Impact of AXL tyrosin kinase inhibitors on migration and resistance mechanisms of cancer cells

PhD thesis

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1. Introduction

Treatment of cancer is one of the biggest challenges of medicine. Based on the results of the past years research it became evident that mutation, malformations in the DNA can lead to the formation/appearance of malignant cancer cells. These errors in the DNA through the regulation of certain signaling pathways can contribute to the proliferation, survival and spreading of the cancer cells. Until now cancer was treated mainly with conventional therapy (chemotherapy, radiation and surgery), but nowadays targeted therapies are widely used, due to their effectiveness. Tyrosine kinases are proved to be one of the most promising targets in the therapy of cancer. As they are main members of malfunctioned signaling pathways and they are druggable.
Currently most of the FDA approved monoclonal antibodies and small molecule inhibitors, target tyrosine kinases.

1.1. AXL receptor tyrosine kinase role in cancer/ as target in cancer therapy

The AXL receptor tyrosine kinase is one member of the TAM receptor tyrosine kinase family. The activation of AXL occurs when the growth arrest specific 6 (Gas6) ligand binds to its extracellular domain, which results in the activation of downstream signaling pathways such as mitogen activated protein kinases (MAPK), phosphatidylinositol 3-kinase (PI3K)- AKT and NF-KB (Nuclear Factor Kappa B). Overexpression of AXL has been reported from several cancer type including breast, gastric, prostate, lung, ovarian, colon, liver and glioma. Overexpression of AXL has been shown to correlate with motility, invasiveness and poorer prognosis.
Recently several studies reported that AXL is overexpressed and is activated in many drug resistant cancer cell lines such as Lapatinib-resistant HER2 positive breast tumor cells, Imatinib-resistant

gastrointestinal stromal tumors, Erlotinib resistant EGFR mutant lung cancer cells and xenografts and Nilotinib-resistant chronic myeloid leukemia cells.
These findings turn AXL receptor tyrosine kinase to a promising target to fight cancer.
Among the known strategies for targeting AXL, specific AXL inhibition with small molecule inhibitors is the most promising and established. Most of the small molecule inhibitors were originally designed for other targets, but they are also target AXL RTK very efficiently.

2. Aim of the study

Although there are several publication showing AXL important role in the migration of cancer cells and drug resistance, the exact role of AXL in cancer cell migration and drug resistance had not been explored in detail. In the first phase of my PhD work I analyzed the impact of AXL tyrosine kinase inhibitors on the migration of triple negative breast cancer cell lines.
With our experiments I wanted to answer the following questions:

1) What is the role of AXL expression and phosphorylation in the migration of triple negative breast cancer cells?
2) What kind of differences are between the mechanism of action and migration inhibition efficacy of the analyzed AXL inhibitors?

In the second part of my PhD work we analyzed the resistance mechanism against AXL inhibition in AXL overexpressing cancer cells from different type of tumors. We focused especially on the effect of AXL inhibition on the activation of other receptor tyrosine kinases and on possible resistance mechanisms.
With our experiments we wanted to answer the following questions:

1) How does AXL kinase inhibition affect the phosphorylation pattern of other receptor tyrosine kinases?

2) What kind of combination treatment is necessary to prevent resistance mechanism towards AXL inhibitors?

3. Methods

Cell lines

In our experiments we used AXL overexpressing cell lines from different type of tumors: breast (MDA-MB-231, MDA-MB-231-D3H2LN, Hs578T, BT549, MDA-MB-436, MDA-MB-468), glioma (A172, U373, SF126. U118), lung (H1975, H1299, H292, A549), pancreas (AsPC-1, BxPC3, Capan2), ovarian (Ovcar8, Scov3), cervix (C4-I) and fibroblast (NIH/3T3).

Compounds and ligands

Lapatinib, SKI606 and MPCD84111 were obtained from Vichem Chemie, BMS777607 and R428 were kind gifts from LDC Lead Discovery Center GmbH. Herceptin and Erbitux were purchased from the Max-Planck Pharmacy in Martinsried. The recombinant human NRG1 (#396-HB-050) and Batimastat (BB94, #2961) were purchased from R&D Systems, GmbH, Germany

Short interfering RNA transfections

AXL, Lyn, Met, EGFR, HER2, HER3 and p130Cas short interfering RNAs were used for siRNA experiments. Ambion control # 1 siRNA was used as a control. Briefly 120000 cells were plated in six well plates and after 24 h transfected with a final concentration of 40 nmol siRNA for 48 h. The Lipofectamine RNAiMAX was used according to the manufacturer`s instruction.

Cell viability and migration assay

Cell viability was determined using CellTiter-Glo assay, according to the manufacturer `s instructions.
The motility of the cells was determined by Boyden chamber assay. Briefly 50,000 cells were plated on top of a membrane with 8 µm pores in 300 µl of medium with 0.1% of FCS. As chemoattractant 1% FCS in 700 µl of medium was used. After 3.5 h incubation the cells which traversed the membrane were fixed and counted.

SDS PAGE and Western blot analysis

Samples were separated on a 4 % to 12 % polyacrylamide gel, transferred to a nitrocellulose membrane, and blocked with NET-gelatine.
Membranes were incubated against p-Lyn (Y507), p-Src (Y 416), p-Pyk (Y 580), pFAK (Y576/577), p130Cas (Y410), pHER3 (Y1289), pHER2 (Y1248), EGFR, pAKT (S473), HER2 HER3 , Met and Tubulin. The secondary antibodies HRP-coupled goat anti-rabbit and goat anti-mouse were used at 1:10,000 dilutions in NET-gelatine. Detection of bound antibodies was performed by using an ECL substrate reaction and exposed to Hyperfilm ECL

Human Phospho-RTK array

Human Phospho-RTK array was utilized according to the manufacturer`s instructions.
500 µg of lysates were incubated with blocked array membranes overnight. Detection was performed using enhanced chemiluminescence.

Immunoprecipitation

HER2 and HER3 antibodies were pre-coupled to A-Sepharose beads.
Lysates and pre-coupled antibody-beads were incubated at 4 °C for 16 hours. The precipitates were analyzed by western blot.

3D spheroid culture

Matrigel™ Matrix Basement membrane was diluted at a concentration of 3 mg/ml. Inhibitor treatments was initiated at the moment of cell seeding into Matrigel.

Quantitative PCR

MDA-MB231 cells were treated with 10 µM of BMS777607 or DMSO for the indicated periods of time. RNA preparation and first-strand cDNA synthesis were performed according to the manufacturer`s protocol. For cDNA synthesis, 1 µg of total RNA and 200 ng of random hexamer primer were used. The PCR was carried out on a StepOnePlus instrument according to the manufacturer`s instruction.

Phospho-Axl ELISA

Starved cells were treated with inhibitor for 1 h and subsequently stimulated with 250 ng/ml Gas6 for 30 min. 96-well plates were coated with homemade anti-AXL capture antibody 2 µg/ml (clone 259/2, IgG1 isotype). Cell lysates were incubated for overnight at 4°C. For detection of phosphorylated tyrosine we used homemade biotinylated 4G10-antibody (0.5 µg/ml) for 2 h at room temperature. For fluorimetric detection of alkaline phosphatase AttoPhos

Substrate Set was used. The fluorimetric signal was quantified at 430/560 nm wavelength.

NRG1-ELISA and Phospho-AKT S473 ELISA

We used a specific human NRG1-ELISA and a Pan AKT-specific ELISA kit to quantify protein amounts according to the manufacturer`s protocol with the following modifications: After the incubation with a biotinylated detection specific antibody we used an alkaline phosphatase-conjugated streptavidin SA110 at room temperature. For fluorometric detection of alkaline phosphatase AttoPhos Substrate Set was used. The fluorometric signal was quantified after 90 minutes at a wavelength of 430/560 nm.

Statistical and Data Analysis

All assays were performed as biological triplicates and values from the inhibitor treated cell always were compared to DMSO treated control cells. IC_{50} values were determined from dose response curve generated by XLfit 5.1.0 software and four parameter curve fitting. For statistical analysis we used the Dunnett`s Multiple Comparison Test, a Mann-Whitney test or a Kruskal-Wallis test in combination with Dunn`s multiple comparison posttest using GraphPad Prism 5. In the case of Dunnett's Multiple Comparison Test at values $p < 0.0001$, whereas in the case of Mann-Whitney test or a Kruskal-Wallis test differences with * = $p<0.05$, ** = $p<0.01$ and *** = $p<0.001$ were considered as statistically significant.

4. Results

4.1. The effect of AXL tyrosine kinase inhibitors on the migration of triple negative breast cancer cells

In order to determine the impact of two potential (BMS777607 and SKI606) and one patented (MPCD84111) AXL tyrosine kinase inhibitors on AXL phosphorylation we selected three AXL-expressing TNBC cell lines (MDA-MB-231, Hs578T and BT549). We examined the effect of AXL tyrosine kinase inhibitors on AXL phosphorylation by treatment of cell lines with different inhibitor concentrations for 1 hour and subsequent stimulation with Gas6 for 30 minutes. The compounds inhibited the phosphorylation of AXL at different concentrations. The strongest inhibition for AXL phosphorylation was achieved by BMS777607, followed by MPCD84111 and SKI606. Among the analyzed cell lines, Hs578T was proven to be the most sensitive cell line to AXL phosphorylation inhibition. In order to analyze the effect of AXL tyrosine kinase inhibitors on the migration we used a Boyden chamber assay. The traversed cells were counted after 3.5 hour incubation. Comparing the inhibitors migration IC_{50} values we discovered that SKI606 had the lowest IC_{50} value for inhibition of migration in all three cell lines.

Among the three analyzed cell lines Hs578T cell was less sensitive to kinase inhibitor treatment than MDA-MB-231 and BT549 cells.

To determine the effect of AXL tyrosine kinase inhibitors on cell viability we treated the cells with ninefold serially diluted inhibitor concentrations and luminescent signal was measured after 72 hour incubation. SKI606 and MPCD84111 had a significant inhibitory effect on Hs578T and BT549 cell viability, whereas BMS777607 exhibited an IC_{50} of 9.43 μM in Hs578T cells and >10 μM in BT549 cells. The inhibitors had no significant effect on MDA-MB-231 cell

line viability, exhibiting IC_{50} values of 3.20 µM for SKI606, 8.50 µM for MPCD84111 and more than 10 µM for BMS777607.
As the half maximum inhibitory concentrations for migration and viability were higher than the IC_{50} value for AXL kinase activity, we concluded that the effect of the inhibitors on migration and viability is not depending on AXL receptor tyrosine kinase. Next, we tried to understand why SKI606 and MPCD84111 more effectively block the migration of the triple negative breast cancer cell lines than BMS777607. Therefore we performed a Cellular Target Profiling assay on Hs578T cell lysates to determine the targets of the compounds. As the target spectrum for BMS777607 was already reported by Schroeder et al. 2009, we focused only on the characterization of SKI606 and MPCD84111. The results from the Cellular profiling assay revealed that SKI606 and MPCD84111 commonly target the SRC family kinases such as Lyn, Src and Yes.
Based on the inhibitors target profile, 84 kinases were selected to further analyze the inhibition potential of the three AXL tyrosine kinase inhibitors in parallel. The Profiler kinase assay data underlined our previous findings, that SKI606 and MPCD84111 efficiently target Src family kinases and few other kinases (Abl, PTK5, EGFR, ErbB4, Lok, Tie2). These results imply that SKI606 and MPCD84111 significant effect on cell migration can be due that they inhibit SRC family members. Among the Src family kinases targeted by SKI606 and MPCD84111, Lyn is the only one being significantly overexpressed in all the three cell lines. Based on this finding we selected Lyn and one migration related scaffold protein, p130Cas, to analyze their effect on the migration and viability of triple negative breast cancer cell lines. The knockdown of Lyn and p130Cas led to a significant decrease in the migration of all studied cell lines. The knockdown of Lyn had no significant effect on the viability of Hs578T and MDA-MB-231. While knockdown of p130Cas significantly reduced the viability of these cell lines. The viability of BT549 cells was attenuated by both siRNA treatments.

To characterize the effect of AXL inhibitors on the migration related signaling pathways, we treated the serum starved cells with 1 μM SKI606, BMS777607 and MPCD84111 for 1.5 and 48 h.
The Western blot results confirmed our previous findings, as SKI606 and MPCD84111 inhibited the phoshorylation of Pyk, FAK, p130Cas and Lyn in all the three cell line. While BMS777607 had no significant effect on the phosphorylation of this kinases.
These data represent strong evidence that SKI606 and MPCD84111 inhibit migration related signaling pathways.

4.2. The effect of AXL inhibition on the activation of other receptor tyrosine kinases

In the second part of our work we analyzed the effect of AXL targeted therapy on the phosphorylation pattern of other receptor tyrosine kinases.
In the first step we validated the efficiency of BMS777607 to inhibit AXL phosphorylation with a phospho AXL ELISA assay. In order to determine the effect of AXL inhibition on the activation pattern of other receptor tyrosine kinase we performed a Human Phospho-RTK array.
Therefore AXL receptor tyrosine kinase was depleted by AXL specific siRNA knockdown, whereas AXL activity was inhibited using 1 μM BMS777607 treatment. Both treatments inhibited the phosphorylation of AXL and Met, while phosphorylation of HER3 was significantly increased. This result was confirmed by immunoprecipitation and Western blot, after AXL specific siRNA and BMS777607 kinase inhibitor treatments. In the next step we examined the effect of Met on the phosphorylation of HER3, as BMS777607 is known as a Met inhibitor. Therefore we depleted Met by Met specific siRNA and we proved that Met knockdown exhibits no effect on the phosphorylation of HER3 compared to control

levels. This result excludes Met involvement in the induction of phospho HER3.

To investigate the activation of HER3 in other AXL expressing cell lines, we blocked activity of AXL by 10 μM BMS777607 and AXL by AXL specific siRNA knockdown. Interestingly the HER3 phosphorylation was induced only in MDA-MB-231 and Ovcar8 cells. To further characterize the HER3 induction after AXL inhibition we selected 20 AXL expressing cell lines originating from brain, breast, ovary, cervix, lung and pancreatic tumors. As AXL inhibition exhibits an impact on AKT signaling, we analyzed the phosphorylation of AKT S473 by an ELISA assay. In parallel we determined the phosphorylation of HER3 by Western blot.

Our results revealed a positive correlation between a low basal phosphorylation of AKT S473 and the induction of HER3 upon AXL inhibition. To explore the levels of HER3 mRNA after AXL inhibition, we performed a quantitative PCR. A significant induction of HER3 was evident after 16 h of 10 μM BMS777607 treatment. The maximum increase of HER3 mRNA was detected after 48 h. However NRG1 mRNA expression levels in MDA-MB-231 was detectable; we excluded NRG1 mRNA expression being responsible for HER3 activation.

Next we determined the NRG1 protein levels from the supernatant of BMS777607 treated MDA-MB-231 cells by NRG1 ELISA assay. Strikingly the levels of NRG1 decreased in the supernatant of 10 μM BMS777607 treated MDA-MB-231 cells in a time dependent manner. Consumption of NRG1 from supernatant correlated with the enhanced HER3 phosphorylation, suggesting a strong correlation between HER3 activation and NRG1.

As we could show that AXL inhibition by BMS777607 induces phospho HER3, we asked whether this is a common characteristic of AXL inhibitors. Therefore we compared the effect of BMS777607 with R428 and MPCD84111. The effect of inhibitors on the phosphorylation of AXL was validated with AXL ELISA assay. In

contrast to BMS777607 and R428, MPCD84111 did not induce the phosphorylation of HER3. In the same time the expression of HER3 protein levels was increased by all the three AXL inhibitors. In order to understand why MPCD84111 in contrast to BMS777607 inhibits the phosphorylation of HER3 we determined the kinase selectivity profile of MPCD84111. We found that MPCD84111 uniquely targets HER2, a possible dimerization partner of HER3. This result was also confirmed in HER2 expressing MCF7 cell line. Although triple negative breast cancer cell lines exhibiting low levels of HER2, we assumed that also in MDA-MB-231 cells HER3 dimerize with HER2. To clarify this hypothesis we examined the effect on HER3 phosphorylation of 10μM of BMS777607 in combination with Erbitux or Herceptin or Lapatinib or Batimastat. The combination treatment with Herceptin as well with Lapatinib or Batimastat inhibited the phosphorylation of HER3, whereas the combination treatment with Erbitux did not. These data present strong evidence that even in triple negative breast cancer cells HER2 is the dimerization partner of HER3. To corroborate these findings, we treated MDA-MB-231 cells with 10 μM of BMS777607 for 24 hour, and we further enhanced the phosphorylation of HER2 by stimulating the cells with 50 ng/ml NRG1. The phosphorylation of HER2 was analyzed with immunoprecipitation using 2 mg protein.
However we used a huge protein amount, the phosphorylation of HER2 was slightly visible but reproducible. This result further underlined our previous findings.
As Lapatinib is known as dual HER2/EGFR inhibitor, we wanted to elucidate the effect of EGFR on the phosphorylation of HER3. Therefore we depleted EGFR, HER2, HER3 and HER2/EGFR proteins by using specific siRNAs, and we induced HER3 phosporylation by treating the cells for 24 h with 10 μM BMS777607. Interestingly only the combination of EGFR and HER2 siRNA treatments blocked completely the phosporylation of HER3, compared to control siRNA treatments. This result implies

that not only HER2 but also EGFR is important in the phosphorylation of HER3.

Although the recovery of AKT S473 was not visible after 1 μM BMS777607 treatment, the HER3 expression and HER3 phosporylation was clearly evident, suggesting that inhibition of AXL/PI3K/AKT pathway leads to the reactivation of HER3. It is known that activation of HER3 supports pro-survival and proliferation pathways, which may limit the efficiency of AXL inhibitors. To investigate this hypothesis we treated the MDA-MB-231 cells with BMS777607 and after 72 h incubation the viability of the cells was measured with CellTiter Glo assay. The stimulation with 50 ng/ml NRG1 compensated the inhibitory effect of 1 μM BMS777607, whereas the untreated cells were not affected by NRG1. This data underlined our hypothesis that activation of HER3 compensates the inhibition of AXL/PI3K/AKT pathway. This result it was also proved with combination treatments performed on Ovcar8 and MDA-MB-231 cells, using 3D spheroid Matrigel test system.

Using the 3D spheroid Matrigel test system, we carried out combination treatment using BMS777607 and Lapatnib. Application of 1 μM BMS777607 and 5 μM Lapatinib in combination significantly reduced the cell viability comparing to the single inhibitor treatments. This data was also confirmed with AXL and HER3 siRNA double knockdown experiments, suggesting that simultaneous blockade of AXL and HER2/3 complex would be more suitable to overcome the resistance against AXL inhibitors.

5. Conclusions

- We showed that the migration of triple negative breast cancer cell lines, which is one of the most aggressive type of breast cancer, efficiently can be inhibited only by the inhibitors which target the Lyn kinase and p130Cas scaffold protein
- Our result show that from the overexpression of AXL protein can not be concluded that cell migration will depend on the activity of AXL tyrosine kinase
- We proved for the first time, that AXL inhibition induce phosphorylation of HER3 receptor tyrosine kinase and this induction depends not only from AXL overexpression but also from the level of AKT S473 level
- We showed that HER3 activation is independent from HER3 and NRG1 transcription but depends on NRG1 ligand
- We proved that even in triple negative breast cancer cell lines the dimerization partner of HER3 is HER2
- We showed that for a total HER3 inactivation is necessary the dual inhibition of HER2 and EGFR
- We observed, that is necessary to target AXL and HER2/HER3 to inhibit efficiently the viability of cancer cell lines with low AKT S473 levels

6. Publications related to the dissertation

1. Torka R, **Pénzes K**, Gusenbauer S, Baumann C, Szabadkai I, Őrfi L, Kéri G, Ullrich A. (2014) Activation of HER3 Interferes with Antitumor Effects of Axl Receptor Tyrosine Kinase Inhibitors: Suggestion of Combination Therapy. *Neoplasia*, 16:(4) 301-318.
   IF: 5,398

2. Szokol B, Gyulavári P, Kurkó I, Baska F, Szántai-Kis Cs, Greff Z, Őrfi Z, Peták I, **Pénzes K**, Torka R, Ullrich A, Őrfi L, Vántus T, Kéri G. (2014) Discovery and biological evaluation of novel dual EGFR/c-Met inhibitors. *ACS Med Chem Lett*, 5: 298-303.
   IF: 3,073

3. **Pénzes K**, Christine B, Szabadkai I, Őrfi L, Kéri Gy, Ullrich, Robert T. (2014) Combined inhibition of AXL, Lyn and p130Cas kinases block migration of triple negative breast cancer cells. *Cancer Biol Ther*, 15:(11) 1571-1582.
   IF: 3,630