**Supplementary Figures**



**Figure S1 Determination of oxygen consumption and ATP levels after VLX600 treatment in U-251 cells. A)** U-251 cells were treated with 5 µM VLX600 for 24 h and the oxygen consumption was measured over the whole time period. Data represents the mean + SEM normalized to t0 of three independent experiments with three replicates. Data represents the oxygen consumption change over the whole time period. Statistical significance was measured with a 2way ANOVA. **B)** Measurement of ATP levels using the BioTracker ATP dye and subsequent flow cytometry of U-251 cells after 1 and 5 µM VLX600 treatment for the indicated time periods (6 h, 16 h, 24 h) or with the withdrawal of glucose (without (w/o) glucose). Data represents mean + SEM of three experiments with one to three replicates. Statistical significance was measured with a 2way ANOVA.



**Figure S2 Evaluation of mitochondrial proteins after VLX600 treatment in NCH644 glioma stem-like cells. A)** Immunoblot analysis of the mitochondrial protein COX4I1 after 1 and 2 µM VLX600 treatment for 6 h, 24 h and 48 h in NCH644 wt cells (left) or for 24 h in NCH644 non-target (control), *ATG5* and *ATG7* KD cells (right). GAPDH was used as a loading control and the experiment was repeated at least three times. **B, C)** Quantification of the protein expression of the western blots shown in **(A)**. Data represents mean + SEM of at least four independent experiments. Statistical significance was calculated with a one-way **(B)** or 2way ANOVA **(C)**. Dashed line represent vehicle (DMSO) treated condition



**Figure S3 Analysis of VLX600-induced cell death and mitophagy after *BNIP3* and *BNIP3L* knockdown**. **A, B)** U-251 cells were treated with 1 nM siRNA against BNIP3 and BNIP3L for 16 h following 5 µM VLX600 treatment for 48 h. Afterwards, immunoblot analysis (A) or an annexin/PI staining with subsequent flow cytometric analysis (B) was performed. Mock served as a control for the transfection substances and the siRNA Universal Negative Control (siCon) as a control for the siRNA. DMSO served as vehicle control. **A)** Immunoblot analysis of BNIP3 and BNIP3L after siRNA and VLX600 treatment. Representative blots of four independent experiments. **B)** Cell death analysis after siRNA and VLX600 treatment (B = BNIP3, N = BNIP3L/NIX). Displayed is the total cell death consisting of annexin V only-positive, PI only-positive and double-positive cells. Data represents mean + SEM of two experiments with three replicates and 5,000 – 10,000 cells measured in each sample. **C)** Immunoblot analysis of the mitochondrial proteins COX4I1 and TOMM20 after siRNA treatment (5 nM each) against BNIP3 (B) and BNIP3L (N) and 24 h 1 µM VLX600 treatment. Two different siRNAs (1 and 2) were used for each gene. GAPDH served as a housekeeper and the experiment was repeated four times. **D, E)** Quantification of the COX4I1 and TOMM20 protein expression of the western blots shown in **(C)**. Data represents mean + SEM of four independent experiments and was normalized to the solvent control. Dashed lines indicate the solvent control. Statistical significance was analysed with a 2way ANOVA.



**Figure S4 *Ex vivo* analysis of VLX600’s tumor growth inhibiting ability.** **A, B)** Organotypic brain slice culture (OTC) model using adult mice brains and tumor spheres consisting of glioma-stem like cells. GS-5 tumor spheres were treated with 1, 5 and 10 µM VLX600 for a total of 9 days. DMSO was used as a vehicle control. Shown is the quantification of the tumor area normalized to day 0 **(A)** or to day 0 and the vehicle control **(B)**. Data represents mean + SEM of 5 – 8 tumors. Statistical significance was measured with a one-way ANOVA. The dashed line indicates the solvent control.



**Figure S5 Extracellular addition of iron protects NCH644 cells from VLX600-induced cell death. A, B)** Cell death analysis of GSC line NCH644 after 2 µM VLX600 treatment with the addition of different concentrations of FeCl2 **(A)** and FeCl3 **(B)** as indicated after 48 h. Total cell death includes only annexin V-positive, only PI-positive and double-positive cells. Data represents mean + SEM of at least two experiments with three replicates and 10,000 cells measured in each sample. Statistical significance was calculated with a 2way ANOVA. Asterisks represent statistically difference to the VLX600 treatment without the addition of FeCl2 or FeCl3.