Supplemental Methods

Maintenance of AML cell lines

Kasumi-1 was obtained from ATCC (Manassas, VA, USA) and maintained in RPMI 1640 (Gibco, Life Technologies, Carlsbad, CA, USA) supplemented with 15% fetal bovine serum (FBS) from PAA Laboratories (Piscataway, NJ, USA). SKNO-1 cells were purchased from DSMZ (Braunschweig, Germany) and maintained in RPMI 1640 supplemented with 20% FBS and 7 ng/ml granulocyte-macrophage colony-stimulating factor (Cell Signaling Technology, Danvers, MA, USA). HL60 was obtained from ATCC and cultured in IMDM (Gibco) supplemented with 10% FBS. All cell lines were cultured at 37°C in the presence of 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco) in an atmosphere of 5% CO₂ in air, and seeded at 5 x 10⁵/ml one day prior to experiments. The cell lines were tested negative for mycoplasma.

Immunoblotting

Treated cells were washed in cold PBS/0.5% FBS and lysed in SDS buffer (0.8% SDS, 50 mM Tris pH 7.5, 1 mM EDTA) supplemented with a mixture of protease inhibitors (Roche, Penzberg, Germany). The lysates were passed through QIAshredders (QIAGEN, Germantown, MD, USA) and the protein concentrations were determined by the BCA Assay (Thermo Scientific, Rockford, IL, USA). 20 μg of protein per sample were loaded and resolved on Criterion 4–20% gradient gels (Bio-Rad, Hercules, CA, USA) followed by electro-blotting to Immobilon-P membranes (EMD Millipore Corporation). The blots were probed with antibodies indicated in the figures, and were detected using standard ECL reagents. The band signal intensities were quantified with the Quantity One software (Bio-Rad) and normalized to the loading control. Antibodies against the following proteins were used: ETO (sc-9737, Santa Cruz Biotechnology, Santa Cruz, CA, USA), Caspase-3 (#9665), PARP (#9542), LC3 (#2775), GAPDH (#2118), and ubiquitin (#3936), all from Cell Signaling Technology, and β-actin (Sigma).
Immunofluorescence

For staining of endogenous LC3, treated cells were adhered to poly-D-lysine coated 8-well Lab-Tek™ II Chambered Coverglass slides (Nunc, Sigma), fixed in methanol, and incubated with an antibody against LC3 (Clone 5F10, Nanotools), followed by Alexa488-labeled donkey anti-mouse IgG (Molecular Probes, Life Technologies). The cells were stained with Hoechst 33342 (1 µg/ml in PBS) for visualization of nuclei. Pictures were taken using an automated Cell Observer microscope (Carl Zeiss, Jena, Germany) equipped with a 40x objective and filters for detection of DAPI and GFP.

Quantification of LC3 spots was performed by the spot-detection protocol in the PhysiologyAnalyst module of the AxioVision ASSAYbuilder software. In the automated analysis, the nuclear Hoechst-staining was used for identification of individual cells, and discrete LC3 spots were identified in the GFP channel upon manual setting of threshold values for gating of proper spot size, shape and intensity. The software then determined the number of spots and the total intensity of the spot pixels per cell using the same threshold values for all samples. LC3 spots were quantified from 1-3,000 cells per condition in each experiment. The images were processed by the use of the microscope software and Adobe PhotoShop.

Quantitative real-time RT-PCR

Total RNA was isolated using the RNeasy Plus Mini Kit (QIAGEN, Germantown, MD, USA), according to the manufacturer’s instructions. 0.8 µg of total RNA was used for cDNA synthesis using the iScript cDNA Synthesis Kit (Bio-Rad). The real-time PCR analysis was run on a CFX96™ Real-time PCR Detection System (Bio-Rad) using SsoFast™ EvaGreen® Supermix (Bio-Rad) and prevalidated Quanti-Tect Primer Assays (QIAGEN). The cycling conditions were 95°C for 1 min, followed by 40 cycles of 95°C 5 sec, 55°C 5 sec, and 72°C 5 sec. Relative quantities of LC3B and AML1-ETO transcripts were determined using the regression analysis method provided in the CFX Manager™ Software (Bio-Rad). Transcript quantities were normalized to the relative quantity of the house-keeping gene GAPDH.
(glyceraldehyde 3-phosphate dehydrogenase) for each condition. The following prevalidated Quanti-Tect Primer Assays were used: Hs_MAP1LC3B_1_SG, and Hs_GAPDH_2_SG. For detection of AML1-ETO the following previously published primers were used: Forward primer 5’-CCACCTACCACAGGCCATCA-3’; reverse primer, 5’-AGCCTAGATTGCCTTCACATC-3’.

**Small interfering RNA transfection**

Kasumi-1 cells (5 × 10⁶) were transfected with 1.5 μmol of small interfering RNA (siRNA) against Atg7 or Ulk1, or non-targeting control siRNA (all from Dharmaco, Lafayette, CO, USA) in a nucleofector device (Amaxa Biosciences, Basel, Switzerland) using the Ingenio® nucleofection solution (Mirus Bio LLC, Madison, WI, USA) and program P-19. Cells were then incubated for 32 hours before further treatment. The siRNA oligos had the following sequences (5’-3’ direction, sense strand): Atg7, GCCCACAGAUGGAGUAGCA; Ulk1, UCACUGACCUGCUUUAA.

**Isolation of normal peripheral blood mononuclear cells**

Buffy coats from healthy blood donors were obtained from the blood bank at Ullevål University Hospital (Oslo, Norway). Peripheral blood mononuclear cells (PBMCs) were isolated using density-gradient centrifugation with Lymphoprep (Nycomed Pharma AS, Oslo, Norway) and maintained in RPMI 1640 supplemented with 15% FCS, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine (all from Gibco).

Supplemental Figure 1. Treatment of Kasumi-1 cells with VPA or vorinostat reduces the protein levels of AML1-ETO, activates caspases and leads to accumulation of LC3II. (A,C-D) Kasumi-1 cells were treated with VPA or vorinostat for 2-24 hours and total cell lysates were prepared for immunoblotting. The blots were probed with antibodies against ETO, PARP and LC3, and actin was used as a loading control. The band intensities of AML1-ETO (A), cleaved PARP (C), and LC3II (D) were normalized to those of actin. (B,E) Kasumi-1 cells were treated with VPA or vorinostat for the indicated times and total RNA was isolated. The mRNA levels of AML1-ETO (B) and LC3B (E) are shown. All bars show mean values ± SEM from at least 3 independent experiments. *, p<0.05; **, p<0.01; ***, p<0.001. The asterisks denote the statistical significances compared to the untreated control.
Supplemental Figure 2. AML1-ETO is not degraded by oridonin-induced autophagy. (A) Kasumi-1 cells were treated with oridonin (5 µM) for the indicated hours and total cell lysates were prepared for immunoblotting. The blots were probed with antibodies against ETO, caspase-3, PARP and LC3, and actin was used as a loading control. Blots from one representative experiment are shown. (B) Kasumi-1 cells were treated with oridonin for the indicated hours and total RNA was isolated. The bars represent the AML1-ETO mRNA levels, and the data shows that AML1-ETO mRNA is unchanged by oridonin. (C) Kasumi-1 cells were treated with oridonin for 16 hours in the absence or presence of autolysosomal inhibitors, and autophagic activity was measured as degradation of long-lived proteins. (D) Kasumi-1 cells were treated with oridonin for 8 hours and then fixed and prepared for immunofluorescence staining of endogenous LC3 (green) and Hoechst staining of nuclei (blue). Scale bar 20 µm. (E) Kasumi-1 cells were treated with the indicated concentrations of oridonin for 16 hours in the presence or absence of Baf or CQ. Cell lysates were prepared for immunoblotting, and the blots were probed with the indicated antibodies. Representative blots are shown. Right panel: The relative band intensities of AML1-ETO at 5 µM oridonin were normalized to GAPDH. All bars show mean values ± SEM quantified from at least 3 independent experiments. *, p<0.05; **, p<0.01.
Supplemental Figure 3. Knockdown of autophagy does not reverse the vorinostat-induced loss of AML1-ETO. (A) Kasumi-1 cells were transfected with siRNA oligoes against Atg7 and Ulk1, and cell lysates were prepared for immunoblotting 48 hours later. Left panel: Representative blots for the indicated antibodies. Right panel: Quantification of knockdown on protein level. The bars show mean values ± SEM quantified from at least three independent experiments. **, p<0.01. (B) Kasumi-1 cells were transfected with siRNA oligoes against Atg7 and Ulk1 and were treated with vorinostat at the indicated concentrations for the last 16 hours. Total cell lysates were prepared for immunoblotting, and AML1-ETO was detected by an ETO-specific antibody. Right panel: The relative band intensities of AML1-ETO at 1 µM vorinostat were normalized to GAPDH. The bars show mean values ± deviation from the mean quantified from 2 independent experiments.

Supplemental Figure 4. The caspase-dependent cleavage fragment of AML1-ETO is hardly visible upon VPA treatment. Kasumi-1 cells were treated with oridonin (5 µM) or VPA (1.5 mM) for 24 hours in the absence or presence of the pan-caspase inhibitor z-VAD-fmk (20 µM). Total cell lysates were prepared for immunoblotting and AML1-ETO was detected by an ETO-specific antibody.
Supplemental Figure 5. Verification of the specificity of the autolysosomal inhibitors Baf and CQ. Kasumi-1 cells were transfected with the siRNA oligo against Ulk1 to inhibit autophagy, and were then treated with or without VPA (1.5 mM) in the presence or absence of Baf (20 nM) or CQ (50 µM) for the final 16 hours. The cell viability was determined by FACS analysis of PI-stained cells and normalized to the untreated control. The bars show mean values ± deviation between duplicates from one representative experiment.

Supplemental Figure 6. Treatment with vorinostat leads to accumulation of ubiquitinated proteins that are further accumulated upon autophagy inhibition. Kasumi-1 cells were treated with the indicated concentrations of vorinostat for 16 hours in the absence or presence of Baf or CQ, and cell lysates were prepared for immunoblotting. The blots were probed with antibodies against ubiquitin and GAPDH. Blots from one representative experiment are shown. Right panel: The relative band intensities of ubiquitin at 1 µM vorinostat were normalized to GAPDH. The bars show mean values ± SEM quantified from at least 3 independent experiments. *, p< 0.05; **, p<0.01; ***, p< 0.001.
Supplemental Figure 7: CQ potentiates the effect of VPA and vorinostat in long-term experiments. (A) Kasumi-1 cells were treated with the indicated concentrations of VPA or vorinostat with or without CQ for 48 hours and cell lysates were prepared for immunoblotting. The blots were probed with antibodies against PARP, LC3 and GAPDH. Blots from one representative experiment are shown. (B) SKNO-1 cells were treated with the indicated concentrations of VPA (mM) with or without CQ for the indicated times, and cell viability was determined by FACS analysis of PI-stained cells. (C) Cells were treated with the indicated concentrations of vorinostat (µM) with or without CQ for the indicated times, and cell viability was determined by FACS analysis of PI-stained cells. The bars represent mean values ± SEM from at least 3 independent experiments. *, p< 0.05; **, p<0.01; ***, p< 0.001.
Supplemental Figure 8: Combined treatment with VPA and CQ shows limited toxicity in PBMC. PBMC isolated from a healthy donor was treated with VPA (1 mM) in the absence or presence of CQ (12.5 µM) for 48 hours, and cell viability was determined by FACS analysis of PI-stained cells. The bars represent mean values ± standard deviation between triplicates.

Supplemental Figure 9: Co-treatment with VPA and CQ has less effect on viability in AML cells negative for t(8;21) than in t(8;21)-positive cells. (A) Primary AML samples negative for t(8;21) were treated or not with VPA (1 mM) for 16 hours and degradation of long-lived proteins was measured. Mean value: 103.1% of untreated control +/- 2.5. (B) The AML samples were treated with VPA (1 mM), CQ (12.5 µM) or both for 48 hours. Cell viability was determined by FACS analysis of PI-stained cells and presented as raw data (left panel) or normalized to the untreated control (right panel). *, p<0.05.
**Supplemental Table 1: Characteristics of AML patient samples included in this study.** Patient samples #6-9 were tested negative for the fusion genes t(8;21), inv16, t(9;11), t(15;17), t(6;9), and t(11;19). Patient samples #2, 4 and 6-9 were tested for the following mutations: FLT3-ITD, FLT3-D835, c-kit D816V, WT1 exon 7 (INDEL mutations), CEBPA (INDEL mutations), IDH1 R132, and NPM1 exon 12 mutation. ND: not determined.

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