn R, Yang G, Rahmeh M, Untch M, Rusnak DW, Spehar G, Mullin RJ, Keith BR, Gilmer TM, et al. Activity of the dual kinase inhibitor lapatinib (GW572016) against HER-2-overexpressing and trastuzumab-treated breast cancer cells. *Cancer Res* 2006;66:1630–9.
45. Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, Levin WJ, Stuart SG, Udove J, Ullrich A, Press MF. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* 1989;244:707–12.
46. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/ neu oncogene. *Science* 1987;235:177–82.
47. Eskens FA, Mom CH, Planting AS, Gietema JA, Amelsberg A, Huisman H, van Doorn L, Burger H, Stopfer P, Verweij J, de Vries EG. A phase I dose escalation study of BIBW 2992, an irreversible dual inhibitor of epidermal growth factor receptor 1 (EGFR) and 2 (HER2) tyrosine kinase in a 2-week on, 2-week off schedule in patients with advanced solid tumours. *Br J Cancer* 2008; 98:80–5.
48. Stopfer P, Schaefer HG, Amelsberg A, Huisman H, Eskens FA, Gietema JA, Briscoe J, Lewis N, Cohen RB, Marshall J, Verweij J. Pharmacokinetic results from two phase I dose escalation studies of once daily oral treatment with BIBW2992, an irreversible dual EGFR/HER2 receptor tyrosine kinase inhibitor, in patients with advanced solid tumors. In: AACR-NCI-EORTC International Conference on "Molecular Targets and Cancer Therapeutics", Philadelphia, PA, USA, November, 2005, no. B172.