Supplementary Materials and Data

Zdzalik-Bielecka *et al.*

Supplementary Materials

Chemicals

AXL inhibitors: R428 (bemcentinib; HY-15150), LDC1267 (HY-12494) and gilteritinib (HY-12432) all from MedChemExpress. DAPI (D9542) and Bafilomycin A1 (B1793) from Sigma-Aldrich.

Antibodies

The following primary antibodies were used: mouse anti-LAMP1 (555798, IF 1:1000) and mouse anti-p62 (610833, WB 1:1000) from BD Biosciences; mouse anti-AKT (2920, WB 1:2000), rabbit anti-phospho-AKT (Ser 473) (4060, WB 1:1000), rabbit anti-phospho-AXL (Tyr702) (5724, WB 1:1000), mouse anti-ERK1/2 (p44/42 MAPK, 9107, WB 1:1000), rabbit anti-phospho-ERK1/2 (p44/42 MAPK; Thr202/Tyr204, 4370, WB 1:1000), rabbit anti-LC3B (2775, WB 1:1000), rabbit anti-MER (4319, WB 1:1000) rabbit anti-TYRO3 (5585, WB 1:1000) all from Cell Signaling Technology; rabbit anti-EEA1 (ALX-210-239, IF 1:1000) from Enzo Life Sciences; mouse anti-AXL (sc-166269, WB 1:1000) and rabbit anti-AXL (sc-20741, WB 1:1000) from Santa Cruz Biotechnology; mouse anti-α-tubulin (T5168, WB 1:5000) and mouse anti-β-actin (A5441, WB 1:5000) from Sigma-Aldrich; goat anti-AXL (AF154, IF 1:500, WB 1:1000) from R&D Systems.

Secondary antibodies used for WB: horseradish peroxidase (HRP)-conjugated anti-mouse-IgG (111-035-062), anti-rabbit-IgG (111-035-144) and anti-goat-IgG (805-035-180) antibodies were from Jackson ImmunoResearch.Secondary antibodies used for IF: Alexa Fluor 488-, 555-, 647-conjugated anti-mouse-IgG and anti-rabbit-IgG were from Thermo Fisher Scientific.

Supplementary Figures and their legends

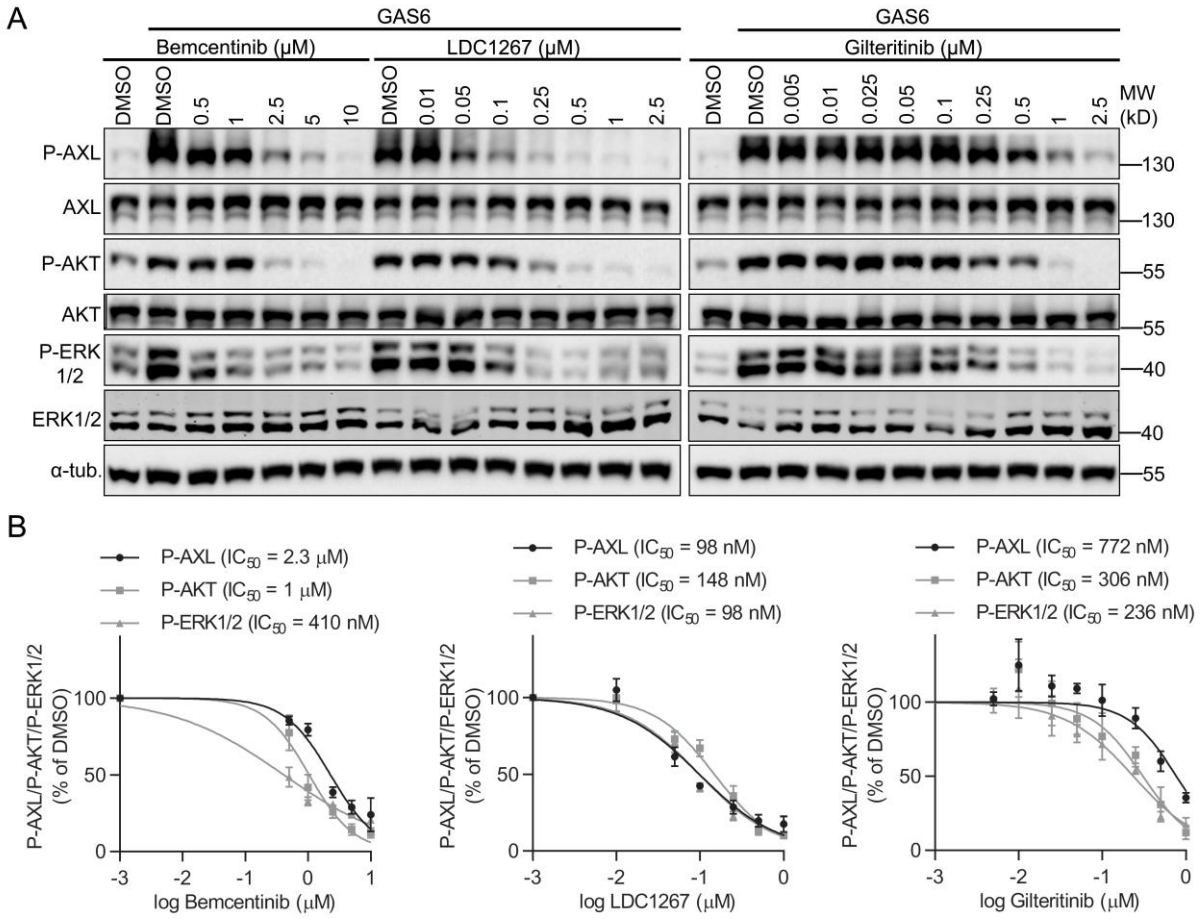

Figure S1. Bemcentinib, LDC1267 and gilteritinib block phosphorylation of AXL and its downstream effectors, AKT and ERK1/2 in LN18 cells. **A,** Western blot showing GAS6-induced AXL (P-AXL, Y702), AKT (P-AKT, S473) and ERK1/2 (P-ERK, T202/T204) phosphorylation upon treatment with AXL inhibitors. Serum-starved LN18 cells were pretreated with DMSO (vehicle control) or increasing concentrations of AXL inhibitors for 30 min prior to stimulation with GAS6 for 10 min. α-tubulin (α-tub.) was used as a loading control. **B,** Quantification of data shown in (A). Data are presented as the mean percentage of the DMSO- and GAS6-treated control ±SEM from at least three independent experiments (n=3-4). IC_{50} values for the inhibition of GAS6-mediated phosphorylation of AXL, AKT and ERK1/2 are shown.

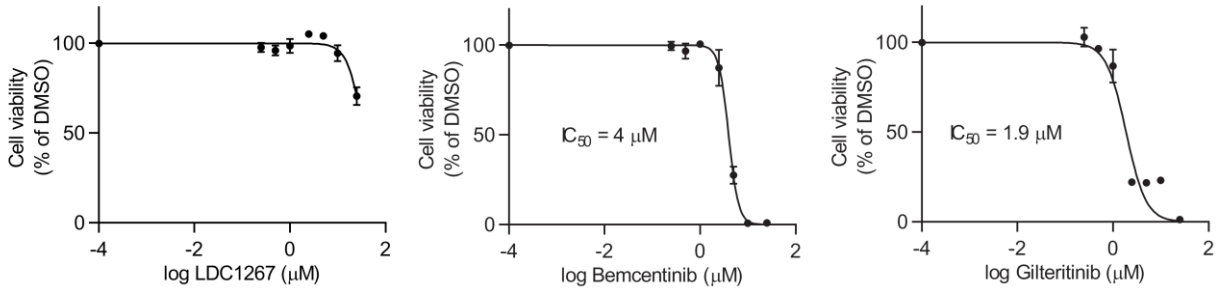

Figure S2. Bemcentinib and gilteritinib inhibit viability of HEK293 cells that do not express AXL. **A,** Dose-inhibition curves for LDC1267, bemcentinib and gilteritinib obtained in HEK293 cells. Cells were treated and analyzed as described in Fig. 3E. Data are expressed as percentage viability relative to DMSO-treated controls, and are presented as means ±SEM from two independent experiments performed in duplicates (n=4).

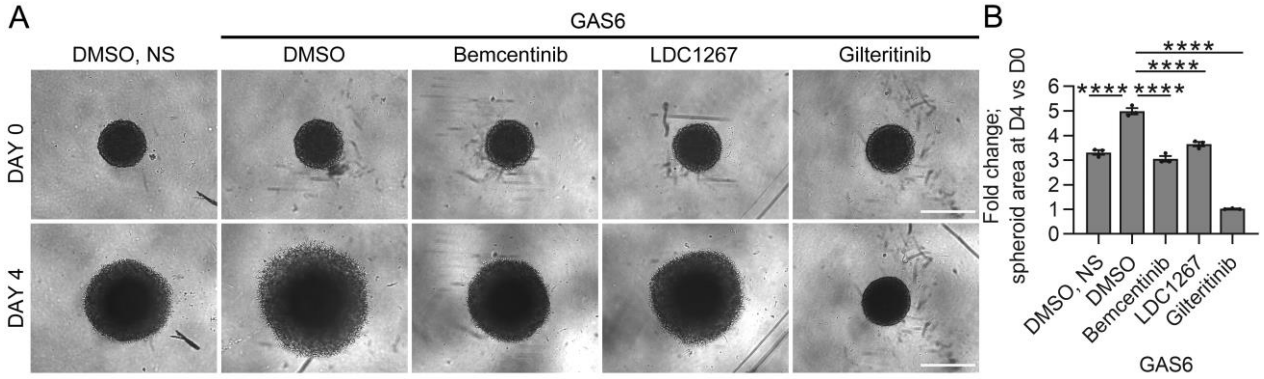

Figure S3. Bemcentinib, LDC1267 and gilteritinib inhibit GAS6-AXL-induced invasion of LN229 cells grown as spheroids. **A,** GAS6-induced invasion of LN229 cells after treatment with AXL inhibitors. LN229 spheroids embedded in Matrigel were incubated with 2.5 μM bemcentinib, LDC1267 or gilteritinib, and after 30 min GAS6 was added. Spheroids were incubated with AXL inhibitors and GAS6 for 4 days. Scale bars: 500 μm. **B,** Quantification of data shown in (A). 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.

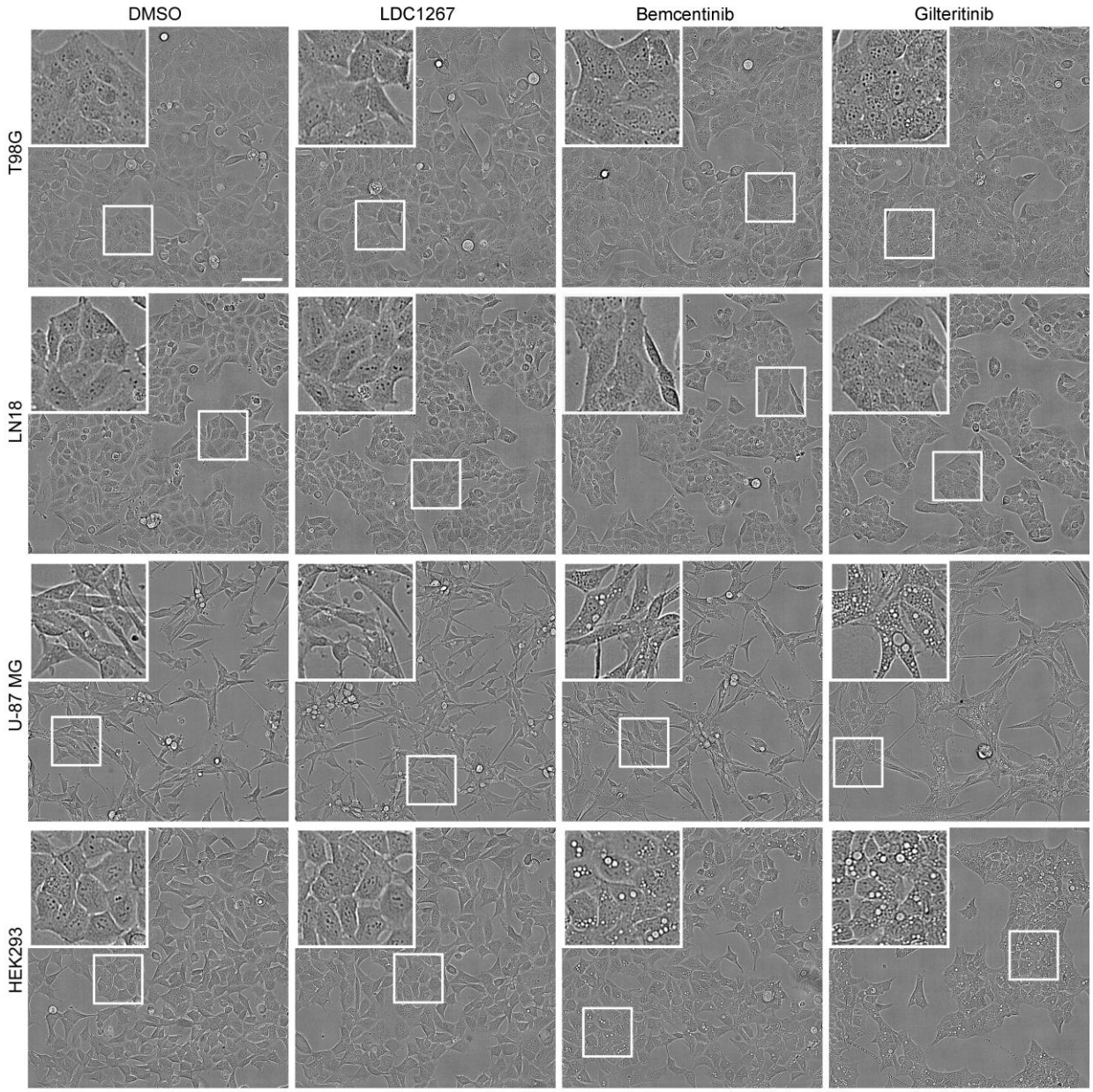

Figure S4. Bemcentinib and gilteritinib trigger cell vacuolization of U-87 MG and HEK293 cells but not of T98G and LN18 cells. Cells were incubated with 2.5 μM bemcentinib, LDC1267 or gilteritinib for 24 h. Representative phase-contrast images from two independent experiments are shown. Scale bar: 100 μm.

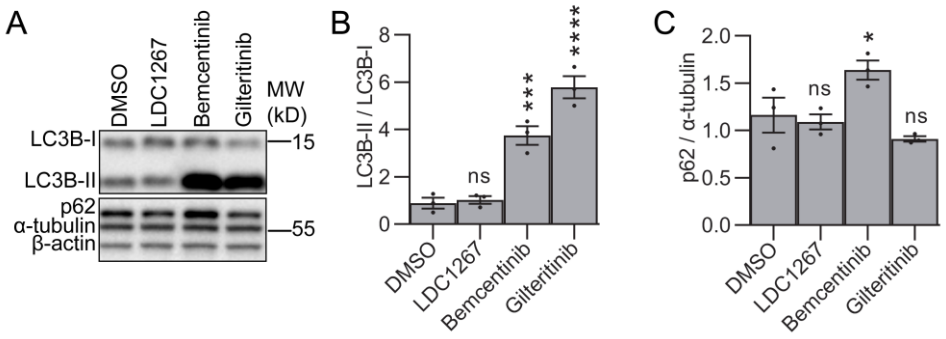

Figure S5. Bemcentinib and gilteritinib display deleterious effects on the autophagy system in HEK293 cells. **A,** Western blot showing levels of autophagy markers, non-lipidated (LC3-I) and lipidated (LC3-II) forms of LC3 and p62, in HEK293 cells treated with AXL inhibitors. Cells were incubated with 2.5 μM bemcentinib, LDC1267 or gilteritinib for 24 h. α-tubulin served as a loading control. **B, C,** Quantification of data shown in (A) performed as described in Fig. 5B-C. Data represent the means ±SEM from three independent experiments (n=3).

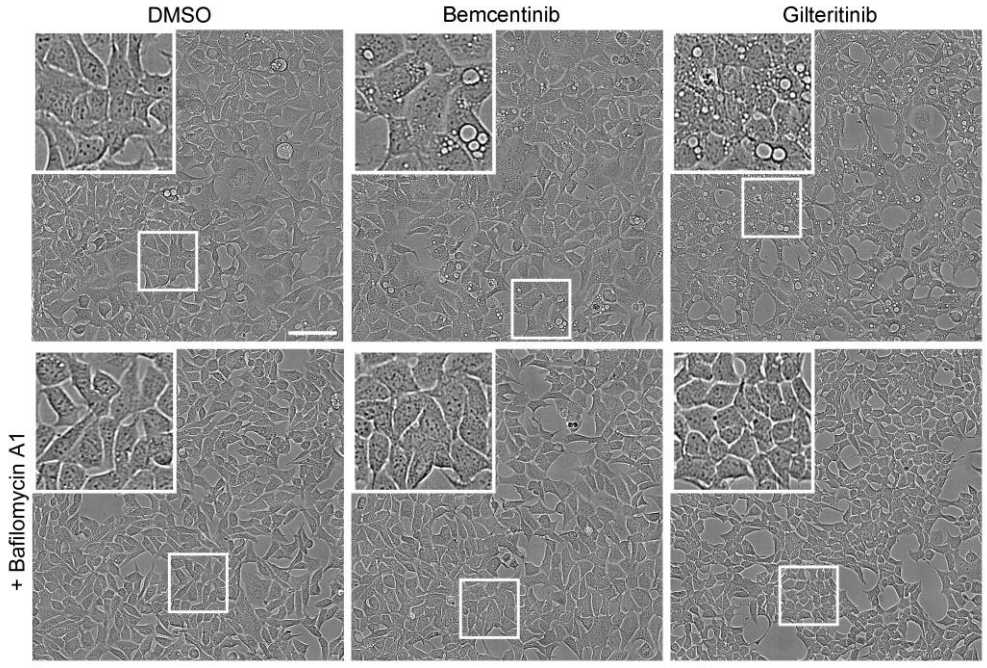

Figure S6. Bemcentinib- and gilteritinib-induced cell vacuolization of HEK293 cells is blocked by autophagy inhibition. HEK293 cells were pretreated with autophagy inhibitor Bafilomycin A1 at 100 nM for 30 min prior to addition of 2.5 μM bemcentinib, LDC1267 or gilteritinib for 24 h. Representative phase-contrast images from two independent experiments are shown. Scale bar: 100 μm.