AXL Is a Potential Target for Therapeutic Intervention in Breast Cancer Progression

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Abstract

Protein kinases play important roles in tumor development and progression. A variety of members of this family of signal transduction enzymes serve as targets for therapeutic intervention in cancer. We have identified the receptor tyrosine kinase (RTK) AXL as a potential mediator of motility and invasivity of breast cancer cells. AXL is expressed in most highly invasive breast cancer cells, but not in breast cancer cells of low invasivity. Ectopic expression of AXL was sufficient to confer a highly invasive phenotype to weakly invasive MCF7 breast cancer cells. Experimental inhibition of AXL signaling by a dominant-negative AXL mutant, an antibody against the extracellular domain of AXL, or short hairpin RNA knockdown of AXL decreased motility and invasivity of highly invasive breast cancer cells. To selectively interfere with cancer cell properties defining the rate of disease progression, we identified 3-quinolinecarbonitrile compounds, which displayed potent inhibitory activity against AXL and showed strong interference with motility and invasivity of breast cancer cells. Our findings validated the RTK AXL as a critical element in the signaling network that governs motility and invasivity of breast cancer cells, and allowed the identification of experimental anti-AXL small molecular inhibitors that represent lead substances for the development of antimetastatic breast cancer therapy. [Cancer Res 2008;68(6):1905–15]

Introduction

Breast cancer is the most common malignant disease in western women. In these patients, it is not the primary tumor, but its metastases at distant sites that are the main cause of death (1). The development of chemotherapy as well as endocrine and radiation therapy, administered as adjuvant treatment after surgery, has led to a reduction in the risk of relapse to 20–40%. However, adjuvant treatment has a wide range of acute and long-term side effects. Over the past 20 years, with the advances in understanding the molecular basis of signaling pathway dysregulation in various cancers, a new era of cancer therapy has begun, which is characterized by the identification of critical regulators of malignant properties of cancer cells as molecular targets (2, 3).

Deregulated expression of protein kinases by gene deletion, mutation, or amplification has been found to be important for tumor initiation and progression, involving cancer cell proliferation, survival, motility, and invasivity, as well as tumor angiogenesis and chemotherapy resistance (4, 5). Because of their critical functions in oncogenesis, protein kinases have been at the forefront of targeted cancer therapy development since the 1980s. Most of the novel targeted cancer therapeutics currently approved by the Food and Drug Administration in clinical use interfere with the signaling action of protein kinases. More than 100 additional protein kinase inhibitors and antibodies are in clinical trials, making kinases, after G protein–coupled receptors, the second most popular drug target class in the pharmaceutical and biotech industries (3).

In breast cancer, the receptor tyrosine kinase (RTK) HER2/neu is overexpressed in ~25% of breast cancer patients, and enhanced expression correlates with lack of response to adjuvant therapy and poor prognosis (6). Based on this discovery, Herceptin, a monoclonal antibody against HER2/neu oncoprotein, has been developed and is in clinical use since 1998 both as a single agent and in combination with chemotherapies for HER2/neu-overexpressing metastatic breast cancer, which has helped to significantly prolong survival of patients (7, 8). However, metastatic breast cancer patients showing no overexpression of HER2/neu do not benefit from this therapy. Therefore, novel therapeutic targets are still urgently needed for intervention in breast cancer metastatic progression.

To identify the genes that mediate progression of breast cancer, we have focused on key elements of the phosphoprotein-mediated signaling system because of its established role in human cancer. After systematically analyzing expression profiles of kinases of 13 weakly invasive and 8 highly invasive breast cancer cell lines and normal mammary epithelia cell lines by cDNA array hybridization analysis, we identified a cluster of genes characteristic for highly invasive cell types. The RTK *AXL* was part of the gene cluster predictive of the aggressiveness of breast cancer cells (data not shown).

The mammalian AXL RTK subfamily includes three closely related members: AXL, SKY, and MER. The subfamily is characterized by an extracellular domain, consisting of two immunoglobulin-like domains followed by two fibronectin type 3–like domains. GAS6, originally isolated as a growth arrest–specific gene, is the common ligand for AXL subfamily receptors (9–11). GAS6 has the highest affinity for AXL, followed by SKY, and finally MER (11). GAS6-AXL signaling has been implicated in a host of discrete cellular responses including cell survival, proliferation, migration, and adhesion (12).

AXL was originally isolated from patients with chronic myelogenous leukemia and was shown to have transforming potential when overexpressed (13, 14). Subsequently, AXL expression has been reported in a wide variety of human cancers (15–20). Especially in breast cancer patients, a significant correlation was

Note: Y-X. Zhang and P.G. Knyazev contributed equally to this work.

Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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found between AXL and tumor stage (15). Moreover, some reports indicated that AXL might be involved in cancer progression (21, 22). These prompted us to further investigate the role of AXL in breast cancer.

Here, we show that AXL has an important role in mediating breast cancer cell motility and invasivity. Moreover, we identified 3-quinolinecarbonitrile compounds that displayed potent inhibitory activity against AXL, and showed strong interference with motility and invasivity of breast cancer cells.

Materials and Methods

Cell lines. Breast cancer cell lines MCF7 and DAL were supplied by Sugen. Other human breast cancer cell lines were obtained from the American Type Culture Collection.

Antibodies and reagents. For the source of antibodies and reagents, see Supplementary Materials and Methods.

Synthesis of compounds. NA80x1 and SKI-606 were synthesized as described (23, 24). The chemical structures of compounds were confirmed by nuclear magnetic resonance and the purity of compounds was >95% according to high-performance liquid chromatography-mass spectrometry (MS) analysis.

cDNA array preparations and hybridization analysis. Gene expression was analyzed by cDNA array hybridization analysis as described before (22). The accession number for AXL is M76125.

Generation of expression constructs and stable cell lines. See Supplementary Materials and Methods.

***In vitro* biological assays.** To study the effects of AXL on cell proliferation, motility, and invasivity of breast cancer cells *in vitro*, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, cell monolayer wound healing assay, Matrigel outgrowth assay, and Boyden chamber migration and invasion assays were done. (For details, see Supplementary Materials and Methods.).

Kinase assay. Kinase assays were carried out according to supplier's instructions. [γ-^{32}P]ATP (10 μCi; Amersham), 100 μmol/L ATP (Sigma), 20 ng AXL kinase, and 250 μmol/L AXL substrate (Upstate) were used for the assay. Radioactivity was measured with a scintillation counter (Beckman LS6500). IC_{50} values were calculated by using a four-parameter log fit.

Immunoblot analysis. See Supplementary Materials and Methods.

***In vitro* association experiments and MS analysis.** See Supplementary Materials and Methods.

Statistical analysis. Statistical analysis was done by using two-tailed Student's *t* test.

Results

AXL expression correlates with motility and invasivity of breast cancer cells. Profiling of kinase gene expression in 21 breast cancer cell lines by cDNA array hybridization analysis led to the observation of high AXL mRNA expression levels in 87.5% highly invasive breast cancer cell lines, but not in weakly invasive breast cancer cell lines (Supplementary Fig. S1). To confirm cDNA array data, Northern blot (data not shown) and immunoblot analyses (Fig. 1*A*) were done to evaluate the expression levels of AXL in breast cancer cell lines. In the highly invasive breast cancer cell lines (BT549, Hs578T, MDA-MB-157, MDA-MB-231, MDA-MB-435s, and MDA-MB-436; refs. 25–28), AXL was highly expressed. In contrast, weakly invasive breast cancer cell lines do not or only weakly express AXL. These results indicate that expression of the RTK AXL correlates with the aggressiveness of breast cancer cells.

To further investigate the involvement of AXL in motility and invasivity of breast cancer cells, we stably overexpressed wild-type AXL (wtAXL) in the weakly invasive breast cancer cell line MCF7 and found that overexpression of wtAXL endowed the cells with a highly invasive phenotype. A significant increase in the motility (1.3-fold) and invasivity (30-fold) of wtAXL-overexpressing cells compared with control cells was observed in Boyden chamber migration and invasion assays (Fig. 1*B*).

To further study the role of AXL in motility and invasivity of breast cancer cells, dominant-negative inhibition of AXL through overexpression of a tyrosine kinase domain deletion mutant (dnAXL) in highly invasive breast cancer cell lines with high endogenous AXL expression was done. As expected, treatment of empty vector–infected MDA-MB-435s cells (control cells) with GAS6 induced tyrosine phosphorylation of AXL; however, in cells overexpressing dnAXL, GAS6-dependent tyrosine phosphorylation was abrogated (Supplementary Fig. S2). Furthermore, we examined the effects of dominant-negative inhibition of AXL on the motility and invasivity of breast cancer cell lines in cell monolayer wound healing, Matrigel outgrowth, and Boyden chamber migration and invasion assays.

When cell motility was examined by the cell monolayer wound healing assay, we observed that MDA-MB-435s control cells migrated into the wound at a higher rate than the dnAXL-overexpressing MDA-MB-435s cells. Moreover, decreased motility of dnAXL cells in comparison with MDA-MB-435s control cells was also confirmed by Boyden chamber migration assay (Fig. 1*C*).

Invasive behavior of cancer cells is reflected in cell culture by their ability to grow into Matrigel. Highly invasive breast cancer cell lines formed stellate colonies with filopodial structures invading the surrounding matrix, and the noninvasive or weekly invasive counterparts did not show this phenotype but grew as spherical clusters on the Matrigel surface. When cell invasivity was examined by the Matrigel outgrowth assay, as shown in Fig. 1*D*, MDA-MB-435s, BT549, and MDA-MB-231 control cells all formed stellate colonies; however, the corresponding dnAXL-overexpressing cells were unable to form such outgrowth structures and grew instead as spherical clusters. From these results, we concluded that overexpression of dnAXL in cells with high endogenous AXL expression converted the highly invasive phenotype of breast cancer cells to a weakly or noninvasive phenotype. Moreover, the dramatic reduction of MDA-MB-435s invasivity by dnAXL overexpression, in comparison with MDA-MB-435s control cells, was further confirmed by the Boyden chamber invasion assay (Fig. 1*D*).

In an alternative approach, we examined the effects of short hairpin RNA (shRNA)–mediated AXL expression knockdown on the motility and invasivity of breast cancer cells. To enrich cells in which the AXL shRNA–mediated knockdown was quite significant, AXL shRNA–infected cells were stained with anti-AXL monoclonal antibody 259-9 and sorted by using FACSVantage; the cell portion with low AXL expression was collected for experiments. As expected, a dramatic decrease in AXL expression was observed in AXL shRNA–expressing cells compared with scrambled shRNA–expressing cells in both MDA-MB-435s and Hs578T cells (Fig. 2*A*).

We further examined the effects of shRNA-mediated AXL knockdown on cell migration and invasion, and found that AXL shRNA–expressing cells have an obviously decreased capacity in motility and invasivity in comparison with scrambled shRNA–expressing cells in the Boyden chamber migration (Fig. 2*B*) and invasion (Fig. 2*C*) assays. Moreover, decreased invasivity of AXL shRNA–expressing cells compared with control cells was also confirmed by Matrigel outgrowth assay with both MDA-MB-435s and Hs578T cells (Supplementary Fig. S3). In contrast, shRNA-mediated AXL knockdown only showed mild effects on cell proliferation under conditions used for migration and invasion

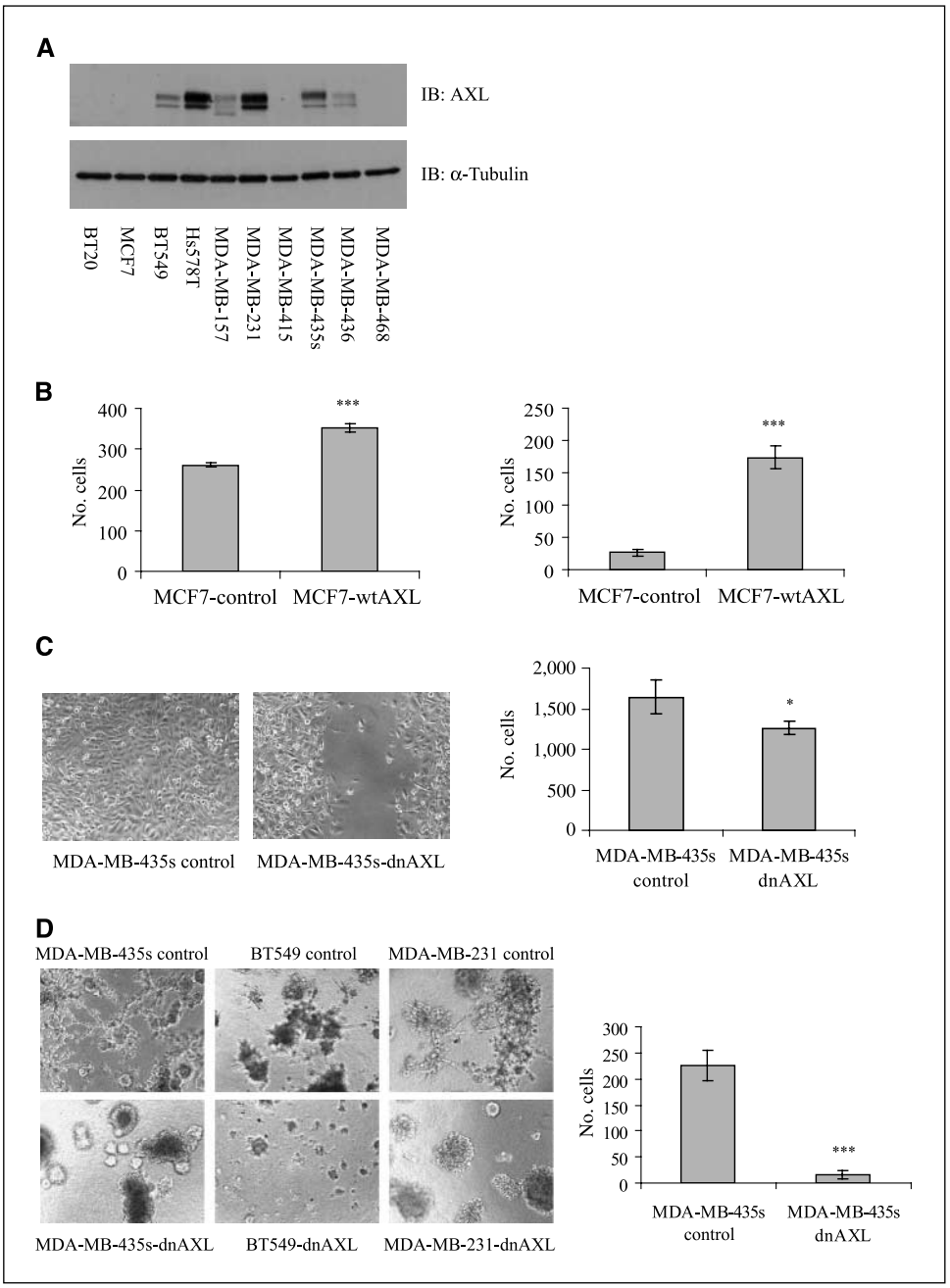


Figure 1. AXL expression correlates with motility and invasivity of breast cancer cells. *A,* expression of AXL protein in invasive and noninvasive breast cancer cells. Whole-cell lysates were used to detect the protein level of AXL by immunoblot analysis (*top*) with α-tubulin as loading control (*bottom*). *B,* motility (*left*) and invasivity (*right*) of MCF7 breast cancer cells stably overexpressing wtAXL or control vector were analyzed by Boyden chamber migration and invasion assays, respectively, for 36 h toward medium containing 10% FCS as a chemoattractant. *Bars,* ±SD ($n = 3$). *C,* the motility of cells was analyzed by cell monolayer wound healing assay (*left*) and Boyden chamber migration assay (*right*). In cell monolayer wound healing assay, cells were allowed to migrate into the wound area for 3 d. Cell migration was visualized at 10× magnification. In Boyden chamber migration assay, motility of cells was analyzed for 36 h toward medium containing 10% FCS as a chemoattractant. *Bars,* ±SD ($n = 3$). *D,* the invasivity of cells was analyzed by Matrigel outgrowth assay (*left*) and Boyden chamber invasion assay (*right*). In Matrigel outgrowth assay, cells were plated on the surface of Matrigel. Colony outgrowth was visualized at 10× magnification. In Boyden chamber invasion assay, invasivity of cells was analyzed for 36 h toward medium containing 10% FCS as a chemoattractant. *Bars,* ±SD ($n = 3$). *, $P < 0.05$; ***, $P < 0.001$, compared with respective control group.

assays (Fig. 2*D*). These findings, in conjunction with those described above, strongly support the conclusion that AXL has an important role in mediating the invasive behavior of breast cancer cells.

Suppression of cancer cell invasiveness by an anti-AXL polyclonal antibody. To evaluate the efficacy *in vitro* of a more therapy-like intervention strategy, we determined the inhibitory potential of a polyclonal AXL antibody. We generated a polyclonal antiserum against the extracellular domain of AXL and studied its effect on the motility and invasivity of MDA-MB-435s breast cancer cells. After 7 days, cells treated with control antibody invaded into the surrounding matrix. However, cells treated with AXL antibody grew as spherical clusters on Matrigel surface (Fig. 3*A*). The inhibitory activity of AXL antibody on motility and invasivity of MDA-MB-435s was also confirmed in the Boyden chamber migration and invasion assays. As shown in Fig. 3*B* and *C*, after AXL antibody treatment, the motility and invasivity of MDA-MB-435s cells were reduced by 50% and 95% respectively, whereas the motility and invasivity of Hs578T cells were decreased by 19.4% and 23.2%, respectively.

Identification of small molecular AXL inhibitors. Based on the results of the target validation experiments with breast cancer cell lines, we initiated efforts to identify small molecular kinase inhibitors with selective activity on the AXL tyrosine kinase. In attempts to identify lead compounds, we applied the Nested Chemical Library technology, which is a novel hit and lead finding method for rational drug design of kinase inhibitors, developed by Keri et al. (29). The Nested Chemical Library was designed on the

platform of a diverse kinase inhibitory library organized around 97 core structures with proven kinase inhibitory activity on various kinase targets. As the first step, we screened chemical validation library, which was built around 97 core structure and includes ~300 compounds, and found several compounds that had inhibitory activity against GAS6-driven autophosphorylation of AXL in intact cells with an IC_{50} <10 μmol/L. The 3-quinolinecarbonitrile compound NA80x1 is shown in Fig. 4*A*. As shown in Fig. 4*B*, GAS6-mediated tyrosine phosphorylation of AXL was inhibited in a dose-dependent manner with an IC_{50} of 4.11 ± 1.47 μmol/L in Hs578T cells; and with an inhibition rate of 67.4% at 25 μmol/L and 30.2% at 6.25 μmol/L in MDA-MB-435s cells. To confirm that NA80x1 inhibits AXL phosphorylation directly, the effects of NA80x1 on AXL kinase activity were assayed *in vitro* with recombinant AXL enzyme. As shown in Fig. 4*C*, NA80x1 inhibited AXL kinase activity in a dose-dependent manner, with an IC_{50} of 12.67 ± 0.45 μmol/L.

NA80x1 has previously been reported to have inhibitory activity against Src kinase (compound 2a in ref. 24). Therefore, we examined the effect of NA80x1 on the phosphorylation of the Src kinase family active site residue Tyr^{416} in breast cancer cells (Fig. 4*B*). In MDA-MB-435s cells, phosphorylation of Src Tyr^{416} was inhibited in a dose-dependent manner. At 3.125 μmol/L inhibition was 50%, and at 12.5 μmol/L it was almost complete. However, in breast cancer cells Hs578T, NA80x1 at 6.25 and 12.5 μmol/L only slightly inhibited phosphorylation. Because the anti–phospho-Src (Tyr^{416}) antibody used in these experiments cross-reacts with all members of the Src family, this result might be due to a different inhibitory activity of NA80x1 against different Src kinase family members or differences in membrane penetration of the compound in different cell lines.

Because AXL has an important role in motility and invasivity of breast cancer cells, we further checked the effects of NA80x1 on these properties of breast cancer cells. As shown in Fig. 4*D*, NA80x1

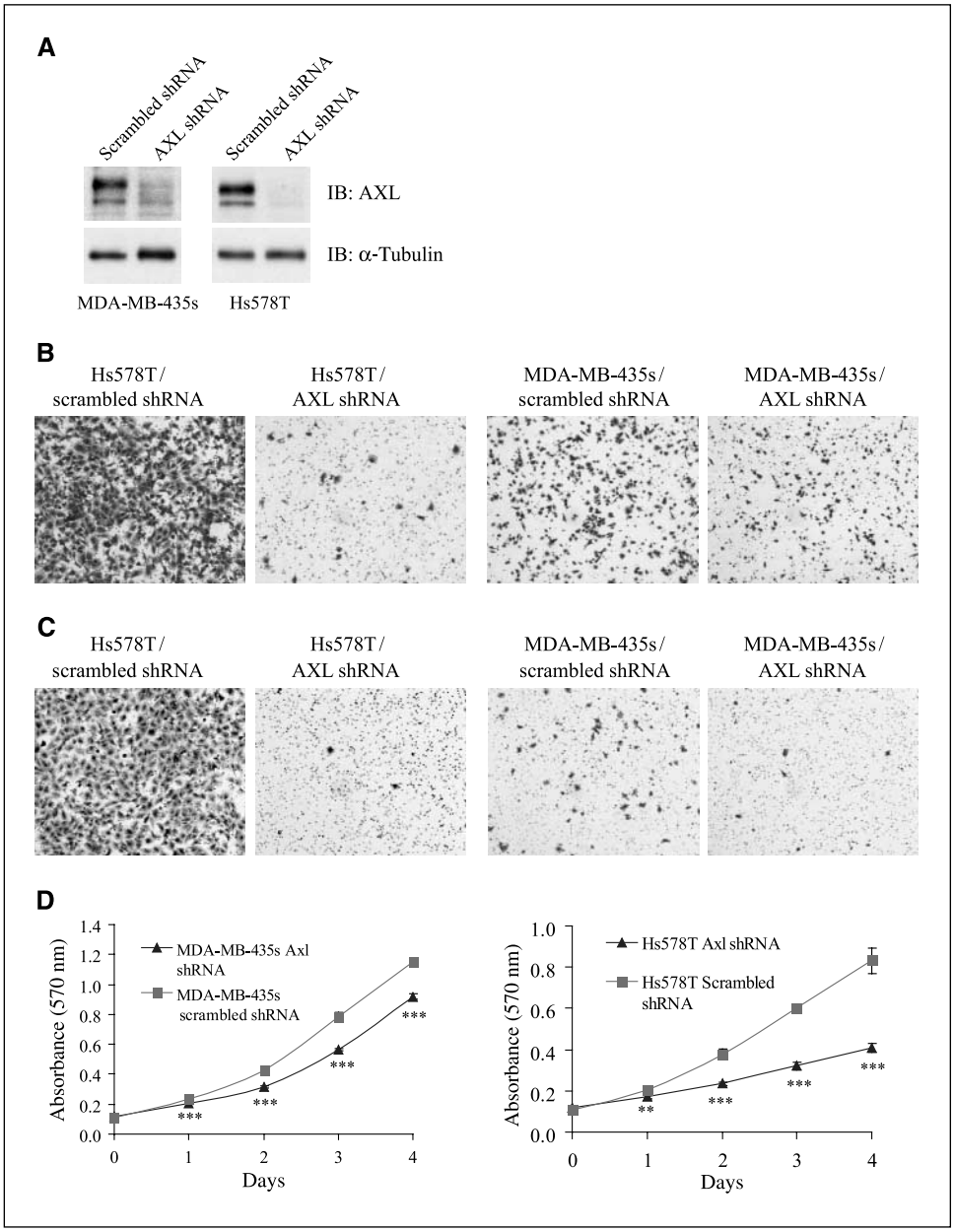


Figure 2. Effects of shRNA-mediated AXL expression knockdown on proliferation, motility, and invasivity of human breast cancer cells. *A,* expression of AXL protein in MDA-MB-435s and Hs578T cells stably expressing pRetrosuper-AXL-shRNA or pRetrosuper-scrambled-shRNA. Whole-cell lysates were used to detect the protein level of AXL by immunoblot analysis (*top*) with α-tubulin as loading control (*bottom*). *B,* motility of cells was analyzed by Boyden chamber migration assay for 12 h toward medium containing 10% FCS as a chemoattractant. The cells that had migrated to the lower surface were fixed and stained with crystal violet. Cells were visualized at 10× magnification. *C,* invasivity of cells was analyzed by Boyden chamber invasion assay, similarly as described above except for seeding the cells into the Matrigel-coated inserts, and cells were permitted to migrate for 36 h. *D,* comparison of cell proliferation by MTT assay. *Bars,* ±SD of absorbance at 570 nm (n = 3). **, $P < 0.01$; ***, $P < 0.001$, compared with scrambled shRNA–expressing cells.

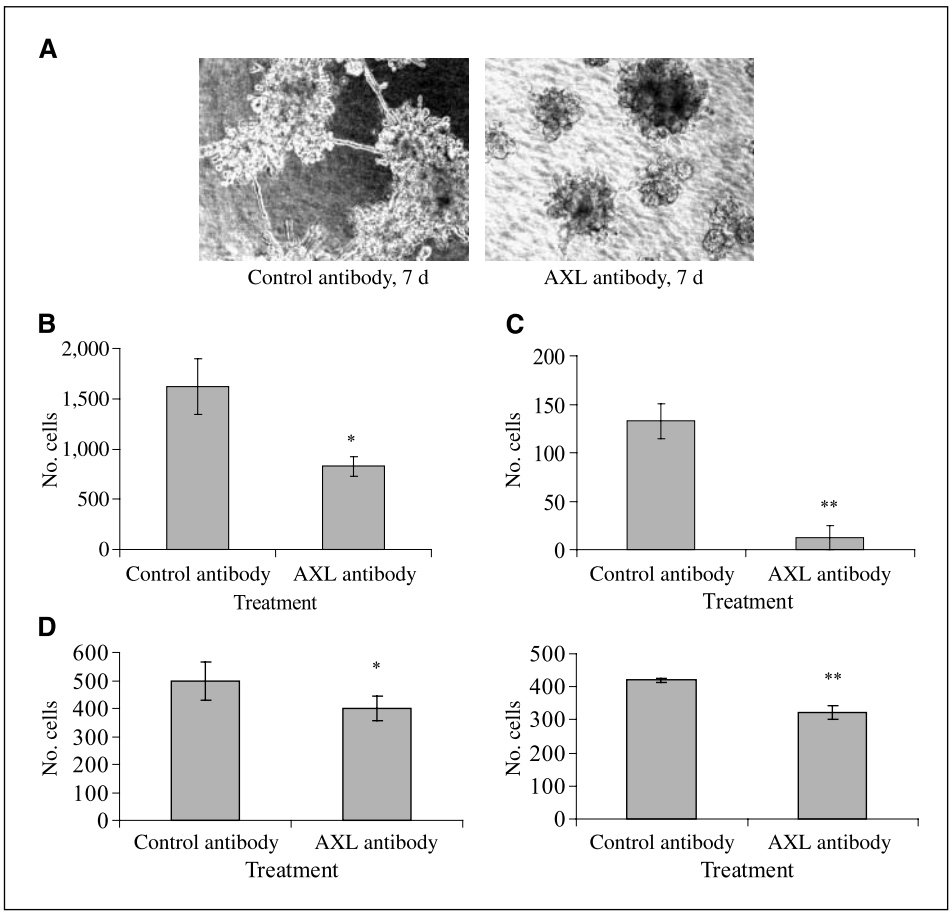


Figure 3. Effects of an anti-AXL polyclonal antibody on the motility and invasivity of human breast cancer cells. *A,* effects of anti-AXL antibody on the invasivity of cells in Matrigel outgrowth assay. Cells were plated on the surface of Matrigel and treated with anti-AXL antibody or control antibody for 7 d. Colony outgrowth was visualized at 10× magnification. *B,* the effects of indicated treatments on the motility of MDA-MB-435s were measured by Boyden chamber migration assay according to the procedure described in Materials and Methods. Data were recorded after 36 h toward medium containing 10% FCS as a chemoattractant. *Bars,* ±SD ($n = 3$). *C,* the effects of indicated treatments on the invasivity of MDA-MB-435s were also evaluated by Boyden chamber invasion assay as described above. *D,* the effects of treatments on the motility (*left*) and invasivity (*right*) of Hs578T were evaluated by Boyden chamber migration and invasion assays as described above. *, $P < 0.05$; **, $P < 0.01$, compared with respective control group treated with control antibody.

inhibited the migration and invasion of cells in a dose-dependent manner as compared with the control in Boyden chamber migration and invasion assays. At 6.25 μmol/L, NA80x1 inhibited cell motility by 70.0% and 46.1%, and cell invasivity by 96.2% and 41.1%, in MDA-MB-435s and Hs578T cells, respectively. At 25 μmol/L, NA80x1 almost completely blocked the motility and invasivity of both MDA-MB-435s and Hs578T cells. In addition, the inhibitory effect of NA80x1 on MDA-MB-435s and Hs578T was confirmed in cell monolayer wound healing experiments (Supplementary Fig. S4*A*) and Matrigel outgrowth assays (Supplementary Fig. S4*B*).

To check if the inhibitory activity of NA80x1 on motility and invasivity of breast cancer cells is partially due to the inhibitory activity of NA80x1 on cell growth, we analyzed the effects of NA80x1 on proliferation of Hs578T and MDA-MB-435s cells by MTT assay and found that NA80x1 did not show obvious interference with this cancer cell function (data not shown).

AXL is a target of the Src/Abl inhibitor SKI-606. In continuing efforts to develop more potent AXL kinase inhibitors than NA80x1, we identified a 3-quinolinecarbonitrile compound, SKI-606 (Fig. 5*A*), with more potent inhibitory activity against AXL autophosphorylation. This compound was developed as an inhibitor of Src and Abl kinases and is currently in phase II clinical trials for the treatment of several cancer types including breast cancer.

As shown in Fig. 5*B*, SKI-606 inhibited AXL kinase activity in a dose-dependent manner, with an IC_{50} of 0.56 ± 0.08 μmol/L (i.e., 20-fold lower than NA80x1). To confirm the measured biochemical activity of SKI-606 in a cell-based assay, the effects on tyrosine phosphorylation of AXL after GAS6 stimulation were examined in MDA-MB-435s and Hs578T breast cancer cells. As shown in Fig. 5*C*, GAS6-mediated tyrosine phosphorylation of AXL was inhibited with an IC_{50} of 0.34 ± 0.04 μmol/L in Hs578T cells, and with an inhibition rate of 83.6% at 6.25 μmol/L and 68.6% at 1.56 μmol/L in MDA-MB-435s cells. Interestingly, the inhibitory activity of SKI-606 on AXL is similar as on Src, the target it was developed for. SKI-606 inhibited the phosphorylation of the Src kinase family phosphorylation site Tyr^{416} in a dose-dependent manner with an IC_{50} of 0.63 ± 0.04 μmol/L in Hs578T cells and 0.55 ± 0.01 μmol/L in MDA-MB-435s cells (Fig. 5*C*).

Furthermore, we determined the effects of SKI-606 on the motility and invasivity of breast cancer cells. As shown in Fig. 5*D*, SKI-606 potently inhibited the migration and invasion of MDA-MB-435s and Hs578T in Boyden chamber migration and invasion assays. At 1.56 μmol/L, SKI-606 inhibited cell motility by 70.7% and 25.4%, and cell invasivity by 85.9% and 63.3%, in MDA-MB-435s and Hs578T cells, respectively. At 6.25 μmol/L, SKI-606 almost completely blocked the motility and invasivity of both MDA-MB-435s and Hs578T cells.

We also checked the effects of SKI-606 on cell growth of MDA-MB-435s and Hs578T cells by MTT assay, and found that SKI-606 can inhibit cell proliferation in a dose-dependent manner, with an IC_{50} of 9.0 ± 1.3 and 5.9 ± 0.3 μmol/L, respectively. However, under the conditions used for migration and invasion assays, SKI-606 had only a weak effect on cell growth (Supplementary Fig. S5).

Identification of cellular protein targets of NA80x1 and SKI-606 by proteomics approach. Because both of SKI-606 and

NA80x1 have previously been reported to be ATP-competitive inhibitors, it is likely that they act on even more targets than the previously reported Src and Abl family kinases and as shown here on AXL. To profile the cellular proteins targets of NA80x1 and SKI-606, we adopted a recently developed proteomics approach, which relies on the use of immobilized inhibitor analogues as capture reagents for selective isolation of drug-interacting protein species in a cellular system (30).

Based on earlier structural and medicinal chemistry data, we reasoned that the *N*-methylpiperazine group of SKI-606 is exposed at the surface of the kinase domain, and therefore synthesized SKI-606 derivative VI17525 with a primary amine at the position of the *N*-methylpiperazine function for directed covalent immobilization and the generation of an affinity purification resin with SKI-606-like binding properties (Fig. 6*A*). Accordingly, we synthesized an immobilizable NA80x1 derivative, VI17614, by replacement of the C-7 methoxy group with a 3-aminopropoxy chain (Fig. 6*A*). Covalent coupling of this compound resulted in an affinity resin with NA80x1-like binding properties.

To identify the specific targets of VI17525 and VI17614, we combined stable isotope labeling by amino acids in cell culture (SILAC) with the chemical proteomics approach. Equal amounts of

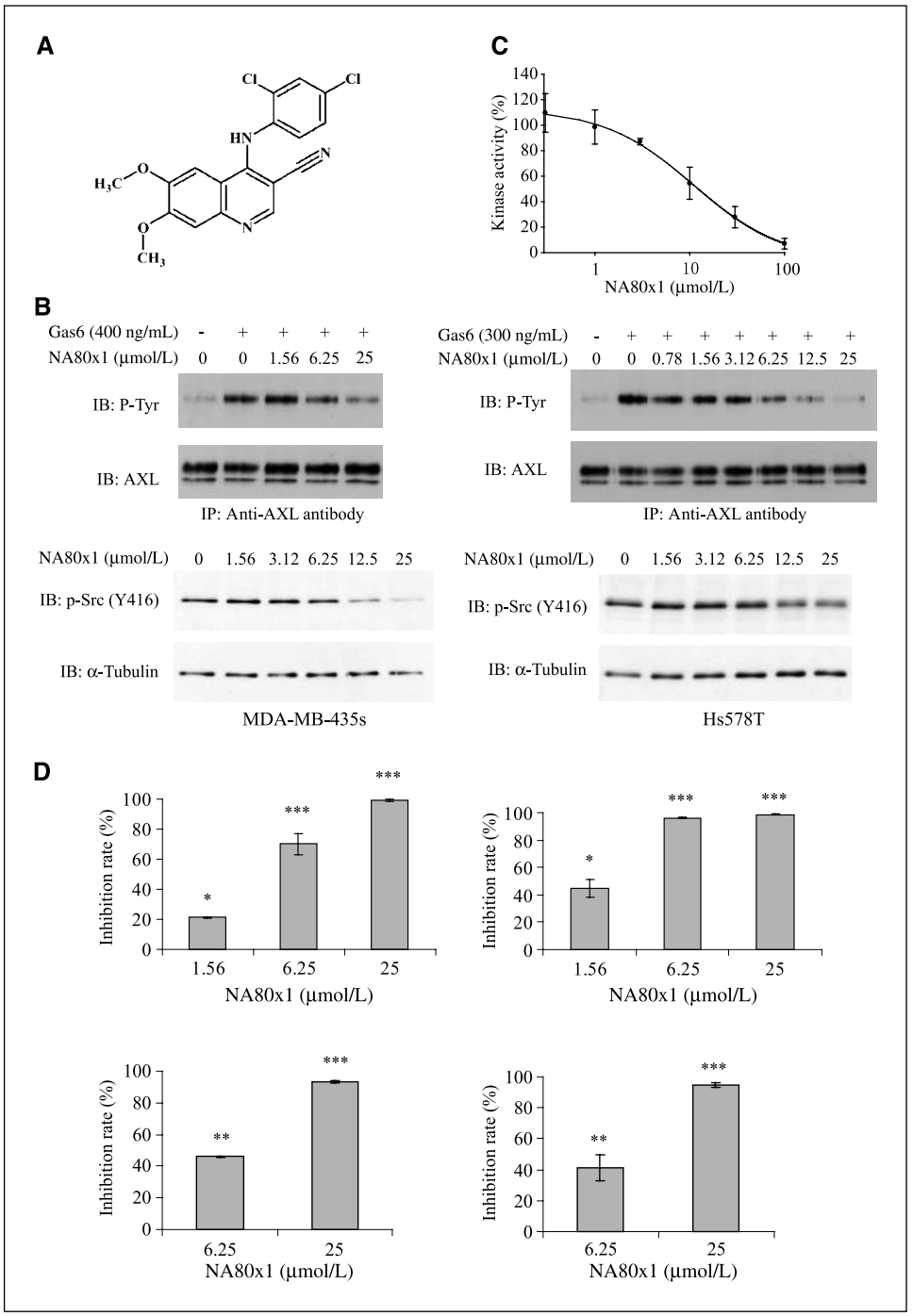


Figure 4. NA80x1 inhibits AXL phosphorylation, cell motility, and invasivity. *A,* chemical structure of NA80x1. *B,* effects of NA80x1 on phosphorylation of AXL and Src kinase family. For AXL, starved MDA-MB-435s (*top left*) or Hs578T (*top right*) cells were pretreated with indicated concentration of NA80x1 for 1 h, and subsequently stimulated with GAS6 for 20 min. Following immunoprecipitation of cell extracts with anti-AXL antibody, proteins were immunoblotted with anti-phosphotyrosine antibody and reprobed with anti-AXL antibody. For Src (*bottom*), MDA-MB-435s and Hs578T cells were treated with indicated concentration of NA80x1 for 2 h. Immunoblots were probed with anti–phospho-Src (Tyr^{416}) antibody and reprobed with anti–α-tubulin antibody. *C,* effects of NA80x1 on AXL phosphorylation in *in vitro* kinase assay. *In vitro* kinase reactions were done with recombinant AXL enzyme by using a synthetic peptide as specific substrate for AXL. Kinase activities in the absence of inhibitor were set to 100%, and remaining activities at different NA80x1 concentrations are expressed relative to this value. *D,* effects of NA80x1 on the motility (*top left,* MDA-MB-435s; *bottom left,* Hs578T; analyzed for 12 h) and invasivity (*top right,* MDA-MB-435s; *bottom right,* Hs578T; analyzed for 36 h) cells were analyzed by Boyden chamber migration and invasion assays, respectively, toward medium containing 10% FCS as a chemoattractant. *Bars,* ±SD ($n = 2$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, compared with respective control group without NA80x1 treatment.

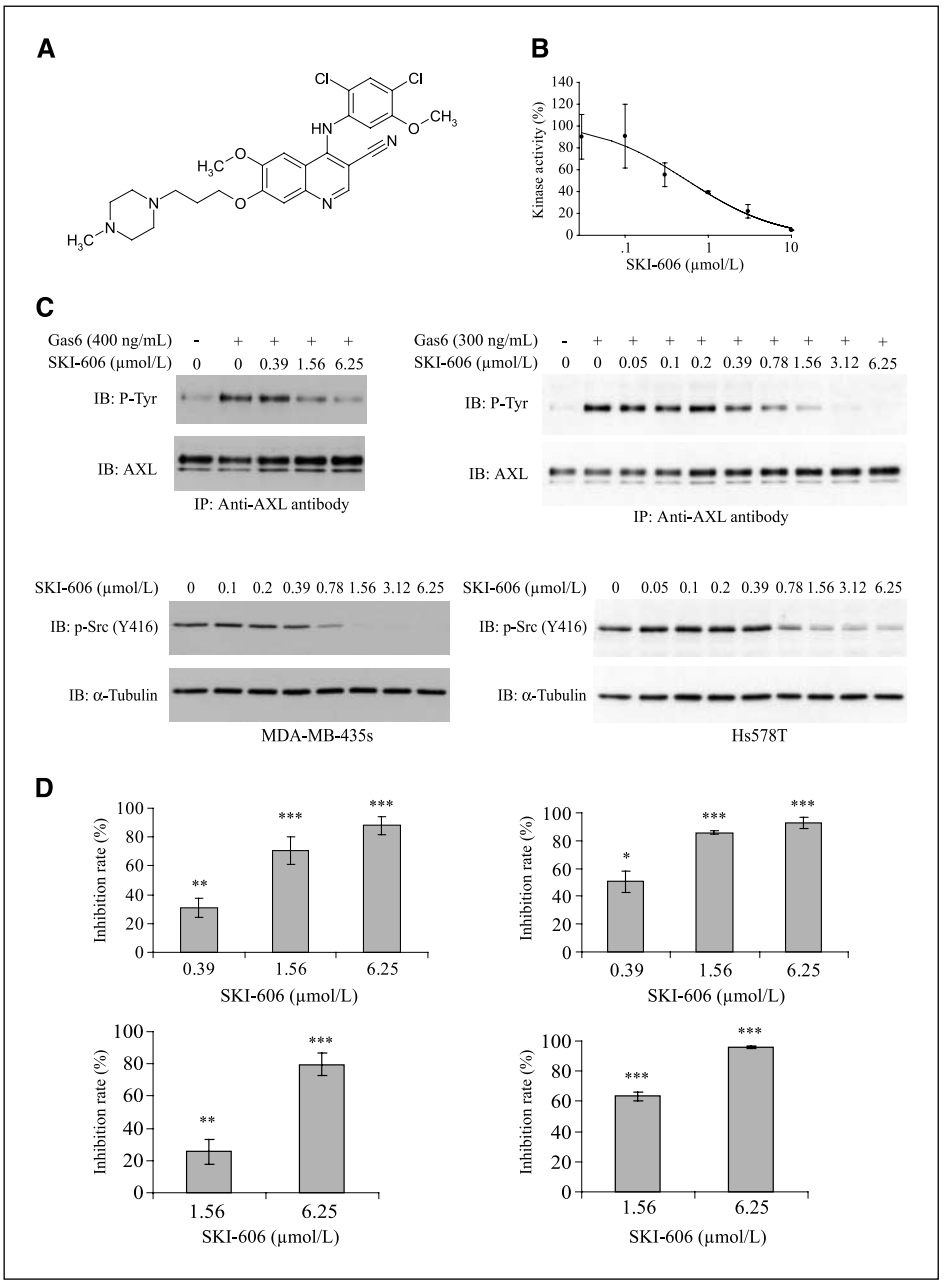
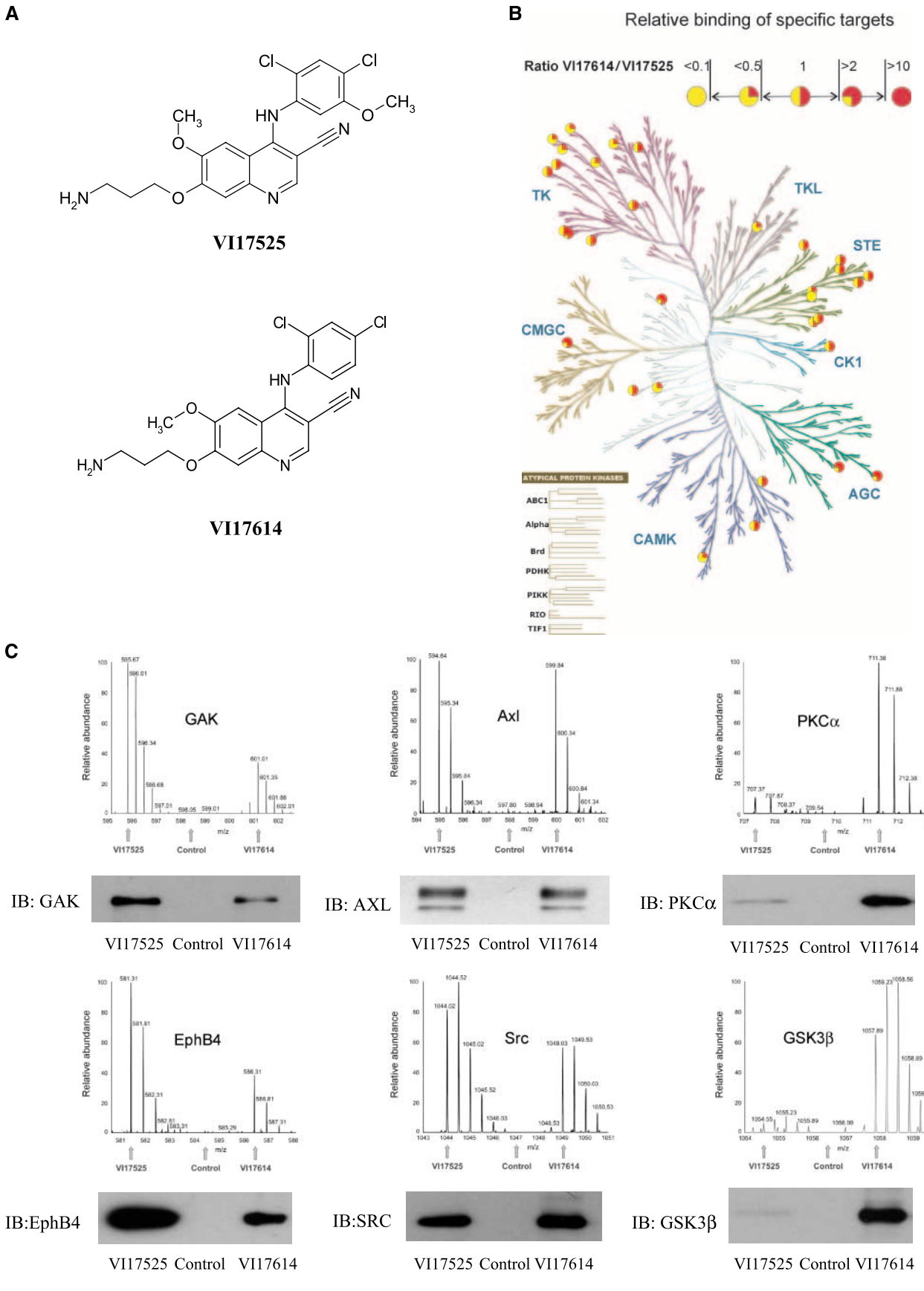


Figure 5. SKI-606 inhibits AXL phosphorylation, cell motility, and invasivity. *A,* chemical structure of SKI-606. *B,* effects of SKI-606 on AXL phosphorylation in *in vitro* kinase assay. *In vitro* kinase reactions were done with recombinant AXL enzyme by using a synthetic peptide as specific substrate for AXL. Kinase activities in the absence of inhibitor were set to 100%, and remaining activities at different SKI-606 concentrations are expressed relative to this value. *C,* effects of SKI-606 on phosphorylation of AXL and Src kinase family. For AXL, starved MDA-MB-435s (*top left*) or Hs578T (*top right*) cells were pretreated with indicated concentration of SKI-606 for 1 h, and subsequently stimulated with GAS6 for 20 min. Following immunoprecipitation of cell extracts with anti-AXL antibody, proteins were immunoblotted with anti-phosphotyrosine antibody and reprobed with anti-AXL antibody. For Src, MDA-MB-435s (*bottom left*) and Hs578T (*bottom right*) cells were treated with indicated concentration of SKI-606 for 2 h. Immunoblots were probed with anti–phospho-Src (Tyr^{416}) antibody and reprobed with anti–α-tubulin antibody. *D,* effects of SKI-606 on the motility (*top left,* MDA-MB-435s; *bottom left,* Hs578T; analyzed for 12 h) and invasivity (*top right,* MDA-MB-435s; *bottom right,* Hs578T; analyzed for 36 h) of cells were analyzed by Boyden chamber migration and invasion assays, respectively, toward medium containing 10% FCS as a chemoattractant. *Bars,* ±SD ($n = 2$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, compared with respective control group without SKI-606 treatment.

lysates from three populations of differentially SILAC-encoded Hs578T cells were used for *in vitro* association experiments with the VI17525, control, and VI17614 resins. The protein eluates from the respective affinity purifications were combined, digested with the protease trypsin, and the resulting peptide fractions were then analyzed by MS. Identified peptides were quantified using the MSQuant software and the respective protein ratios for binding to the different resins were then determined (see Supplementary Table). The Arg^6/Lys^4 versus Arg^0/Lys^0 and Arg^6/Lys^4 versus Arg^{10}/Lys^8 ratios represent the relative binding of proteins to control resin (Arg^6/Lys^4) compared with VI17525 (Arg^0/Lys^0) and VI17614 (Arg^{10}/Lys^8) resins. In total, 146 different proteins were identified with at least two unique peptides in the MS experiments. Among them, 43 proteins were found to specifically bind to the immobilized compounds because they showed <10% binding to control matrix in comparison with the binding observed with either VI17525 or VI17614 matrix.

Among the 43 putative target proteins, 32 proteins were kinases (Fig. 6*B*; Supplementary Table). In addition to known targets such as Src/Abl family kinases Src, Lyn, Arg, and the RTK AXL, which we functionally characterized as a cellular target in this study, we identified a variety of other inhibitor-interacting proteins including eight more tyrosine kinases (such as focal adhesion kinase and four Eph receptor kinase family members) as well as nine members from the STE group of kinases involved in mitogen-activated protein kinase (MAPK) signaling (including six MAP4K/STE20 kinase family members and two MAP2K family members).

We further subgrouped the putative protein targets by the ratio Arg^{10}/Lys^8 versus Arg^0/Lys^0 (VI17614:VI17525), which represents the relative affinity of proteins to VI17614 resin compared with



VI17525 resin. Fourteen proteins have a ratio of <0.5, which indicates that these proteins might have higher affinity toward VI17525 compared with VI17614; eight proteins have a ratio of >2, which indicates that these proteins might have higher affinity to VI17614 compared with VI17525. Another 21 proteins have a ratio between 0.5 and 2, which indicates that these proteins either have similar affinity to these two resins or, alternatively, have high binding affinities beyond a certain threshold that results in quantitative retention by both VI17614 and VI17525 under the conditions of the *in vitro* association experiments (Fig. 6*B*).

To validate the MS data, we investigated the interaction of several putative target proteins with VI17525, VI17614, and control matrix *in vitro*. Immunoblot analysis confirmed that all of six putative target proteins specifically interacted with VI17525 and VI17614 resins. In addition, the binding pattern of target proteins to VI17525 compared with VI17614 is the same as what is obtained from MS analysis, thus verifying the identifications by MS (Fig. 6*C*).

Discussion

Metastasis is the primary death cause of breast cancer patients. The process is complex and consists of a series of sequential steps, including intravasation of cells from a primary tumor into the blood circulation, survival of cells in the bloodstream, arrest in a new organ, extravasation into the surrounding tissue, initiation and maintenance of growth, and vascularization the tumor metastasis. Obviously, the capability to migrate and invade through tissue barriers is essential for cancer cells to successfully complete the process of metastasis (1, 31).

In this study, we identified the RTK AXL as a potential mediator of motility and invasivity of breast cancer cells. Based on our systematic approach, we found that the expression of AXL was correlated with the invasive properties of breast cancer. Ectopic overexpression of wtAXL can endow nonmetastatic MCF7 human breast cancer cells with a highly invasive phenotype. Conversely, inhibition of endogenous AXL signaling in highly invasive breast cancer cells by a dominant-negative mutant or shRNA knockdown results in decreased capacity to migrate toward attractants and loss of the capacity to invade the surrounding matrix. Furthermore, a previous report had shown that there is a significant relation between AXL and tumor stage in breast cancer patients, which is in agreement with our hypothesis that AXL plays a pivotal role in breast cancer metastasis. Interestingly, recently AXL was also reported to be a mediator of invasion in lung cancer (17) and glioblastoma (22). These reports support and emphasize an important role of AXL signaling in the metastatic progression of various cancer types.

In addition to its function in the control of cancer cell motility and invasivity that we investigated in this study, AXL may also have a role in tumorigenesis of breast cancer. Using a human MDA-MB-231 breast cancer xenograft model, Holland et al. (21) showed that RNA interference–mediated inhibition of AXL expression in tumor cells blocked tumor growth *in vivo*.

Besides its metastasis-promoting activity, AXL might play a role in other cell biological properties relevant to breast cancer progression. This was suggested in a previous study that showed the ability of GAS6/AXL signaling to protect NIH3T3 cells from serum starvation or tumor necrosis factor-α–induced apoptosis (32). In the progression of cancer, cells have to face the challenge of nutrient starvation and apoptosis induced by death signals. Whether GAS6/AXL signaling has an antiapoptotic function to facilitate survival of cancer cells in circulating bloods and the formation of micrometastases in distant tissues remains to be investigated. Moreover, AXL might support vital processes required for neovascularization because silencing of AXL in an *in vivo* angiogenesis model produced a phenotype similar to vascular endothelial growth factor receptor-2/Flk-1 function knockdown (21, 33).

Taken together, AXL seems to have multiple roles in tumorigenesis and progression of breast cancer. Therapeutic intervention in AXL signaling function offers a novel avenue toward the multifunctional treatment of metastatic breast cancer.

In the development of targeted agents against RTKs, monoclonal antibodies that target the extracellular domain of RTKs and small-molecular inhibitors that target the intracellular kinase domains of the RTKs are two main cancer treatment strategies (34). Currently, all of the kinase-targeting anticancer agents that are approved for clinical use belong to these two classes.

Antibodies, by virtue of the fact that they are highly specific, represent an ideal approach for selectively interfering with a single target molecular. In this study, we performed experimental therapy against breast cancer *in vitro* by using a polyclonal AXL antibody, and showed that the antibody successfully inhibited the motility and invasivity of MDA-MB-435s breast cancer cells, which proved that developing a therapeutic monoclonal anti-AXL extracellular domain antibody is a promising strategy for the treatment of invasive breast cancer. The recently characterized crystal structure of the immunoglobulin-like domains of AXL ectodomain and first laminin G–like domain of GAS6 complex (35) should be helpful for the rational development of therapeutic antibodies against AXL.

Small molecular inhibitors represent an alternative approach for developing kinase-targeted cancer therapeutics, which can block the function of RTKs by binding to the ATP-binding site and thereby blocking the kinase activity and downstream signaling pathways. Previous studies have shown that AXL signaling is quite complex. Besides GAS6-triggered AXL signaling pathways, AXL could be activated by a ligand-independent mechanism (36) or by cross–talk with other signaling pathways (36, 37). For example,

Figure 6. Cellular targets of compounds VI17525 and VI17614 identified by quantitative chemical MS approach. *A,* chemical structures of compounds VI17525 (SKI-606 derivative) and VI17614 (NA80X1 derivative), which were immobilized via their primary amino groups to epoxy-activated Sepharose beads. *B,* cellular specificity profile of compounds VI17525 and VI17614. The kinase targets of VI17525 and VI17614 are represented as circles in the figure. As indicated in the figure, different colors in circles indicate the relative binding (VI17614/VI17525 ratio obtained from quantitative MS) of specific kinase targets to VI17614 and VI17525 resins (for details, see Supplementary Table). The kinase dendrogram was adapted with permission from Cell Signaling Technology, Inc. (http://www.cellsignal.com/). *C,* representative LTQ-Orbitrap FT-MS scans indicating the differential binding patterns of various targets (more, equal, or less binding to VI17525 resin compared with VI17614 resin). *Arrows,* monoisotopic peaks used for quantitation. To confirm the results obtained from MS analysis, the total lysates from Hs578T cells were subjected to *in vitro* association experiments with VI17525, VI17614, or control resins. The bound proteins were analyzed by immunoblotting with indicated antibodies: AXL and Src (equal binding to VI17525 and VI17614 resins), EphB4 and cyclin G–associated kinase (*GAK*; higher binding to VI17525 resin), and protein kinase Cα (*PKCα*) and glycogen synthase kinase 3β (*GSK3β*; higher binding to VI17614 resin).

Budagian et al. (37) uncovered a novel alliance between interleukin-15 (IL-15) receptor α and AXL RTK, which enables IL-15 to transactivate AXL and its associated signaling pathways without involvement of GAS6. Therefore, it seems to be necessary to develop AXL small molecular inhibitors for complete blocking of all activation modes of AXL signaling. In our efforts to develop small molecular AXL inhibitors, we found that 3-quinolinecarbonitrile compounds have potent inhibitory activity against AXL autophosphorylation activity, which provides a good scaffold for further development of clinically useful AXL inhibitors.

SKI-606 is currently in phase II clinical trials for the treatment of several cancer types including breast cancer. In our study, we have found that SKI-606 strongly interfered with the motility and invasivity of invasive breast cancer cells. In addition, Jallal et al. (38) recently reported that SKI-606 significantly inhibited the invasion, growth, and metastasis of MDA-MB-231 breast cancer cells *in vitro* and *in vivo*. All of these data indicate that SKI-606 might be a potential therapeutic agent for blocking breast cancer growth and metastasis. Because more than 500 proteins kinases identified in the human genome share a similar ATP pocket, there is great potential for SKI-606 and NA80x1 to interact with multiple kinases, in addition to the known targets including Src/Abl kinase family and AXL, the target reported here. To better understand which targets might be significant or responsible for the biological efficacy of these inhibitors, an analysis of the target profile of SKI-606 and NA80x1 was done by using a chemical proteomics method. Forty-three proteins were identified as potential targets, including 32 kinases. In accordance with our results, Bantscheff et al. (39) recently used a quantitative chemical proteomics approach to identify the cellular targets of SKI-606 in K562 leukemia cells, and obtained a comparable protein kinase target profile. Notably, in addition to Src (40) and AXL, the approach identified another two targets, focal adhesion kinase (41, 42) and MAP4K4 (43, 44), which are known to play an important role in motility and invasivity of cancer cells. Additionally, several members of Eph receptor family, which is reported to be involved in tumor growth and angiogenesis (45), were identified as targets for both of the inhibitors. Whether and how these potential targets contribute to the biological efficacy of NA80x1 and SKI-606 require further investigation.

Taken together, our study shows that AXL is a potential target for therapy development against invasive breast cancer. It was validated by the development of anti-AXL polyclonal antibodies and small molecular kinase inhibitors. 3-Quinolinecarbonitrile compounds can target multiple kinases in addition to Src, for which they were originally validated, and therefore provide new chemical leads toward more potent cancer therapies that interfere with multiple signaling pathways that are vital for cancer cells and critical for cancer progression.

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References

1. Weigelt B, Peterse JL, van't Veer LJ. Breast cancer metastasis: markers and models. Nat Rev Cancer 2005;5:591–602.
2. Shawver LK, Slamon D, Ullrich A. Smart drugs: tyrosine kinase inhibitors in cancer therapy. Cancer Cell 2002;1:117–23.
3. Sebolt-Leopold JS, English JM. Mechanisms of drug inhibition of signalling molecules. Nature 2006;441:457–62.
4. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100:57–70.
5. Blume-Jensen P, Hunter T. Oncogenic kinase signalling. Nature 2001;411:355–65.
6. Slamon DJ, Clark GM, Wong SG, et al. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 1987;235:177–82.
7. Slamon DJ, Leyland-Jones B, Shak S, et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N Engl J Med 2001;344:783–92.
8. Cobleigh MA, Vogel CL, Tripathy D, et al. Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. J Clin Oncol 1999;17:2639–48.
9. Varnum BC, Young C, Elliott G, et al. Axl receptor tyrosine kinase stimulated by the vitamin K-dependent protein encoded by growth-arrest-specific gene 6. Nature 1995;373:623–6.
10. Stitt TN, Conn G, Gore M, et al. The anticoagulation factor protein S and its relative, Gas6, are ligands for the Tyro 3/Axl family of receptor tyrosine kinases. Cell 1995;80:661–70.
11. Nagata K, Ohashi K, Nakano T, et al. Identification of the product of growth arrest-specific gene 6 as a common ligand for Axl, Sky, and Mer receptor tyrosine kinases. J Biol Chem 1996;271:30022–7.
12. Hafizi S, Dahlback B. Signalling and functional diversity within the Axl subfamily of receptor tyrosine kinases. Cytokine Growth Factor Rev 2006;17:295–304.
13. Janssen JW, Schulz AS, Steenvoorden AC, et al. A novel putative tyrosine kinase receptor with oncogenic potential. Oncogene 1991;6:2113–20.
14. O'Bryan JP, Frye RA, Cogswell PC, et al. axl, a transforming gene isolated from primary human myeloid leukemia cells, encodes a novel receptor tyrosine kinase. Mol Cell Biol 1991;11:5016–31.
15. Berclaz G, Altermatt HJ, Rohrbach V, et al. Estrogen dependent expression of the receptor tyrosine kinase axl in normal and malignant human breast. Ann Oncol 2001;12:819–24.
16. Craven RJ, Xu LH, Weiner TM, et al. Receptor tyrosine kinases expressed in metastatic colon cancer. Int J Cancer 1995;60:791–7.
17. Shieh YS, Lai CY, Kao YR, et al. Expression of axl in lung adenocarcinoma and correlation with tumor progression. Neoplasia 2005;7:1058–64.
18. Sun W, Fujimoto J, Tamaya T. Coexpression of Gas6/Axl in human ovarian cancers. Oncology 2004;66:450–7.
19. Green J, Ikram M, Vyas J, et al. Overexpression of the Axl tyrosine kinase receptor in cutaneous SCC-derived cell lines and tumours. Br J Cancer 2006;94:1446–51.
20. Ito T, Ito M, Naito S, et al. Expression of the Axl receptor tyrosine kinase in human thyroid carcinoma. Thyroid 1999;9:563–7.
21. Holland SJ, Powell MJ, Franci C, et al. Multiple roles for the receptor tyrosine kinase axl in tumor formation. Cancer Res 2005;65:9294–303.
22. Vajkoczy P, Knyazev P, Kunkel A, et al. Dominant-negative inhibition of the Axl receptor tyrosine kinase suppresses brain tumor cell growth and invasion and prolongs survival. Proc Natl Acad Sci U S A 2006;103:5799–804.
23. Boschelli DH, Ye F, Wang YD, et al. Optimization of 4-phenylamino-3-quinolinecarbonitriles as potent inhibitors of Src kinase activity. J Med Chem 2001;44:3965–77.
24. Boschelli DH, Wang YD, Ye F, et al. Synthesis and Src kinase inhibitory activity of a series of 4-phenylamino-3-quinolinecarbonitriles. J Med Chem 2001;44:822–33.
25. Thompson EW, Paik S, Brunner N, et al. Association of increased basement membrane invasiveness with absence of estrogen receptor and expression of vimentin in human breast cancer cell lines. J Cell Physiol 1992;150:534–44.
26. Tong D, Czerwenka K, Sedlak J, et al. Association of *in vitro* invasiveness and gene expression of estrogen receptor, progesterone receptor, pS2 and plasminogen activator inhibitor-1 in human breast cancer cell lines. Breast Cancer Res Treat 1999;56:91–7.
27. Sommers CL, Byers SW, Thompson EW, et al. Differentiation state and invasiveness of human breast cancer cell lines. Breast Cancer Res Treat 1994;31:325–35.
28. Draffin JE, McFarlane S, Hill A, et al. CD44

potentiates the adherence of metastatic prostate and breast cancer cells to bone marrow endothelial cells. Cancer Res 2004;64:5702–11.
29. Keri G, Szekelyhidi Z, Banhegyi P, et al. Drug discovery in the kinase inhibitory field using the Nested Chemical Library technology. Assay Drug Dev Technol 2005;3:543–51.
30. Godl K, Wissing J, Kurtenbach A, et al. An efficient proteomics method to identify the cellular targets of protein kinase inhibitors. Proc Natl Acad Sci U S A 2003; 100:15434–9.
31. Steeg PS. Tumor metastasis: mechanistic insights and clinical challenges. Nat Med 2006;12:895–904.
32. Bellosta P, Zhang Q, Goff SP, et al. Signaling through the ARK tyrosine kinase receptor protects from apoptosis in the absence of growth stimulation. Oncogene 1997;15:2387–97.
33. Millauer B, Shawver LK, Plate KH, et al. Glioblastoma growth inhibited *in vivo* by a dominant-negative Flk-1 mutant. Nature 1994;367:576–9.
34. Imai K, Takaoka A. Comparing antibody and small-molecule therapies for cancer. Nat Rev Cancer 2006; 6:714–27.
35. Sasaki T, Knyazev PG, Clout NJ, et al. Structural basis for Gas6-Axl signalling. EMBO J 2006;25:80–7.
36. Zhang QK, Boast S, de los Santos K, et al. Transforming activity of retroviral genomes encoding Gag-Axl fusion proteins. J Virol 1996;70:8089–97.
37. Budagian V, Bulanova E, Orinska Z, et al. A promiscuous liaison between IL-15 receptor and Axl receptor tyrosine kinase in cell death control. EMBO J 2005;24:4260–70.
38. Jallal H, Valentino ML, Chen G, et al. A Src/Abl kinase inhibitor, SKI-606, blocks breast cancer invasion, growth, and metastasis *in vitro* and *in vivo*. Cancer Res 2007;67:1580–8.
39. Bantscheff M, Eberhard D, Abraham Y, et al. Quantitative chemical proteomics reveals mechanisms of action of clinical ABL kinase inhibitors. Nat Biotechnol 2007;25:1035–44.
40. Yeatman TJ. A renaissance for SRC. Nat Rev Cancer 2004;4:470–80.
41. McLean GW, Carragher NO, Avizienyte E, et al. The role of focal-adhesion kinase in cancer—a new therapeutic opportunity. Nat Rev Cancer 2005;5: 505–15.
42. Mon NN, Ito S, Senga T, et al. FAK signaling in neoplastic disorders: a linkage between inflammation and cancer. Ann N Y Acad Sci 2006;1086:199–212.
43. Wright JH, Wang X, Manning G, et al. The STE20 kinase HGK is broadly expressed in human tumor cells and can modulate cellular transformation, invasion, and adhesion. Mol Cell Biol 2003;23:2068–82.
44. Collins CS, Hong J, Sapinoso L, et al. A small interfering RNA screen for modulators of tumor cell motility identifies MAP4K4 as a promigratory kinase. Proc Natl Acad Sci U S A 2006;103:3775–80.
45. Surawska H, Ma PC, Salgia R. The role of ephrins and Eph receptors in cancer. Cytokine Growth Factor Rev 2004;15:419–33.

AXL Is a Potential Target for Therapeutic Intervention in Breast Cancer Progression

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