Supplementary Figure Legends

Supplementary Figure S1.

U87EGFRvIII, LN229 and U251 cell lines have been tested in immunoblotting analysis after time course dependent experiment with CC214-1 (2 μM) and rapamycin (5 nM).

Supplementary Figure S2.

Assessment of proliferation and immunoblotting analysis in U87EGFRvIII cells following combinatory treatment with low doses of CC214-1 plus Rapamycin (A; 24 hours treatment in the western blot test, 4 days treatment in the proliferation assay). Reduction of mTORC1 and mTORC2 biomarkers followed CC214-1 treatment (shown in Fig. 2B) was quantified (B). Schematic representation of DEAL capture with Cetuximab (C). DEAL captured GBM39 EGFR wt positive, EGFRvIII positive cells were stained by immunofluorescence for PTEN (green) after 16 and 48 hours of CC214-1 (10 μM) treatment (C). Ten percent of the CC214-1 treated GBM39 cells resulted positive for PTEN, whereas PTEN positive cells constituted only 2-3% of the population in the DMSO, indicating a reduced sensitivity of this cell phenotype to the drug. Images of the 16 hours CC214-1 and DMSO treatments are shown. Scale bar corresponds to 100 μm. Data represent average of three independent experiments. * indicates $P<0.05$ (Student's t-Test). Rapa.: Rapamycin.

Supplementary Figure S3.

Immunoblot showing that doxycycline induction (induction of 32 hours) of EGFRVIII expression leads to increased P-4E-BP1 in genetically modified LN229 cells, indicating that P-4E-BP1 level is under the regulation of EGFR signaling (A). CC214-1 (6 hours treatment) inhibits the phosphorylation of 4E-BP1 in a concentration dependent manner in the LN229 cells with or without doxycycline induction (A). U87EGFR cells have been tested in immunoblotting analysis after treatment with a number of agents specific for EGFR/PI3K/Akt/mTOR cascade for 6 hours (B; EGF concentration was 20 ng/mL).

Supplementary Figure S4.

Proliferation comparison in U373 Tet OFF system plus or minus doxycycline (5 μg/mL) upon CC214-1 treatment (A). Proliferation inhibition was 27% for EGFRvIII positive cells and only 16% for EGFRvIII negative cells (A). CC214-1 induces high level of autophagy in

U87EGFRvIII cell line, as indicated by the degradation of LC3B protein and decrease in free Atg-12 (B). Autophagic flux in U87EGFRVIII cells, assessed by LC3B-II and p62 protein levels (C). Cells were treated with chloroquine (20 μM) plus or minus CC214-1 (2 μM) and assessed in a time course of 2-24 hours (C). * indicates $P<0.05$; N.S.= Not Significant (Student's t-Test).

Supplementary Figure S5.

U373 tetracyclin OFF system shows that the tunable expression of EGFRvIII correlates with corresponding enhancement of autophagy's markers p62 and LC3B under CC214-1 (2 μM) treatment (A). After 18 hours of CC214-1 exposure, LC3B-II shows higher expression level in U373 EGFRvIII positive cells (as indicated in the graph). In LN229 tetracyclin ON model, the expression of EGFRvIII increases the CC214-1 (2 μM) mediated autophagy induction, registered as the level of p62 and LC3B proteins (B). LC3B-II level was higher in EGFRvIII positive cells for all of the time points assessed (as indicated in the graph). Chloroquine and doxycycline concentration in the medium were respectively 20 μM and 5 μg/mL. Chl.: Chloroquine.

Supplementary Figure S6.

Immunoblotting analysis, after 16 and 24 hours treatment with CC214-1 (5 μM), shows LC3B-I lipidation to LC3B-II as a sign of autophagy, whereas p62 level remains constant as a consequence of the chloroquine mediated inhibition of the auophagic flux (A). The quantified ratio of LC3B-II vs LC3B-I is shown (A). Atg-5 knock down induces significant apoptosis after CC214-1 (2 μM) treatment as shown in immunoblotting (B), crystal violet assay (C) and flow cytometry analysis for Annexin V, PI with corresponding % graphs (D). * indicates $P<0.05$ (Student's t-Test). Chl.: Chloroquine.

Supplementary Figure S7.

Immunoblotting analysis after combinatory treatment of CC214-1 (2 μM) plus chloroquine (20 μM) significantly enhanced apoptosis as assessed by cleaved PARP expression in U87EGFRvIII cell line (A). Trypan blue test in U87EGFRvIII and U87 cells (B). Representative IHC images of the intracranial xenografts shown in Fig.6 (C). mTORki effectors and ki67 resulted inhibited upon CC214-2 treatment in both, single and combinatory treatment with chloroquine (C). (Incubation time was 3 days for the trypan blue tests and chloroquine concentration was 10 μM; data represent average of three independent experiments. Ve: Vehicle, CC: CC214-2; Chl: Chloroquine. P-S6-S235-236, P-4E-BP1-T37-46 have been chosen as mTORC1 biomarker's activity; P-Akt-S473 was the selected mTORC2 effector; scale bar corresponds to 40 μm).