Combined inhibition of AXL, Lyn and p130Cas kinases block migration of triple negative breast cancer cells

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Abbreviations: AKT, RAC-α serine/threonine-protein kinase; FAK, focal adhesion kinase; EGFR, epidermal growth factor receptor; ELISA, enzyme-linked immunosorbant assay; Gas6, growth arrest specific 6; MAPK, mitogen activated protein kinases; PI3K, phosphatidylinositol 3-kinase; Pyk2, proline-rich tyrosine kinase 2; RTK, receptor tyrosine kinase; siRNA, short interfering RNA; TKI, tyrosine kinase inhibitor; TNBC, triple negative breast cancer

Blocking the migration of metastatic cancer cells is a major goal in the therapy of cancer. The receptor tyrosine kinase AXL is one of the main triggers for cancer cell migration in neoplasia of breast, colon, skin, thyroid and prostate. In our study we analyzed the effect of AXL inhibition on cell motility and viability in triple negative breast cancer cell lines overexpressing AXL. Thereby we reveal that the compound BMS777607, exhibiting the lowest IC50 values for inhibition of AXL kinase activity in the studied cell lines, attenuates cell motility to a lower extent than the kinase inhibitors MPCD84111 and SKI606. By analyzing the target kinases of MPCD84111 and SKI606 with kinase profiling assays we identified Lyn, a Src family kinase, as a target of both compounds. Knockdown of Lyn and the migration-related CRK-associated substrate (p130Cas), had a significant inhibitory effect on cell migration. Taken together, our findings highlight the importance of combinatorial or multikinase inhibition of non-receptor tyrosine kinases and AXL receptor tyrosine kinase in the therapy of triple negative breast cancer.

Introduction

The formation of metastasis resulting from migration of cells out of the primary tumor to invade distant sites in the organism is the number one reason for cancer patient mortality.1-3 Cell migration is complex and it is precisely regulated by multiple factors such cell-cell and cell-substrate contacts but also by soluble mediators, the binding of which to their receptors on the cell surface triggers a network of interconnected signaling pathways. There is a plethora of mediators and effectors that represent potential targets for inhibitors with the capacity to influence cell migration.4 The receptor tyrosine kinase (RTK) AXL (also known as UFO) is one of these targets and known to play an important role in cancer progression, invasion, metastasis, drug resistance and is correlated to patient mortality.5,6 AXL belongs to the TAM (Tyro3, AXL, Mer) RTK family,7 with all 3 members of the family being highly homologous in their extracellular and catalytic domains.6 The activation of AXL occurs when the growth arrest specific 6 (Gas6) ligand binds to its extracellular domain,8 which results in the activation of downstream signaling pathways such as mitogen activated protein kinases (MAPK), phosphatidylinositol 3-kinase (PI3K) RAC-α serine/threonine-protein kinase (AKT) and NF-KB (Nuclear Factor Kappa B).9,10 AXL is frequently overexpressed in human cancers including lymphocytic leukemia, breast, colon, skin, thyroid and prostate cancer.11-17 Down-regulation of AXL by siRNA attenuates the migration and invasion of breast,18 ovarian,19 hepatocellular carcinoma,20 mesothelioma,21 prostate22 and pancreatic cancer.23 Furthermore it has been shown that the inhibition of AXL results in the reduction of cancer progression and metastatic potential and induces apoptosis in cancer cells.19,24

These observations suggest that AXL is an excellent target to counteract cancer and recently several AXL inhibitors have been described.25

The aim of our study was to elucidate the impact of 3 tyrosine kinase inhibitors (TKI), namely SKI606, BMS777607 and MPCD84111 on Axl phosphorylation and the migration as well as viability of triple negative breast cancer (TNBC) cell lines.

SKI-606 (bosutinib) was described as a dual Src/Ab1 inhibitor exhibiting a significant effect on proliferation of colon cancer and chronic myelogenous leukemia.26 It was also demonstrated that...
SKI606 inhibits AXL at 0.58 μmol/L concentrations and blocks migration and invasion of breast cancer cell lines.12

BMS-777607 was described as a selective MET, RON (also known as MST1R) inhibitor but also shown to target AXL and Tyro3 kinase at low nanomolar concentrations.27 BMS777607 inhibits AXL at 1.1 nM in a biochemical enzyme assay.28

MPCD84111 is a quinolinylxynphenylsulfonamides tyrosine kinase inhibitor (TKI) patented as an AXL inhibitor by the Max Planck Society under patent application example 12 from WO2011045084.

Our results prove that inhibition of migration by AXL inhibitors is independent of AXL kinase phosphorylation. Additionally, we highlight the importance of multikinase inhibition in the therapy of TNBC.

Results

BMS777607, SKI606 and MPCD84111 exhibit different potential on AXL RTK inhibition

Overexpression of the RTK AXL and its implication in the migration of TNBC cell lines has already been demonstrated in publications and also by our studies (Fig. S2 and S3).12,18,29 To determine the impact of 2 potential (BMS777607 and SKI606) and one patented (MPCD84111) AXL TKI on AXL phosphorylation we used 3 AXL-expressing TNBC cell lines.12,30,31

The IC_{50} values of AXL phosphorylation, were measured after 1 h of inhibitor treatment and subsequent Gas6 (250 ng/ml) stimulation. MPCD84111 displayed IC_{50} values of 0.21 μM in MDA-MB-231, 0.30 μM in BT549 and 0.04 μM in Hs578T cells (Fig. 1A, D, G; Fig. S1A-C). SKI606 was less efficient in comparison to MPCD84111 and BMS777607 having an IC_{50} value of >1 μM in MDA-MB-231 and BT549 and 0.94 μM in Hs578T cells (Fig. 1B, E, H; Fig. S1A-C). BMS777607 inhibited the phosphorylation of AXL with IC_{50} values of 0.16 μM in MDA-MB-231, of 0.17 μM in BT549 and of 0.005 μM in Hs578T cells (Fig. 1C, F, I; Fig. S1A-C). In conclusion, the strongest inhibition of AXL phosphorylation was achieved by BMS777607 followed by MPCD84111 and SKI606.

The most potent AXL inhibitor BMS777607 does not inhibit significantly the migration and viability of TNBC cells

We determined the inhibitory effect of BMS777607, MPCD84111 and SKI606 on cell migration using Boyden chamber assays. The IC_{50} value for MPCD84111 was 0.535 μM in MDA-MB-231 (Fig. 2A), 2.9 μM in Hs578T (Fig. 2D) and 0.862 μM in BT549 (Fig. 2G) cells. SKI606 inhibited the migration with an IC_{50} value of 0.421 μM in MDA-MB-231 (Fig. 2B) of 0.971 μM in Hs578T (Fig. 2E) and 0.627 μM in BT549 (Fig. 2H) cells. In contrast to SKI606 and MPCD84111, BMS777607 had an IC_{50} value of 1.39 μM in MDA-MB-231 (Fig. 2C), >3.125 μM in Hs578T (Fig. 2F) and >3.125 μM in BT549 (Fig. 2I) cells. Comparing the IC_{50} values we found that Hs578T cell were less sensitive to TKI treatment than MDA-MB-231 cells. Among the 3 analyzed AXL TKIs SKI606 had the lowest IC_{50} value for inhibition of migration followed by MPCD84111 and BMS777607.

Additionally we analyzed the effect of AXL TKIs on cell viability. As illustrated in Figure 3, SKI606 and MPCD84111 had a significant inhibitory effect on Hs578T and BT549 cell viability. MPCD84111 exhibited an IC_{50} value of 1.14 μM in Hs578T and 2.03 μM in BT549 (Fig. 3D and G), SKI606 showed an IC_{50} value of 2.85 μM in Hs578T and 1.27 μM in BT549 cells (Fig. 3E and H) and BMS777607 had an IC_{50} value of 9.43 μM in Hs578T cells and >10 μM in BT549 cells (Fig. 3F and I). The MDA-MB-231 cell line was less sensitive to TKI treatment than Hs578T, with IC_{50} values of 8.50 μM for MPCD84111 and 3.20 μM for SKI606, more than 10 μM for BMS777607 (Fig. 3A-C).

The inhibitory concentrations for migration and viability were higher than the IC_{50} value for inhibition of the AXL kinase activity, suggesting that the effect on migration and viability is not mediated through AXL RTK.

SKI606 and MPCD84111 display similarities in their target profile

Next, we wanted to understand why SKI606 and MPCD84111 stronger block the migration of the TNBC cell lines than BMS777607. Therefore we performed a Cellular Target Profiling assay in Hs578T cell lysates to determine the targets of the compounds. The target spectrum of BMS777607 was reported by Schroeder et al.,28 therefore we focused on the characterization of SKI606 and MPCD84111.

The Cellular-Target-Profiling assay revealed that SKI606 mainly binds to the SRC-family kinases (Lyn, Src, Fyn and Yes), MAPK-family members, Ephrins and some of the PKC-family members. MPCD84111 displays a strong affinity for AXL, MET and Aurora B kinase and similarly to SKI606 binds to Lyn, Yes and Src (Table 1). Between the 2 analyzed inhibitor SKI606 has with 40% more target than MPCD84111. This result is not surprising in the context that SKI606 potentially inhibits 148 kinases with a K_{i}-value below 1 μM.32 After comparing these data to the target profile of BMS777607,28 we concluded that BMS777607 and MPCD84111 possess common targets such as Met and Aurora B. Binding to AXL kinase was confirmed for all 3 TKIs.

On the basis of Cellular Target Profiling assay and Ambit phage based competition binding assay data, 84 kinases were selected to further examine the compounds inhibitory potential in Kinase Profiler assay by Millipore, at 1 μM concentration. As expected from the results of the Cellular target profiling assay results, the Src family kinases and few other kinases (Abl, PTK5, EGFR, ErbB4, Lok, Tie2) had less than 50% activity after treatment with SKI606 and MPCD84111, whereas BMS777607 and MPCD84111 generally affected the Met-related kinases (Met, Ron) and the Aurora kinases (Aurora A/B/C) (Fig. 4). In conclusion, the profiling assays revealed that SKI606 and MPCD84111 commonly affect migration related kinases such as Src-family kinases, which might explain their significant effect on cell migration.
Lyn and p130Cas affect the migration of triple negative breast cancer cells

Among the Src-family kinases targeted by SKI606 and MPCD84111, Lyn is the only one being significantly overexpressed in TNBC cell lines. Based on these results, we selected Lyn to analyze its function on the migration and viability of MDA-MB-231, Hs578T and BT549 cells. It is known that Lyn interacts with one of the members of the focal adhesion complex, namely p130Cas. Therefore, we analyzed p130Cas function in the migration and viability of MDA-MB-231, Hs578T and BT549 cells. To evaluate a functional connection between the selected proteins and cell migration, we used a siRNA knockdown. The siRNA knockdown efficiency was validated by Western blot analysis (Fig. 5C).

The knockdown of Lyn and p130Cas led to a significant decrease in the migration of all cell lines (Fig. 5A). In MDA-MB-231 and Hs578T cells, knockdown of Lyn had no significant impact on cell viability while the knockdown of p130Cas showed a clear effect (Fig. 5B). In BT549 cells, all siRNA treatments had a significant effect on cell viability (Fig. 5B).
Effect of AXL receptor tyrosine kinase inhibitors on intracellular migration related pathways in triple negative breast cancer cells

In the next step we characterized the effect of AXL TKIs on migration related signaling pathways. Serum starved cells were treated with 1 μM of SKI606, BMS777607 or MPCD84111 for 1.5 or 48 h in serum free medium.

As illustrated in Figure 6, MPCD84111 caused an inhibition of phosphorylation of Lyn, Pyk2, FAK, p130Cas in all cell lines. There was no significant impact on phosphorylation of c-Src, showing that the inhibition of the FAK-p130Cas-complex is not dependent on phospho-c-Src in these cell lines. SKI606 had an inhibitory effect on the phosphorylation of Pyk2, FAK, p130Cas and Lyn comparable to

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**Figure 2.** Effect of AXL TKIs on the migration of TNBC cell lines. The impact of MPCD84111, SKI606 and BMS777607 on cell motility was analyzed with Boyden chamber assay. The cells were allowed to migrate toward to the 1% FCS containing lower chamber for 3.5 h. MDA-MB-231 (top) and BT549 (bottom) were more sensitive to TKI treatment than Hs578T cells (middle). Among the 3 analyzed AXL TKIs SKI606 had the lowest IC50 value for migration inhibition. The IC50 values were determined as percentage of the untreated DMSO control. The results shown are means and standard deviation calculated from 3 independent experiments.
In contrast to MPCD84111, SKI606 inhibited c-Src Tyr 416. There were no significant changes in the phosphorylation of Lyn after 1.5 h of treatment with MPCD84111 and SKI606 in Hs578T and BT549 cells. BMS777607 led to a less significant inhibition of Lyn, Pyk2, FAK and p130Cas phosphorylation in all cell lines. At the same time BMS777607 was proven to inhibit c-Src in Hs578T cells after 1.5 h of treatment but finally the phosphorylation of c-Src recovered after 48 h (Fig. 6B).

These results are consistent with our findings that SKI606 and MPCD84111 inhibit cell migration due to inhibition of essential migration related kinases such as p130Cas or Lyn.
Discussion

Triple negative breast cancer, which accounts up to 10% to 27% of breast cancer cases, is characterized by the absence of HER2, estrogen and progesterone receptor and by elevated phosphorylation of AXL, FAK, Met, Lyn, p130Cas and EGFR.19,36,37

In this study we analyzed the efficacy of 3 AXL TKIs BMS777607, MPCD84111 and SKI606 exhibiting different selectivity profiles28,32 on the migration and viability of AXL overexpressing TNBC cell lines,30,31,38 namely MDA-MB-231, Hs578T and BT549.

Several studies demonstrated that the inhibition of AXL expression by AXL specific siRNAs attenuates the migration of TNBC cell lines.12,18,39 Although these studies established the role of AXL protein in promoting TNBC cell migration, the phosphorylation status of AXL in breast cancer cell migration has not been clarified. In this study we elucidate the function of the AXL phosphorylation on the migration of TNBC cells. Therefore we used a selective AXL inhibitor BMS777607 and 2 AXL targeting multikinase inhibitor, SKI606 and MPCD84111 in our experiments. We show that in contrast to SKI606 and MPCD84111, the most specific and efficient AXL inhibitor BMS777607 was not capable to block the migration of TNBC cells. The inhibition values of migration were significantly higher than those determined for inhibition of AXL phosphorylation, suggesting that migration of the 3 studied TNBC cell lines does not depend on AXL phosphorylation. Similar to our study Tan et al. analyzed the inhibitory potential of SKI606 on the migration of non small lung cancer cell lines. They showed that the inhibitory effect of SKI606 on migration was independent from AXL RTK.40 This study is in line with our conclusion that efficiency of SKI606 and MPCD84111 is not correlated with the migration and invasion inhibition by the AXL inhibitor R428 can be observed in the study of Holland et al., were the IC50 value for invasion was 3000 nM whereas the IC50 value for AXL phosphorylation inhibition was more than 200 times lower exhibiting a value of 14 nM.41

Table 1. Target profiles of SKI606 and MPCD84111. Tables show the Kd values of the proteins targeted by SKI606 (A) and MPCD84111 (B) in Hs578T cell lysates

| Protein names SKI606 | Protein names MPCD84111 |
|----------------------|------------------------|
| ABL1            | ABL1                   |
| ACRV1/ALK2       | ACRV1                 |
| AURKA            | AURKA                 |
| AURKB            | AURKB                 |
| AXL              | AXL                    |
| BMP2R            | BMP2R                 |
| CaMK2γ           | CAMK4                 |
| CaMK2b           | CSK                   |
| CDK1             | DDR1                  |
| CDK5             | DDR2                  |
| CDK5L            | EIF2AK4/GCN2          |
| CK1α             | FAK                   |
| CK1b             | FER                   |
| CK1c             | FYN                   |
| CLK1             | LIMK1                |
| CSK              | LOK                   |
| DDR2             | LYN                   |
| EphA2            | MAP3K11/MLK3          |
| EphA5            | MAP5K2               |
| EphB2            | MAP3K5               |
| EphB3            | MAP3K4/MEK4K         |
| EphB4            | MAP4K2               |
| FAK              | MAP4K3/MLK3          |
| FER              | MAP4K4/NIK           |
| FRK              | MAP4K5/KHS1           |
| FYN              | MARK2                |
| GAK              | MARK3                |
| GSK3α            | MET                   |
| GSK3β            | MINK1                |
| ICK              | MST1/STK4            |
| ILK              | MST2/STK3            |
| JAK1             | PDGFβB               |
| LYN              | PLK4                 |
| MAP2K1           | PRKAA1               |
| MAP2K2           | PRKAA2               |
| MAP2K3           | RIK2                 |
| MAP2K6           | SRC                  |
| MAP3K1           | TAOK1                |
| MAP3K2           | TAOK2                |
| MAP3K4           | TGFBR1               |
| MAP4K2/GCK       | TGFBR2               |
| MAP4K3/GLK/KHS2  | TNK2                 |
| MAP4K4/HKG       | YES1                 |
| MAP4K5/KHS1      | ZAK                  |
| MER              | 9.41              |
| MINK1            | 0.106             |
| MLK3             | 14.35            |
| MST1             | 3.57              |
| MST2             | 12                 |
| MYT1             | 0.965             |
| p38/MAPK14       | 13.47           |
| PFTK1            | 9.66              |
| PKα a            | >12.62           |
| PKα b            | >14.82           |
| PKC α            | >8.04            |
| PKC δ            | 5.72             |
| PKC ε            | >13.05          |

(Continued on next page)
However association and transactivation of AXL with EGFR and c-Met would limit the effects of targeted therapies.\(^{44,45}\)

To comprehend the efficiency of SKI606 and MPCD84111 on the migration of TNBC cells we examined the targets of SKI606 and MPCD84111. Based on our experiments we concluded that SKI606 has 40% more cellular targets than MPCD84111 but still both compounds commonly affect the migration related Src family kinases (Table 1 and Fig 4). This could explain their significant inhibitory effect on the migration of TNBC cell lines. Among the Src family kinases targeted by SKI606 and MPCD84111 (Yes, Fyn, Hck, Fgr, c-Src, Blk, Lyn), we analyzed the role of Lyn. Lyn has been described as an important target in hematopoietic and solid tumors including TNBC, and was proven to be one of the most overexpressed kinase in TNBC cell lines.\(^{29,33}\) Lyn plays an important role in the migration of cancer cells and was shown that can interact with p130Cas, one of the member of the focal adhesion complex.\(^{34}\) Association of p130Cas with motility, invasion and survival of TNBC cells has been shown before by several publications.\(^{29,46,47}\)

Therefore additionally we analyzed the function of p130Cas in the migration and viability of TNBC cells. We proved that the siRNA-mediated knockdown of Lyn and p130Cas attenuated the migration of all 3 TNBC cell lines. Consequently our results underline the importance of Lyn and p130Cas in the migration of TNBC cell lines. At the same time the AXL TKIs SKI606 and MPCD84111 inhibit the phosphorylation of Pyk2, FAK, p130Cas and Lyn all 3 TNBC cell lines, while BMS777607 had no effect on these signaling molecules. This might explain why BMS777607, being the most efficient AXL TKI published until now, does not inhibit migration of TNBC cells. Despite other reports we show that Src inhibition attenuates breast cancer cell migration,\(^{48,49}\) our study proves that the strong inhibitory effect on migration exerted by MPCD84111 does not depend on the inhibition of c-Src phosphorylation. Thus, the migration of TNBC cell lines described here does not depend on the activity of c-Src kinase but on other tyrosine kinases such as p130Cas and Lyn.

We also demonstrate that viability inhibition is not correlated with the migration attenuation, as the growth inhibitory concentrations of the investigated inhibitors (MPCD84111, BMS777607 and SKI606) were significantly higher than the IC\(_{50}\) of migration inhibition. Thus we exclude viability inhibition being responsible for the reduction of cell migration in TNBC cells by the analyzed AXL inhibitors.

The present study reveals that efficacy of SKI606 and MPCD84111 to block TNBC cell migration is independent from AXL kinase phosphorylation. Although we cannot exclude AXL important role in modulation of tumor associated vasculature and immune cell function\(^{43}\) and modulation of natural killer cells by the TAM receptors in vivo.\(^{50}\) Our data show that SKI606 and MPCD84111 inhibit breast cancer cell migration through migration related kinases, namely Lyn and p130Cas. However tumor cell migration can not be blocked completely by inhibition of Lyn or p130Cas (Fig. 5). Our results underline previous findings that TNBC cell lines are not addicted to a single signaling pathway.\(^{29}\) Based on these findings we conclude that the use of multi-kinase inhibitors such as SKI606 and MPCD84111 which target migration related kinases might improve the treatment of TNBC.

### Table 1. Target profiles of SKI606 and MPCD84111.

| Protein names | SKI606 | Protein names | MPCD84111 |
|--------------|--------|--------------|-----------|
| QIK          | 0.281  |              |           |
| QSK          | 0.64   |              |           |
| RPS6KA1      | >13.17 |              |           |
| SLK          | 0.3    |              |           |
| SRC          | 0.00018|              |           |
| TBK1         | 5.38   |              |           |
| TNNK2/ACK    | 0      |              |           |
| TVK2         | 13.42  |              |           |
| ULK3         | 5.89   |              |           |
| VRK2         | >6.7   |              |           |
| YES1         | 0.0029 |              |           |
| ZAK          | 0.55   |              |           |

### Material and Methods

**Cell lines**

Experiments were performed on 3 different triple negative basal breast cancer cell lines. MDA-MB-231 cells were obtained from Stefano Iacobelli (Department of Oncology and Experimental Medicine, University Foundation ‘G. D’Annunzio’ Chieti-Pescara, Chieti, Italy) Hs578T cells were a kind gift from U3 Pharma (Martinsried, Germany) and BT549 cells were obtained from the American Type Culture Collection. MDA-MB-231 cells were grown at 37°C in Dulbecco’s modified Eagle’s medium (GIBCO, Invitrogen, Cat no: 41965-039) supplemented with 1% sodium pyruvate (GE Healthcare, Cat no: 11360-039.), Hs578T and BT549 cells in RPMI 1640 medium (GIBCO, Invitrogen, Cat no: 31870-025), supplemented with 1% glutamine (GIBCO, Invitrogen Cat no: 25030-081) and the medium of the BT549 cells with 0.001% human insulin (GE Healthcare). All media were supplemented with 10% fetal calf serum (FCS, GIBCO, Cat. no. 16000-044), 100 U/ml penicillin, and 100 μg/ml streptomycin (PAA, Cat. no: P11-010). All cells were maintained at 37°C in a humidified atmosphere containing 5% CO\(_2\).
Figure 4. The kinase selectivity profile of MPCD84111, BMS777607 and SKI606. The heatmap shows the activity of 84 kinase after treatment with 1 µM of MPCD84111, BMS777607 and SKI606. The scale indicates colors corresponding to kinase activities.
antisense: 5’AUAGUCAUAAACUUCGUUAGt3’ for BCAR1 18372 siRNA was sense: 5’GGAUGGAGGACUAUGACUAtt3’; antisense: 5’UGAGUGAUUGCUUACUGAGCCAGAtt3’; antisense: 5’UCUGGAGAUGCUUACUGAGCCAGAtt3’; for Lyn 8356 siRNA was sense: 5’CAAGAGGAUAAUUGAGAUAAtt3’; antisense: 5’UUAGUCUAAACUUCGUUAGt3’ for Lyn 277 siRNA was sense: 5’GGAACAGAGACAUUUUGGt3’; antisense: 5’CAACAGAGACAUUUUGGt3’. Ambion control # 1 siRNA was used as a control. Briefly 120,000 cells were plated in 6 well plates (Corning) and after 24 h transfected with a final concentration of 40 nmol siRNA for 48 h. The Lipofectamine RNAiMAX was obtained from Invitrogen (Cat. no. 13778-075) and used according to the manufacturer’s instruction.

Tyrosine kinase inhibitors
SKI606 and MPCI84111 were obtained from Vichem Chemie (Budapest, Hungary). MPCI84111 is patented under application example 12 from WO2011045084. BMS777607 was a kind gift from LDC Discovery Center GmbH (Dortmund,
Germany). All inhibitors were dissolved in DMSO and stored at room temperature in 10 mM stock solution.

**Cellular profiling assay**

The assay was performed by Kinaxo on s578T cell lysates. The assay combines different technology platforms, such as the stable isotope labeling by amino acids (SILAC) in cell culture or isobaric tags for relative and absolute quantification (ITRAQ). These technologies are used together with affinity-based separation methods with data showing the binding affinities of the compound to the corresponding targets. The assay is described in detail in the publication of Contradt et al., published in 2011.51

**Kinase profiler assay**

The assay was performed according to the manufacturer instruction by Millipore on 84 kinases with 1 μM compound concentration.

**Cell viability and migration assay**

We measured cell viability using a luciferase-coupled ATP quantitation assay (CellTiter-Glo; Promega, Cat no: G7571). Cells were dispensed at 1000 cells/100 μl/well in tissue-culture treated 96-well white bottom assay plates (PerkinElmer). After 24 h incubation the cells were treated with the indicated inhibitor concentration from the inhibitor dilution, except the siRNA treated cells. After inhibitor addition, plates were incubated for 72 h at 37°C in humidified atmosphere containing 5% CO2.

At the end of the incubation period, CellTiter-Glo reagent was added, to the contents of the plates, mixed by an orbital shaker for 2 min and incubated for 15 min. The luminescent signal was recorded using a Microplate Luminometer LB96V (Berthold Technologies). The experiments were performed at least 3 times.

The motility of the cells was determined by Boyden chamber assay. 50,000 cells were plated on top of a membrane with 8 μm pores (BD Transduction Laboratories, Heidelberg, Germany) in 300 μl of medium with 0.1% of FCS. As chemoattractant 1% FCS in 700 μl of medium was used. After incubation for 3.5 h at 37°C the cells remaining in the insert were removed with a cotton swab and the cells on the bottom of the filter were fixed with 0.05% Crystal violet/20% methanol (Sigma, Cat no: 32213-2). The cells which had traversed the membrane were counted and images were taken with 10 x objectives under brightfield illumination using a charge-coupled device camera-mounted on a Axiovert 300 Microscope (Zeiss) and analyzed with MetaVue software. At least 5 random fields were counted per well.

**Western blot analysis**

Starved cells were incubated with 1 μM of indicated inhibitor concentration for 1.5 or 48 h 100 micrograms of total protein lysate were denatured with sample buffer containing SDS and 2-mercaptoethanol at 95°C for 5 min and electrophoretically separated on a 4% to 12% polyacrylamide gel, transferred to a nitrocellulose membrane, and blocked with NET-gelatine. Antibodies raised against p-Lyn (Tyr507) # 2731, Lyn # 2732, p-Src (Tyr 416) #2101, p-Pyk (Tyr 416) #3291, p FAK (Tyr576/577) # 3281, p130Cas (Tyr 410) #4011, p130 Cas # 13846 were obtained from Cell Signaling Technologies. Anti AXL antibody # sc-1096 was purchased from Santa Cruz. Anti tubulin # T1926 was used from Sigma- Aldrich. The antibodies were used at 1:2,000 dilutions with overnight incubation at 4°C in NET-gelatine. The secondary antibodies HRP-coupled goat anti-rabbit (Bio-Rad) and goat anti-mouse (Sigma) were used at 1:10,000 dilutions in NET-gelatine. Detection of bound antibodies was performed by using an ECL substrate reaction (Perkin Elmer, Cat no: NEL103001EA) and exposed to Kodak X-Omat (Amersham Biosciences) film.

**Immunoprecipitation**

The homemade anti AXL capture antibody was pre-coupled to 40 μl A-Sepharose beads (GE Healthcare) in lysis buffer for 1 hour. After incubation were washed 3 times with lysis buffer. Cell lysates and pre-coupled antibody-beads were incubated at 4°C for 16 hours. The precipitates were washed 3 times with 1 ml lysis buffer, suspended in Laemmli buffer and boiled for 10 minutes. The AXL protein phosphorylation was analyzed by western blot using the homemade anti-p-Tyr clone 4G10 antibody. The total level of AXL was detected by using the anti-AXL

![Figure 6. Effect of AXL RTK inhibitors on intracellular signaling pathways. Immunoblots of lysates of MDA-MB-231 (A), Hs578T (B) and BT549 (C) starved cells treated with 1 μM of MPCD84111, SKI606 or BMS777607 for 1.5 or 48 h using antibodies against phospho-Pyk2 (pY580), phospho-FAK (pY576/577), phospho-p130Cas (pY410), phospho-Lyn (pY507), phospho-Src (pY416) and Tubulin (represents the loading control).](image-url)
concentrations for 1 h. After 1 h of the incubation with the inhibitors the cells were stimulated with 250 ng/ml Gas6 (R&D Systems) for 30 min. Cells were lysed on ice in 400 µl lysis buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EGTA, 10% Glycerol (Roht, Cat. no: 4043.2), 1% Triton X-100 (Roht, Cat. no. 668), 100 mM NaF, 10 mM Na₃P₂O₇·10 H₂O, 1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, and 10 mg/ml aprotinin) for 15 min.

96-well Nunc MicroWell™ plates (Fischer Scientific GmbH) which had been coated overnight with homemade anti-AXL capture antibody 2 µg/ml (clone 259/2, IgG1 isotype) in PBS (100 µl/well) were blocked with PBS-0.05% Tween®20 (Sigma, P1379-1) + 10% FCS for 4 h at 37°C. Plates were washed 5 times with PBS/0.05% Tween®20 and 95 µl of lysis was transferred per well for overnight incubation at 4°C. Plates were washed 5 times with PBS/0.05% Tween®20 (Sigma) For detection of phosphorylated tyrosine we used homemade biotynilated antibody 2 µg/ml (clone 259/2, IgG1 isotype) in PBS 10% FCS (100 µl/well) and incubated overnight with homemade anti-AXL capture antibody 2 µg/ml (clone 259/2, IgG1 isotype) in PBS (100 µl/well) for 15 min at room temperature. The anti-phosphotyrosine mouse monoclonal antibody 4G10 was biotinylated with Sulfo-NHS™-Biotin according to the suppliers protocol (Pierce) and purified by Micro Bio-Spin 6 Chromatography Columns (BIO RAD Laboratories) using PBS as diluent. Plates were washed 5 times with PBS/0.05% Tween®20. For binding to biotin alkaline phosphatase-conjugated streptavidin SA110 (Millipore) (1:4,000) was used in PBS/0.05% Tween®20 + 10% FCS (100 µl/well) and incubated for 30 min at room temperature. Plates were washed 5 times with PBS-0.05% Tween®20. For fluorometric detection of alkaline phosphatase AttoPhos Substrate Set (Roche Diagnostics GmbH) was used (100 µl/well). The fluorimetric signal was quantified after 90 min at 430/560 nm wavelength using a TECAN Ultra Evolution plate reader (Tecan Deutschland GmbH).

**Statistical and data analysis**

Assays were performed as biological triplicates, comparing inhibitor treated cell to DMSO control. IC₅₀ values were determined from dose response curve generated by XLfit5.1.0 software (IDBS). For statistical analysis Dunnett’s Multiple Comparison Test was performed using GraphPad Prism 5 (GraphPad Software, Inc.). At values p < 0.0001 differences were considered statistically significant.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**Supplemental Material**

Supplemental data for this article can be accessed on the publisher’s website.

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