Bemcentinib and Gilteritinib Inhibit Cell Growth and Impair the Endo-Lysosomal and Autophagy Systems in an AXL-Independent Manner

Daria Zdżalik-Bielecka, Kamila Kozik, Agata Poźniata, Kamil Jastrzębski, Marta Jakubik, and Marta Miączyńska

ABSTRACT

AXL, a receptor tyrosine kinase from the TAM (TYRO3, AXL, and MER) subfamily, and its ligand growth arrest-specific 6 (GAS6) are implicated in pathogenesis of a wide array of cancers, acquisition of resistance to diverse anticancer therapies and cellular entry of viruses. The continuous development of AXL inhibitors for treatment of patients with cancer and COVID-19 underscores the need to better characterize the cellular effects of AXL targeting.

In the present study, we compared the cellular phenotypes of CRISPR–Cas9-induced depletion of AXL and its pharmacological inhibition with bemcentinib, LDC1267 and gilteritinib. Specifically, we evaluated GAS6–AXL signaling, cell viability and invasion, the endo-lysosomal system and autophagy in glioblastoma cells. We showed that depletion of AXL but not of TYRO3 inhibited GAS6-induced phosphorylation of downstream signaling effectors, AKT and ERK1/2, indicating that AXL is a primary receptor for GAS6. AXL was also specifically required for GAS6-dependent increase in cell viability but was dispensable for viability of cells grown without exogenous addition of GAS6. Furthermore, we revealed that LDC1267 is the most potent and specific inhibitor of AXL activation among the tested compounds. Finally, we found that, in contrast to AXL depletion and its inhibition with LDC1267, cell treatment with bemcentinib and gilteritinib impaired the endo-lysosomal and autophagy systems in an AXL-independent manner.

Implications: Altogether, our findings are of high clinical importance as we discovered that two clinically advanced AXL inhibitors, bemcentinib and gilteritinib, may display AXL-independent cellular effects and toxicity.

Introduction

AXL together with TYRO3 and MER (MERTK) belongs to the TAM (TYRO3, AXL, and MER) receptor tyrosine kinase (RTK) family. The best characterized ligands for the TAM receptors are two vitamin K-dependent proteins, anticoagulant protein S (PROS1) and growth arrest-specific 6 (GAS6). GAS6 was proposed to be able to bind all three TAMs, albeit with the highest affinity for AXL, whereas PROS1 binds TYRO3 and MER (1, 2). In an adult organism, TAMs are considered as homeostatic regulators that are involved in phagocytic clearance of apoptotic cells and dampening of innate immune response (3–5). Moreover, TAMs play also a role in viral infections and AXL was proposed to facilitate entry of viruses such as Zika and SARS-CoV-2 (3, 6–12).

AXL signaling has been implicated in many human cancers, including gliomas, melanomas, and lung, ovarian, and breast cancers, and its overexpression was shown to correlate with poorer prognosis and metastasis (13, 14). Moreover, AXL and GAS6 were reported to be frequently overexpressed in malignant human gliomas, and patients with glioblastoma with high levels of AXL and/or GAS6 showed a significantly shorter time to tumor progression (15). Recently, we demonstrated that at the cellular level GAS6-mediated AXL activation triggered multiple actin-dependent processes such as membrane ruffling, macropinocytosis, and focal adhesion turnover that jointly contributed to invasion of glioblastoma cells (16). An activation of similar actin-dependent processes by GAS6–AXL signaling was also reported in other cancer cells (16, 17). Importantly, AXL activation constitutes a mechanism of acquired resistance to both conventional and targeted cancer therapies, and increased AXL level has been reported in many drug-resistant cancer cell lines (13, 14).

Because of multiple implications of AXL in cancer progression, its inhibition constitutes a promising strategy for the development of targeted anticancer therapies (13, 18). A first-in-class AXL kinase inhibitor, bemcentinib (R428, BGB324), is in the second phase of clinical trials for advanced and metastatic cancers (19, 20). Bemcentinib was shown to inhibit cancer cell migration and invasion in vitro as well as to suppress angiogenesis, metastasis and prolong survival in in vivo models of breast cancer metastasis (21). It also re sensitized different resistant cancer cells to tyrosine kinase inhibitors or standard-of-care chemotherapy drugs (22–26). Several other inhibitors selective for AXL/TAMs, like LDC1267, are also under preclinical development (18, 27). Moreover, many multikinase inhibitors, currently under different stages of clinical development, are also potent inhibitors of AXL (14, 18). For example, gilteritinib, an Fms-related RTK 3 (FLT3) inhibitor that was recently approved by the FDA for the treatment of relapsed and/or refractory patients with acute myelogenous leukemia harboring FLT3 mutations, also effectively blocks AXL activity (18, 28–30). Importantly, pharmacological inhibition of AXL shows also a promise as a potential COVID-19 therapy, and bemcentinib is currently tested for the treatment of...
patients with COVID-19 (12, 31). Moreover, gilteritinib was reported to have an antiviral activity against SARS-CoV-2 (32). Strikingly, Lotsberg and colleagues have recently shown that pharmacological inhibition of AXL with bemcentinib impairs autophagic flux and triggers immunogenic cell death in non–small cell lung cancer (NSCLC) cells resistant to first- and third-generation EGFR inhibitors (33). However, prior research of Chen and colleagues revealed that the suppression of autophagic flux is not a result of AXL inhibition but rather an off-target effect of bemcentinib (34). The discrepancy between those studies together with the fact that AXL is a therapeutic target and its inhibitors are currently being tested in clinical trials underscore the need to better characterize the impact of AXL depletion and its inhibitors on cell signaling, various intracellular processes, and cell growth. This is particularly important for ensuring patient safety and reducing risk of drug withdrawal due to toxic side effects. Here, we evaluated the effects of CRISPR-Cas9–mediated knockout (KO) of AXL and its pharmacological inhibition on GAS6–AXL signaling, cell proliferation, endo-lysosomal compartment and autophagy in glioblastoma cells. To this end, we compared two selective AXL/TAM inhibitors, bemcentinib and LDC1267, and a dual FLT3/AXL inhibitor, gilteritinib.

Materials and Methods

Antibodies and chemicals

Antibodies and chemicals are listed in Supplementary Material.

Cell culture

LN18, LN229, T98G, U-87MG, and HEK293 cells were purchased from the ATCC and maintained in DMEM medium supplemented with 10% FBS and 2 mmol/L L-glutamine (all from Sigma-Aldrich). Cells were cultured at 37°C and 5% CO₂ and regularly tested for Mycoplasma contamination.

Isolation of CRISPR-Cas9–induced AXL KO clones of LN229 cells

AXL KO clones were isolated from nonselected population of AXL KO LN229 cells generated using sgRNA target sequence 5’CCCCGAAGCCAATGTACCTCG-3’ as described previously (16). To isolate single clones, 100 cells of nonselected population of AXL KO in 20 mL of DMEM medium were seeded into a 96-well plate (200 μL/well), and single clones were picked when colonies had formed. Then, the colonies were expanded and subjected to Western blot analysis to verify AXL KO.

Purification of GAS6–MycHis

GAS6-MycHis was purified as described previously (16).

Western blot analysis

HEK293 (3 × 10⁵ cells/well), LN229, T98G (3.5 × 10⁵ cells/well), LN18 and U-87 MG (4 × 10⁵ cells/well) cells were seeded into 6-well plates. For analysis of GAS6-mediated phosphorylation of AXL, AKT, and ERK1/2, 24 hours after seeding, cells were washed with PBS and incubated in serum-free medium for 16 hours. Next, cells were incubated with DMSO (vehicle control) or AXL inhibitors (at indicated concentrations) for 30 minutes at 37°C before stimulation with 400 ng/mL GAS6 for 10 minutes at 37°C. For assessing the level of autophagy markers, 24 hours after seeding, cells were incubated with DMSO (vehicle control) or 2.5 μmol/L bemcentinib, LDC1267 or gilteritinib for 24 hours. Cells were lysed and analyzed by Western blot as described elsewhere (16, 35).

Cell viability

LN18 and U-87 MG (3 × 10³ cells/well), HEK293 and LN229 (2–3 × 10³ cells/well for inhibitor treatment or 4 × 10³ cells/well for siRNA transfection) cells were seeded into 96-well plates. 24 hours after seeding, cells were exposed to increasing doses of bemcentinib, LDC1267 or gilteritinib, or cells were transfected with siRNAs targeting AXL and TYRO3 as described previously (16). 24 hours after transfection, cells were washed in PBS and incubated in DMEM medium; with 10% FBS or without FBS or without FBS with 400 ng/mL GAS6. Cell viability was assessed 96 hours after seeding using the ATPlute assay (PerkinElmer) according to the manufacturer’s protocol. IC₅₀ values were calculated using GraphPad Prism version 8. The curves were fit using a nonlinear regression model with a log (inhibitor) versus normalized response formula.

Spheroid invasion assay

Spheroid invasion assay was performed as described previously (16).

Phase-contrast microscopy

LN229 cells were seeded into 24-well plates at a density of 5 × 10⁴ cells/well. Next day, cells were exposed to DMSO (vehicle control) or 2.5 μmol/L bemcentinib, LDC1267 or gilteritinib. After 24 hours incubation, cells were washed twice with ice-cold PBS and fixed with 3.6% paraformaldehyde in PBS for 10 minutes at room temperature. The phase-contrast images were collected using Olympus IX70 with LCPlanFI 20x/0.4 Ph1 objective. HEK293, T98G (1 × 10⁴ cells/well), LN18 and U-87 MG (1.5 × 10⁴ cells/well) cells were seeded into 96-well plates. Next day, cells were exposed to DMSO (vehicle control) or 2.5 μmol/L bemcentinib, LDC1267 or gilteritinib, and live cells were imaged under phase contrast every 5–10 minutes for 24 hours using the Opera Phenix microscope (PerkinElmer) as described below.

Immunofluorescence staining and image analysis

LN229 cells were seeded into μClear 96-well plates (Greiner Bio-One International GmbH; #655090) at a density of 5.5 × 10⁴ cells/well. After 24 hours incubation, cells were treated with DMSO (DMSO vehicle control) or AXL inhibitors and fixed as described above. Next, fixed cells were stained according to the immunofluorescence protocol with saponin permeabilization described previously elsewhere (35). The images were acquired using Opera Phenix microscope (PerkinElmer) with 40x/NA 1.1 water immersion objective. Harmony software (version 4.9; PerkinElmer) was used for image acquisition and analysis. At least twenty 16-bit images with resolution 2,048 × 2,048 were acquired per experimental condition. Flat-field correction was applied to all images before analysis. Pictures were assembled in ImageJ and Photoshop (Adobe) with only linear adjustments of contrast and brightness (36).

Statistical analysis

Data are provided as means ± SEM from at least three independent experiments, unless stated otherwise. Statistical analysis was performed using one-way ANOVA, unless indicated otherwise, using GraphPad Prism version 8. The significance of mean comparisons annotated as follows: ns, non-significant (P > 0.05); *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001; and ****, P ≤ 0.0001.

Data availability

The data generated in this study are available within the article and its Supplementary Data Files.
Results

GAS6-mediated activation of AKT and ERK is solely dependent on AXL

As AXL and GAS6 are overexpressed in human gliomas and AXL mediates glioma growth and invasion (15, 37), we decided to study the consequences of AXL depletion or inactivation in glioblastoma cells. First, we evaluated the AXL level in four glioblastoma cell lines and selected the LN229 line with the highest amounts of endogenous AXL as our experimental model (Fig. 1A). In addition, LN229 cells displayed the highest phosphorylation of AXL and its downstream signaling effectors, AKT and ERK1/2 upon GAS6 stimulation among the tested glioblastoma cell lines (Fig. 1B and C). To ensure complete AXL depletion in this line, we isolated single clones from the previously established CRISPR-Cas9–induced AXL KO cell population (16). Western blot analysis confirmed lack of AXL protein in three selected clones in comparison to wild-type (WT) LN229 cells (Fig. 1D). Next, we evaluated the impact of AXL KO on GAS6-mediated activation of downstream signaling effectors, AKT and ERK1/2. As shown in Fig. 1E, although LN229 cells expresses AXL and TYRO3 (Fig. 1A), AXL KO was sufficient to prevent GAS6-mediated activation of both AKT and ERK1/2.

Taken together, we showed that GAS6 induces robust activation of AXL and its downstream effectors, AKT and ERK1/2, in glioblastoma cells. Moreover, a complete inhibition of GAS6-mediated phosphorylation of AKT and ERK1/2 upon KO of AXL further underscores that AXL is a primary receptor for GAS6.

LDC1267 is the most potent and specific inhibitor of AXL activation

To test the impact of AXL inhibitors on the activation of GAS6–AXL signaling, we investigated GAS6-mediated phosphorylation of AXL and its downstream effectors, AKT and ERK1/2, upon pretreatment of...
LN229 cells with increasing concentrations of bemcentinib, LDC1267 or gilteritinib. As shown in Fig. 2A, all tested inhibitors blocked GAS6-activated phosphorylation of AXL, AKT and ERK1/2, with LDC1267 showing the highest inhibitory activity against AXL. Specifically, LDC1267 displayed IC50 values of 26, 25, and 48 nmol/L for the inhibition of phosphorylation of AXL, AKT, and ERK1/2, respectively (Fig. 2B). Gilte ritinib was a slightly less potent inhibitor of AXL activation than LDC1267, and it inhibited phosphorylation of AXL, AKT, and ERK1/2, respectively (Fig. 2B). Bemcentinib was the lowest potency among the tested compounds as it inhibited phosphorylation of AXL, AKT, and ERK1/2 with IC50 values of 53, 21, and 36 nmol/L, respectively (Fig. 2B). Of note, IC50 values of LDC1267 and bemcentinib for the inhibition of ERK1/2 are approximate values as the corresponding dose–response curves did not fit to a sigmoidal dose–response curve. Similar to LN229 cells, in LN18 cells, bemcentinib was also the least potent inhibitor of GAS6-induced phosphorylation of AXL and its downstream effectors, whereas LDC1267 was the most potent one (Supplementary Fig. S1A and S1B). Importantly, in contrast to LDC1267, both bemcentinib and gilteritinib reduced phosphorylation of AKT and ERK1/2 at considerably lower concentrations compared with the concentrations that inhibited AXL phosphorylation (Fig. 2A and B, Supplementary Fig. S1A and S1B). This suggests that these two inhibitors display AXL-independent inhibitory effects on the phosphorylation of AKT and ERK1/2.

Cumulatively, we showed that bemcentinib, LDC1267, and gilteritinib inhibit GAS6-induced phosphorylation of AXL and its downstream effectors, AKT and ERK1/2. Moreover, we demonstrated that LDC1267 is considerably more potent and specific inhibitor of AXL activation in comparison with bemcentinib and gilteritinib.

**Bemcentinib and gilteritinib inhibit cell growth in an AXL-independent manner**

The dependence on AXL for cell growth was proposed to be cell type-specific as AXL targeting was shown to inhibit proliferation of some cancer cells but not the others (37–41). Thus, we first assessed whether AXL depletion had any impact on growth of glioblastoma...
that bemcentinib but not LDC1267 inhibited growth of human non-small cell lung carcinoma H1299 cells, and it induced apoptosis also in AXL-silenced H1299 cells. Moreover, the authors showed that in contrast to siRNA-mediated AXL silencing, bemcentinib also triggered extensive cell vacuolization and dysfunction of late endosomes/lysosomes (34). To determine whether bemcentinib- and gilteritinib-mediated growth inhibition of glioblastoma cells observed by us is also accompanied by cell vacuolization, we assessed the morphology of WT and AXL KO LN229 cells upon 24-hour incubation with the tested AXL inhibitors at 2.5 μmol/L concentration. As shown in Fig. 4A, bemcentinib and gilteritinib induced vacuolization of LN229 cells, and this vacuolization was also triggered in AXL KO cells. On the contrary, KO of AXL and cell treatment with LDC1267 did not induce vacuolization of LN229 cells (Fig. 4A). Moreover, bemcentinib and gilteritinib induced vacuolization of glioblastoma U-87 MG cells but not of T98G and LN18 cells (Supplementary Fig. S4). Importantly, vacuolization triggered by the treatment with these inhibitors was also observed in HEK293 cells, non-cancer cells that do not express AXL (Supplementary Fig. S4). The study of Chen and colleagues revealed that bemcentinib and gilteritinib significantly reduced the number of EE1-positive early endosomes and LAMP1-positive late endosomes/lysosomes in both WT and AXL KO cells (Fig. 4C and D). This reduction in the endosome number was accompanied by significant increase in their area (Fig. 4C and D). At the same time, we neither observed a decrease in the number of early endosomes and late endosomes/lysosomes nor an increase in their area in vehicle-treated AXL KO LN229 cells (Fig. 4C and D).

Cumulatively, we demonstrated that bemcentinib and gilteritinib trigger cell vacuolization and changes in the endo-lysosomal compartment independently of AXL inhibition. Importantly, we showed that CRISPR-Cas9–mediated depletion of AXL itself does not induce such changes within the cell.

**Bemcentinib and gilteritinib impede autophagic flux independently of AXL**

The study of Chen and colleagues revealed that bemcentinib-induced vacuolization was accompanied by increased autophagy initiation and blockade of its progression, and all these effects were not the consequence of the inhibition of AXL by bemcentinib (34). On the contrary, in a recent study Lotsberg and colleagues reported that bemcentinib-induced disturbance in autophagic flux was dependent on AXL, and siRNA-mediated silencing of AXL also disrupted autophagic flux (33). Given this discrepancy, we checked whether bemcentinib- and gilteritinib-induced vacuolization of glioblastoma LN229 cells observed here was associated with impairment of autophagic flux and whether AXL deficiency by itself induced any changes in autophagy. The term of autophagy flux refers to the entire process of autophagy, including formation and maturation of autophagosomes, their fusion with lysosomes, and final degradation of autophagic substrates in lysosomes (42). Upon induction of autophagy, the cytosolic forms of microtubule-associated proteins 1A/1B light chain 3A/B (LC3A/B) are lipidated to autophagosome-bound forms, and

**Bemcentinib and gilteritinib induce cell vacuolization and disrupt the endo-lysosomal compartment independently of AXL**

In line with our findings, Chen and colleagues (34) demonstrated that bemcentinib but not LDC1267 inhibited growth of human non-
Figure 3.
Bemcentinib and gilteritinib inhibit cell growth in an AXL-independent manner. A, Efficiency of siRNA-mediated AXL and TYRO3 depletion. LN229 cells were transfected with non-targeting siRNAs (siCTR#1–2), siRNAs targeting AXL (siAXL#1–3) or TYRO3 (siTYRO3#1–3), and protein levels were assessed by Western blot. α-Tubulin (α-tub.) was used as a loading control. B, Viability of LN229 cells cultured in the presence of serum upon depletion of AXL or TYRO3. Cells were transfected as in (A), and cell viability was measured after 72 hours using an ATPlite assay. Data are expressed as the percentage of viability relative to siCTR#1-treated control, and are presented as means ±SEM from five independent experiments performed in duplicates (n = 10); ns, non-significant; P > 0.05 and *P ≤ 0.05 by one-way ANOVA (with Dunnett’s multiple comparisons test). C, Viability of LN18 and U-87 MG cells cultured in the presence of serum upon depletion of AXL. Cells were transfected as in (A) and their viability was measured as in (B). Data are expressed as the percentage of viability relative to siCTR#2-treated controls, and are presented as means ±SEM from two independent experiments performed in triplicates (n = 6); ns, non-significant P > 0.05 by one-way ANOVA (with Dunnett’s multiple comparisons test). D, Viability of LN229 cells cultured in the absence of serum upon depletion of AXL or TYRO3. Cells were transfected as in (A), serum-starved with or without GAS6 24 hours after transfection, and then their viability was measured after 48 hours using an ATPlite assay. Data are expressed as the percentage of viability relative to siCTR#1 cultured without GAS6, and are presented as means ±SEM from five independent experiments performed in duplicates (n = 10); ns, non-significant P > 0.05; ***P = 0.01 and ****P ≤ 0.0001 by one-way ANOVA (with Tukey’s multiple comparisons test). E and F, Dose-inhibition curves for bemcentinib (E) and gilteritinib (F) obtained in wild-type (WT) and AXL KO clones of LN229 cells. Cells were treated and analyzed as described previously in (E). Data are expressed as the percentage of viability relative to DMSO-treated controls, and are presented as means ±SEM from three independent experiments performed in duplicates (n = 6). H, GAS6-induced invasion of LN229 cells grown as spheroids after treatment with AXL inhibitors. Spheroids embedded in Matrigel were incubated with 1µmol/L bemcentinib, LDC1267 or gilteritinib, and after 30 minutes GAS6 was added. Spheroids were incubated with AXL inhibitors and GAS6 for 4 days; scale bars, 500 µm. I, Quantification of data shown in (H). The area of spheroids was measured by ImageJ software. Data are expressed as fold changes of the spheroid area on the 4th day (day 4, D4) with respect to the spheroid area before Matrigel addition (day 0, D0). Each dot represents data from one independent experiment whereas bars represent the means ± SEM from 3 experiments (n = 3). NS, non-stimulated cells; GAS6, GAS6-stimulated cells; *P ≤ 0.05; **P ≤ 0.001 and ***P ≤ 0.0001 by one-way ANOVA (with Dunnett’s multiple comparisons test).
LC3 lipidation is widely used as a marker of autophagosome formation. Thus, to monitor autophagic flux, we assessed the conversion of the cytosolic form of LC3B (LC3-I) to its lipidated form (LC3-II) and degradation of autophagy-selective substrate p62 (42). As shown in Fig. 5A–C, none of the tested AXL KO clones displayed changes in the levels of LC3-II and p62, indicating that depletion of AXL does not have any impact on autophagy. In addition, to assess an impact of GAS6-mediated AXL activation on autophagy, we incubated LN229 cells with GAS6 for prolonged periods of time. As shown in Fig. 5D–F, GAS6 reduced conversion of LC3-I to LC3-II as well as autophagic...
degradation of p62. This indicated that the activation of GAS6–AXL signaling inhibits autophagy at an early-stage of autophagosome formation.

Similarly to AXL KO, pharmacological inhibition of AXL by LDC1267 did not influence autophagy (Fig. 5A–C). In contrast, treatment of LN229 cells with bemcentinib and gilteritinib induced a significant increase in the LC3-II/LC3-I ratio and inhibited autophagic degradation of p62 both in WT and AXL KO LN229 cells (Fig. 5A–C). This indicates that bemcentinib and gilteritinib blocked later stages of autophagy as they increased the formation of autophagosomes and decreased degradation of autophagic substrate p62 in lysosomes. Moreover, this bemcentinib- and gilteritinib-mediated impairment of autophagic flux was not a result of AXL inhibition especially that these inhibitors impeded autophagy also in HEK293 cells devoid of AXL (Supplementary Fig. S5A–S5C). Moreover, in line with data obtained by Chen and colleagues (34), autophagy inhibition by Bafilomycin A1 prevented cell vacuolization triggered by these inhibitors (Supplementary Fig. S6; ref. 34).

In summary, we showed that GAS6-induced activation of AXL triggers early suppression of autophagy; however, targeting of AXL by its pharmacological inhibition with LDC1267 or CRISPR-Cas9–mediated KO does not affect the process of autophagy. Importantly, our data revealed that bemcentinib and gilteritinib abrogate autophagic flux in an AXL-independent manner that reflects off-target effects of these inhibitors.

Discussion

AXL inhibitors are in clinical testing, thus, it is essential to carefully examine their cellular mechanisms of action and possible off-target effects to ensure patient safety. Here, we systematically characterized and compared the activity of two selective AXL/TAM inhibitors (bemcentinib and LDC1267) and one multikinase inhibitor (gilteritinib) in glioblastoma cells. All three AXL inhibitors blocked activation of the GAS6–AXL signaling pathway; however, our data demonstrated that LDC1267 is overall a more potent and specific AXL inhibitor in comparison with bemcentinib and gilteritinib. In contrast, bemcentinib turned out to be the least potent AXL inhibitor. Moreover, both, bemcentinib and gilteritinib, displayed off-target effects as they evoked changes in the endo-lysosomal and autophagy systems and inhibited the growth of cells independently of whether they expressed AXL or not.

GAS6-mediated AXL activation was shown to induce autophagy in murine macrophages (43); however, the role of AXL in the...
regulation of autophagy in cancer cells is poorly characterized. Here, we found that GAS6 stimulation reduced autophagy initiation in glioblastoma cells. This is in line with recent findings of us and others that GAS6 induces macropinocytosis, which allows cancer cells to acquire extracellular nutrients (16, 44). Thus, it seems reasonable that at the same time autophagy, which serves as a scavenging pathway to derive nutrients from intracellular compartments, is blocked (42, 45). The recent study of Lotsberg and colleagues, based mostly on AXL inhibition with bemcentinib, suggested that AXL was implicated in autophagy in NSCLC cells resistant to EGFR inhibitors (33). On the contrary, an earlier study of Chen and colleagues demonstrated that bemcentinib impaired autophagic flux independently of AXL (34). Importantly, our study has addressed the discrepancy between these two reports. In line with the data obtained by Chen and colleagues (34), we showed that AXL depletion had no effect on autophagic flux, and bemcentinib triggered cell vacuolization and impeded autophagic flux in an AXL-independent manner. In addition, we discovered that bemcentinib not only affected late endosomes, but also induced changes in early endosomes. In addition, we showed that gilteritinib displayed the same negative effects on both endosomes and autophagy. Notably, although LDC1267 potently blocked AXL activation, it did not trigger changes in the endo-lysosomal and autophagy systems. Altogether, in line with the study of Chen and colleagues (34) but in contrast to Lotsberg and colleagues (33), our results imply that AXL depletion does not impair the endo-lysosomal and autophagy systems, and the negative impact of bemcentinib and gilteritinib on endo-lysosomal compartment and autophagic flux is the result of off-target effects of these inhibitors. Interestingly, Chen and colleagues proposed that bemcentinib acts as a lysosomotropic agent that in its protonated state accumulates extensively in the acidic compartment of lysosomes (34). Because lysosomotropic agents are known to inhibit viral infection, including SARS-CoV-2, it is highly possible that the anti–SARS-CoV-2 effects of bemcentinib and gilteritinib result from alkalescent properties of these inhibitors but not from AXL inhibition, and such possibility requires further investigation (10, 32, 46–49).

Several types of cancer cells have been shown to depend on AXL for proliferation, whereas proliferation of others was unaffected upon targeting of AXL (37–41). These differences may result from off-target toxicity of the method used to target AXL, or reflect cell-type specificity. Thus, it is still a matter of debate whether AXL is needed for proliferation of cancer cells or it is rather implicated in triggering and/or supporting a more aggressive and invasive phenotype (41). In line with this, our previous and current studies showed that AXL depletion and pharmacological inhibition of its kinase activity (by LDC1267, the AXL inhibitor not inhibiting cell growth) blocked GAS6-induced invasion of LN229 cells in a sphereoid model (16). Moreover, here, we revealed that although LN229 glioblastoma cells express high level of AXL, its siRNA-mediated depletion did not inhibit proliferation when cells were cultured in full medium without exogenous addition of GAS6. However, knockdown of AXL specifically blocked increase in cell viability induced by exogenous GAS6. Similarly, Li and colleagues found that AXL depletion abolished GAS6-induced increase in viability of H1299 lung carcinoma cells whereas it did not affect viability of cells grown in the absence of the ligand (39). These findings indicate that GAS6 exerts a beneficial impact on growth of at least some cancer cells and AXL is specifically required for this GAS6-triggered viability improvement. Because GAS6 has been shown to be produced by some cancer cells (15, 37, 50), it is possible that the observed different dependency on AXL for growth of diverse types of cancer cells stems from their ability to secrete the ligand.

Prior studies indicated that GAS6 is able to bind all three TAMs (1, 2). However, we recently showed that depletion of AXL but not of TYRO3 inhibited GAS6-induced membrane ruffling, macropinocytosis, and invasion of LN229 cells (16). Here, we additionally discovered that AXL KO was sufficient to completely block GAS6-mediated activation of downstream signaling effectors, AKT and ERK1/2, and increase in viability of glioblastoma LN229 cells. Because these cells express also TYRO3, the results obtained here provide yet additional experimental evidence that AXL is a primary receptor for GAS6. Thus, our data imply that the role of GAS6 in activating TAM receptors should be re-examined in diverse cellular models as it seems highly possible that GAS6 specifically activates AXL but not TYRO3.

In summary, we reported that GAS6-mediated AXL activation has a positive effect on the growth of glioblastoma LN229 cells; however, AXL is not essential for the proliferation of these cells. Importantly, our study revealed that LDC1267 is a more potent and specific AXL inhibitor in comparison with bemcentinib and gilteritinib, which display off-target effects on cell growth as well as the endo-lysosomal and autophagy systems. Thus, our data imply that any further conclusions on the role of AXL based on experiments performed with the usage of bemcentinib or gilteritinib should be drawn very carefully and require validation in AXL-depleted cells. Moreover, the off-target toxicity and effects of bemcentinib and gilteritinib identified in our study have important implications for ongoing clinical trials.

Authors’ Disclosures

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Authors’ Contributions

D. Zdzialik-Bielecka: Conceptualization, formal analysis, supervision, funding acquisition, investigation, visualization, methodology, writing—original draft, project administration, writing–review and editing. K. Koziak: Formal analysis, investigation, visualization, writing–review and editing. A. Poziwata: Investigation, visualization, writing–review and editing. K. Jastrzębski: Formal analysis, investigation. M. Jakubiak: Investigation. M. Mięczyńska: Conceptualization, supervision, writing–review and editing.

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