Supplementary results

We performed cell viability and clonogenic assays to assess the effect of MCL-1 knock-down by RNA interference in GC-resistant ALL cells. Reduction of MCL-1 expression levels resulted in a partial increase in dexamethasone sensitivity of GC-resistant ALL cells. This was reversed by zVAD.fmk but not with 3-MA (Figure S7A), as assessed using 7AAD stainings by flow cytometry (upper panel) and in clonogenic assays (lower panel). Moreover, MCL-1 knock-down suppressed the marked GC-sensitization effect by obatoclax that was observed in cells transfected with scrambled siRNA. In addition, reduction of MCL-1 levels resulted in activation of caspase-9 and in induction of mitochondrial membrane depolarization in cells treated with dexamethasone and obatoclax (Figure S7B and C), confirming that the partial GC-sensitization effect occurs by activation of apoptosis. As reported recently, apoptosis can suppress autophagy by enhancing caspase-mediated cleavage of beclin-1 (27). Indeed, exposure to dexamethasone and obatoclax resulted in a decrease of beclin-1 levels after knockdown of MCL-1 (Figure S7D). The decrease in beclin-1 levels did not occur in cells with intact MCL-1 levels and was prevented by zVAD.fmk or in cells devoid of caspase-9. Taken together, our results show that MCL-1 is involved in preventing apoptosis induction in GC-resistant ALL cells, and that in absence of MCL-1, dexamethasone and obatoclax trigger mitochondrial apoptosis, which overrides the autophagy-dependent pathway.

Supplementary methods

Reagents

Dexamethasone was purchased from Mepha Pharma, rapamycin from Calbiochem, and 3-MA from Sigma. Obatoclax was provided by GEMIN X, ABT-737 by Abbott Laboratories, and rabbit antibodies were from Cell Signaling Technology (anti-Beclin-1, 1:1000 and anti-Bcl-X_L, 1:1000), Sigma (anti-actin, 1:3000 and anti-Mcl-1, 1:3000), ProSci (anti-APG7, 1:1000) and Abgent (anti-APG5L, 1:1000). Mouse-anti-LC3 (1:1000) was purchased from Axxora. FITC-labelled anti-human CD45, PE-labelled anti-human CD19 and Alexa 647-labelled anti-mouse CD45 antibodies were from Serotec.

Cell culture

The ALL cell lines, CEM-C1 and -C7, 697 (ATCC #CRL-7433), MOLT-4 (ATCC #CRL-1582), Jurkat (ATCC #TIB-152) and REH (DSMZ, ACC22) were maintained in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, and 100 IU/mL penicillin/streptomycin (Invitrogen). The RS4;11 (ATCC # CRL-1873) cell line was maintained in alpha MEM medium containing 10% heat-inactivated fetal bovine serum and 100 IU/mL penicillin/streptomycin (Invitrogen). Human mesenchymal stroma cells (MSC) were maintained in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 IU/mL penicillin/streptomycin and 50µM hydrocortisol (Sigma). Wild-type primary mouse embryonic fibroblasts and those deficient for BAX and BAK were maintained in DMEM containing 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, and 100 IU/mL penicillin/streptomycin. For all compounds, the effect of treatment at low dose was used to normalize values for the calculation of survival curves.

Immunoprecipitations and western analysis

For immunoprecipitation experiments, treated cells were lysed in CHAPS-buffer (20 mM Tris-Cl, pH 7.5, 137 mM NaCl, 2 mM EDTA, 10% glycerol and 2% CHAPS). Lysates were incubated with anti-MCL-1 (Stressgen), anti-Beclin-1 (Santa Cruz) combined with protein-G sepharose (Sigma). Washed beads were processed for western blotting.

For Western blotting, whole-cell extracts were prepared from 1 x 10^6 cells using radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-Cl, pH 6.8, 100 mM NaCl, 1% Triton-X-100, 0.1% SDS) supplemented with complete mini protease inhibitor cocktail (Roche Applied Sciences) and 1 mM sodium orthovanadate (Sigma) for 20 minutes on ice. After SDS-PAGE, proteins were blotted onto nitrocellulose membranes. Membranes were blocked in 5% non-fat dry milk and incubated with primary antibodies in 3% bovine serum albumin

(Sigma). Horseradish peroxidase-labeled goat anti-rabbit or anti-mouse antibodies were used for signal detection with chemiluminescence substrate (Pierce) and direct scanning (Fujicolor).

Fluorescence microscopy

ALL cells and MEFs were transfected with the full-length rat pEGFP-LC3 as described above ALL cells were directly co-cultured with MSCs on coverslips for 24h and treated with DMSO, 1 μM DEX, 100 nM obatoclax, or in combination, in the presence or absence of 3-MA (Sigma, 200 μM) for 4 hours. The cells were fixed with methanol for 20 min, washed three times with PBS, mounted with Vectashield on glass slides, and analyzed using a Zeiss META confocal microscope. Mouse embryonic fibroblasts were attached to coverslips for 24 h after transfection and treated with obatoclax or rapamycin for 4 h and then fixed with PFA 4% for 20 min. Nuclei were stained with DAPI in PBS for 10 min. The number of cells with punctate staining per 100 GFP-LC3 transfected cells was determined in two independent experiments. Counting was performed in six random fields, each representing 100 GFP-positive cells and expressed as the mean ± SD.

Transmission electron microscopy

Jurkat cells were treated either with vehicle, dexamethasone and obatoclax (for 72h), TRAIL (for 24h) or with NaAzide (3% for 15min). Cells were harvested, washed with PBS 1X and fixed over night at 4°C in 3% glutaraldehyde in PBS. Cells were then washed and the pellet post-fixed for 1 h at RT with 2% osmium tetroxide and embedded into 2.5% agar in 50 mM sodium cacodylate buffer (pH 7.3), cooled down over night at 4°C and dehydrated in an ethanol series. The samples were diluted with EPON in increasing concentration and embedded in fresh EPON in capsules or flat silicone rubber moulds in the oven at 60°C for at least 12h. The samples were then analyzed using a CM 100 transmission electron microscope.

Viability and Apoptosis Assays

Cell culture was performed in 96-well plates with 2 x 10^4 ALL cells in cell suspension, or as co-culture of 10^5 primary ALL cells on 10^4 MSC, or with 10^5 MEF cells per well. Ten percent of the IC50 concentration of obatoclax or rapamycin was used for combination treatment with DEX, unless stated otherwise. Cell viability for ALL cell lines and MEF experiments was assessed using the MTT cell proliferation kit (Roche Applied Sciences) and, for primary ALL cells, by flow cytometry using 7AAD (BD biosciences). The minimal absorbance of control wells was OD 0.8. Caspase activation was detected by flow cytometry using the CaspGLOW

Red Active Caspase-3 Staining Kit (Alexis) according to the manufacturer's instructions, incubating1 µL specific substrate for caspase-3 (Red-DEVD-FMK) for 10^5 treated cells.

Ratiometric Measurement of Mitochondrial Membrane Potential

The mitochondrial membrane potential was assessed using the JC-1 mitochondrial membrane sensor (Invitrogen) according to the manufacturer's instructions.

Xenograft model

10^6 primary ALL cells were recovered on dry ice in small portions from cryopreserved presentation samples, washed in PBS, and transplanted intrafemorally into Nod/LtSzScid IL2γ^{null} mice. Xenografted ALL cells were recovered from the spleen and bone marrow, yielding more than 96% human CD45 and C19 positive cells per harvest as verified by flow cytometry, and were cryopreserved for cell culture experiments.
For in vivo treatment, ALL cells recovered from a first xenotransplantation in NSG mice with cells from a precursor B-ALL patient with poor prednisone response were injected intrafemorally into cohorts of NSG mice (10^6 cells per animal). Leukemia progression was assessed by flow cytometry of the mouse peripheral blood using anti-mouse and human CD45 and CD19 antibodies. When 1% human cells were detectable in the peripheral blood, animals were randomized into 4 treatment groups and treated with either vehicle intramuscularly (i.m.; n=11 for patient 4 and n=6 for patient 2), 3 mg/kg/day obatoclax i.m. (n=8 for patient 4 and n=6 for patient 2), 5 µg/g/day DEX intraperitoneally (i.p; n=10 for patient 4 and n=6 for patient 2), or the combination of DEX i.p. and obatoclax i.m. (n=10 for patient 4 and n=6 for patient 2). Animals were treated 5 days per week for three consecutive weeks. Leukemia progression was monitored by flow cytometry.

Supplementary table S1: Cytotoxicity of obatoclax and ABT-737 in ALL cells.
Indicated ALL cell lines were incubated with vehicle, 1 μM DEX, 10% IC_{50} dose of obatoclax, or the combination of DEX and obatoclax for 48h, and caspase activation was measured using flow cytometry. IC50 values obtained from MEF treatment are also presented.

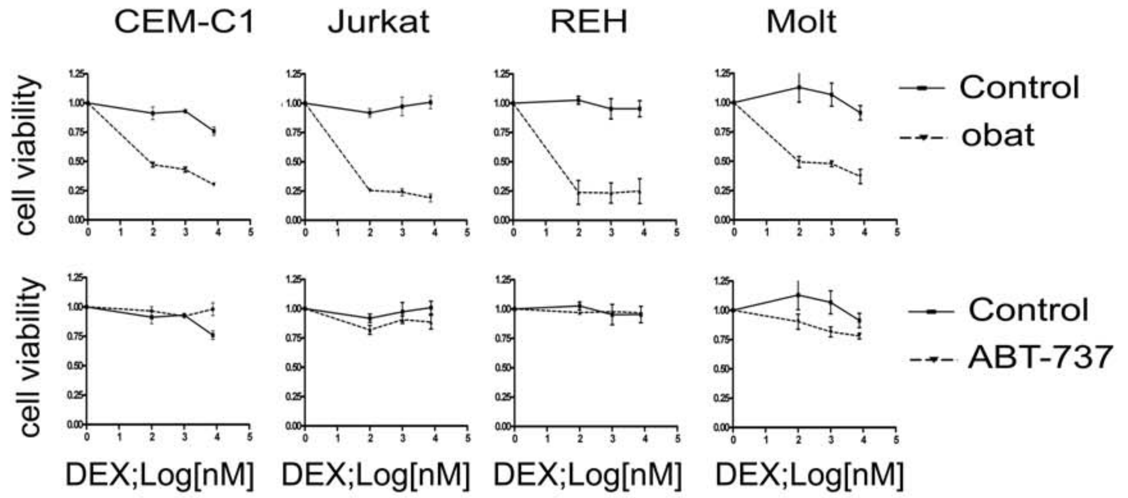
Cell line	IC50 ABT-737, μM	IC50 Obatoclax, μM
CEM-C1	0.242 ± 0.036	0.980 ± 0.145
Jurkat	0.103 ± 0.033	0.500 ± 0.037
REH	1.564 ± 0.13	0.450 ± 0.097
MOLT-4	0.146 ± 0.045	0.055 ± 0.009
CEM-C7	0.058 ± 0.030	0.380 ± 0.079
697	0.182 ± 0.018	0.122 ± 0.023
RS4;11	0.014 ± 0.005	0.113 ± 0.011
wt MEFs	1.234 ± 0.213	0.108 ± 0.025
DKO MEFs	N.D.	0.119 ± 0.022

IC50 values were calculated using GraphPad prism software with triplicate values from MTT assays.

Supplementary Table 2. Patient characteristics

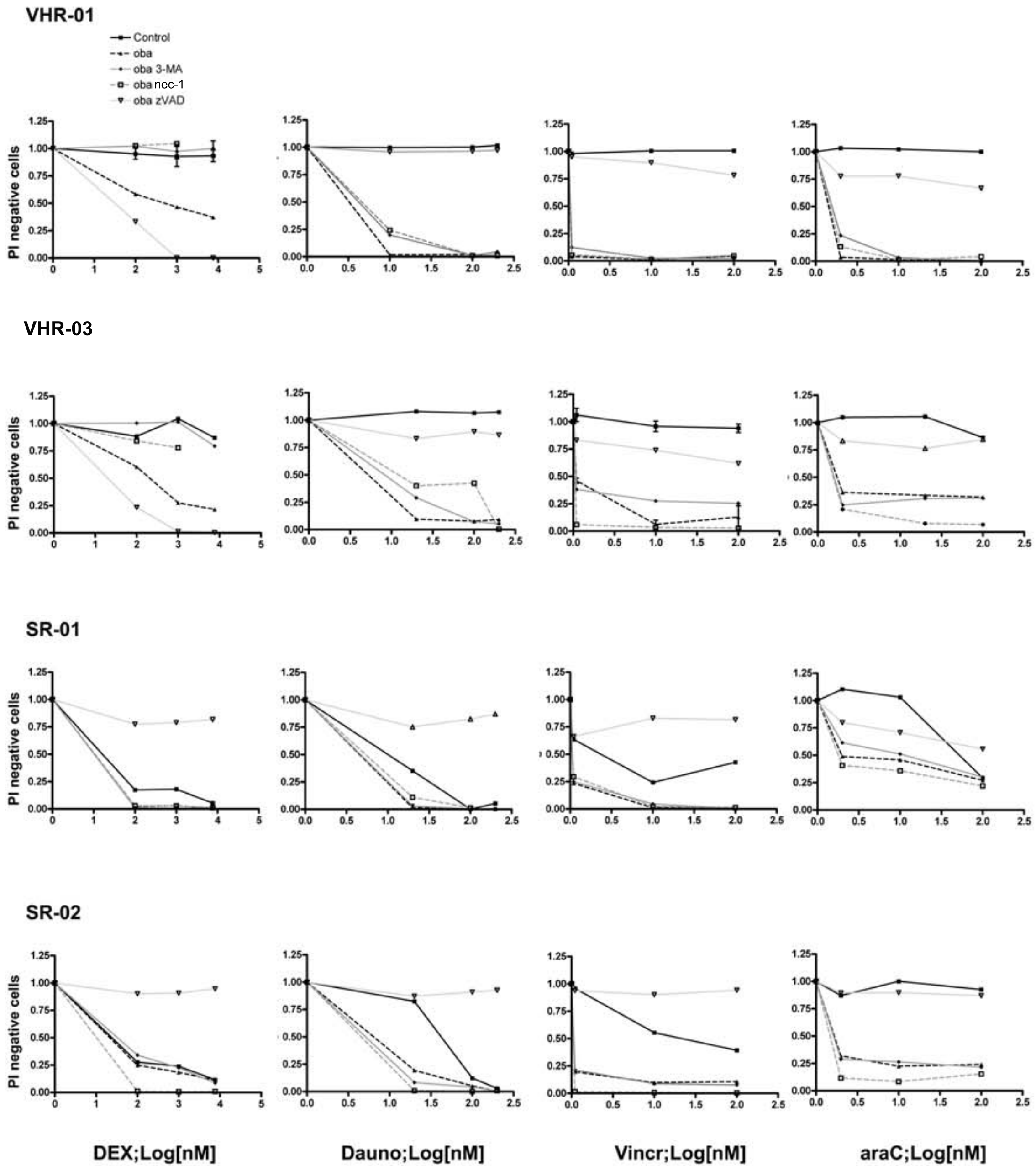
Characteristic	patient no.										
	VHR-01	VHR-02	VHR-03	VHR-05	VHR-06	VHR-07	HR-01	HR-02	SR-01	SR-02	SR-03
Age, y	14	13	17	16	17	12	9	11	11	12	4
Subtype	pc-B	pc-B	pc-B	pc-B	pc-B	pc-B	T	T	pc-B	pc-B	pc-B
Pred.resp.	poor	poor	poor	good	good	poor	poor	poor	good	good	good
Blast, d0	6	80	33	13	16	529	207	75	18	447	11
BM blast d8	1.8	5.7	2.5	0.024	0.164	4.3	127	179	0.017	0.07	0.042
BM blast d0	82%	90%	73%	84%	80%	80%	92%	70%	85%	96%	82%
MRD	VHR	VHR	VHR	VHR	VHR	VHR	HR	HR	SR	SR	SR
Oba IC_{50} (nM)	1284	1521	983	993	1174	1317	1238	1535	923	875	1024

Figure S1



Supplementary Figure S1.
Dose response curves are shown for four different ALL cell lines. Cells were incubated with obatoclax or ABT-737 each at 10% IC50 and increasing concentrations of DEX, and cell survival was assessed with the MTT assay.

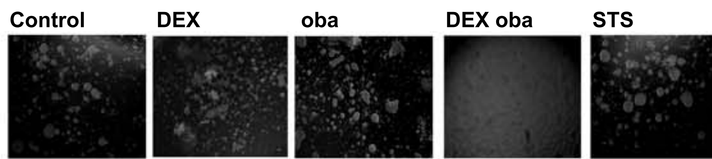
Figure S2



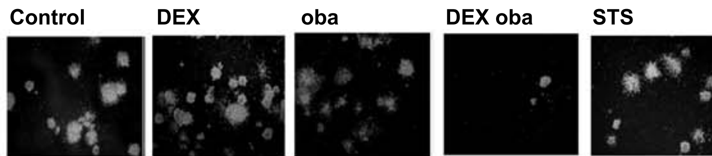
Supplementary Figure S2.
Dose response curves are shown for primary ALL cells from very high-risk (VHR)-ALL and standard-risk (SR)-ALL patients. Cells were cultured on hTERT-immortalized MSCs and incubated for 72h with indicated drugs (obatoclax at 10% IC50, nec-1 at 300 nM, 3-MA at 200 nM and zVAD.fmk at 1 μM) together with increasing concentrations of indicated cytotoxic agents. Cell viability was assessed by 7-AAD stainings using flow cytometry.

Figure S3

Jurkat *caspase 9-/-*

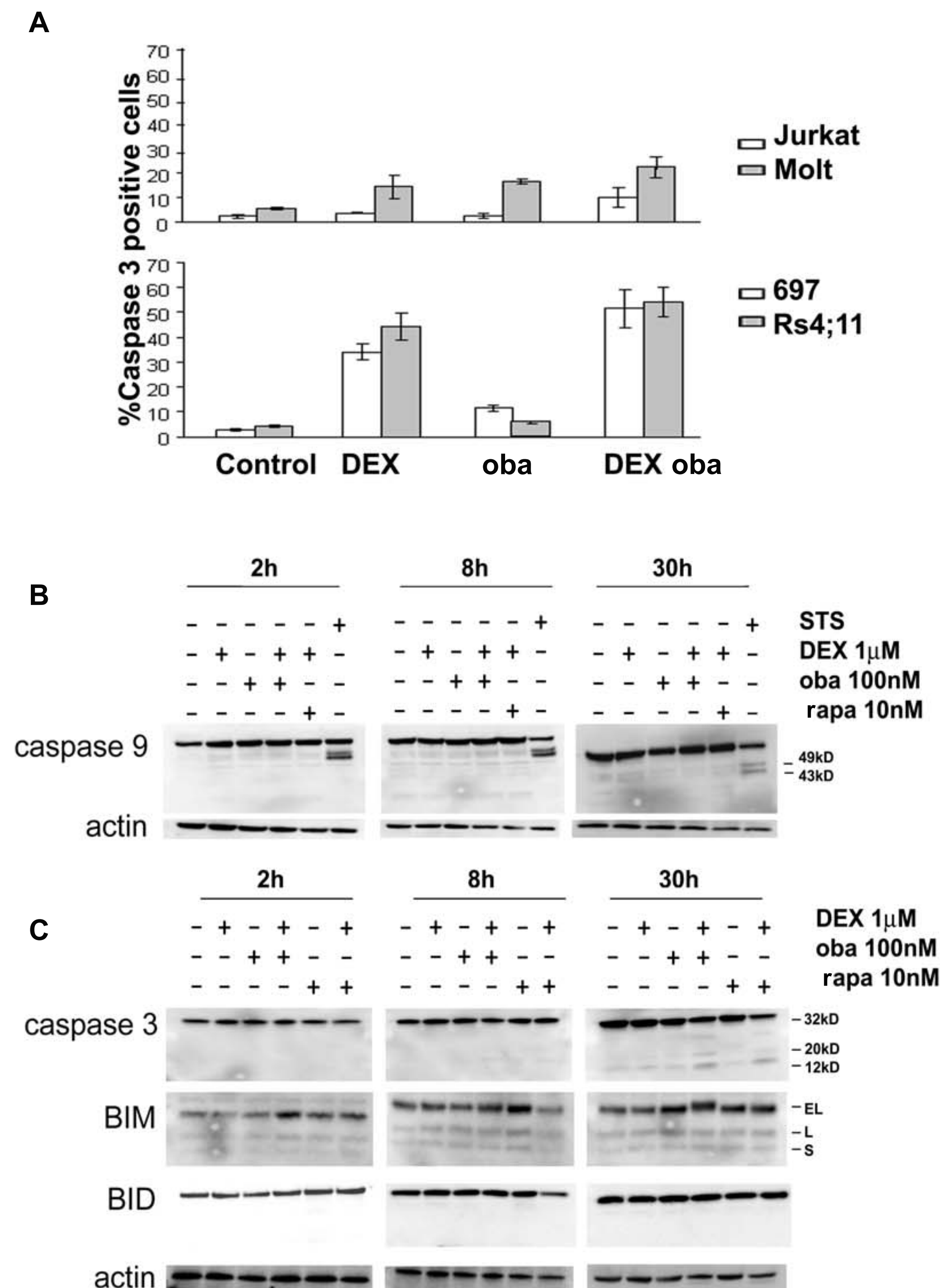


Jurkat *Bax-/-Bak-/-*



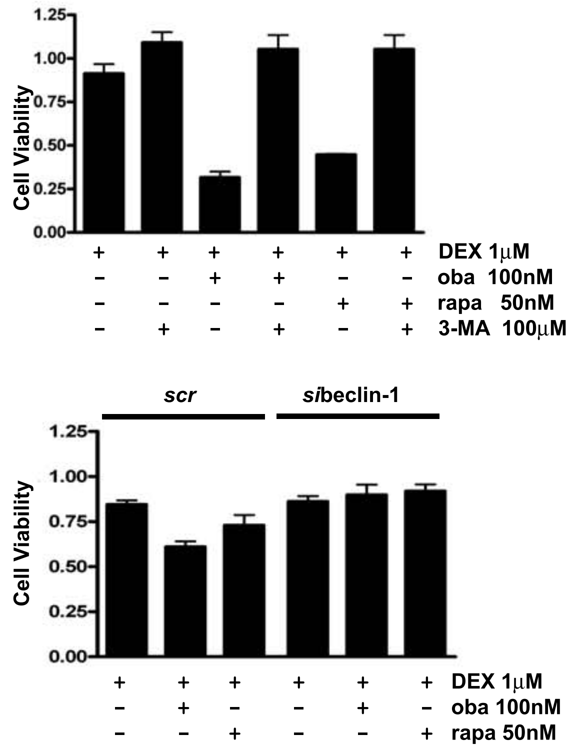
Supplementary Figure S3.
Phase contrast pictures of clonogenic survival assays of Jurkat cells deficient for *caspase-9* (upper panel) or *Bax* and *Bak* (lower panel). Clonogenic survival was assessed after treatment of cells with either vehicle, obatoclax at 100 nM, DEX at 1 μM, their combination or staurosporine at 100 nM for 72h, washing and incubation in methylcellulose for 7 days. Images of representative visual fields, taken on an inverted Leica microscope, are presented.

Figure S4



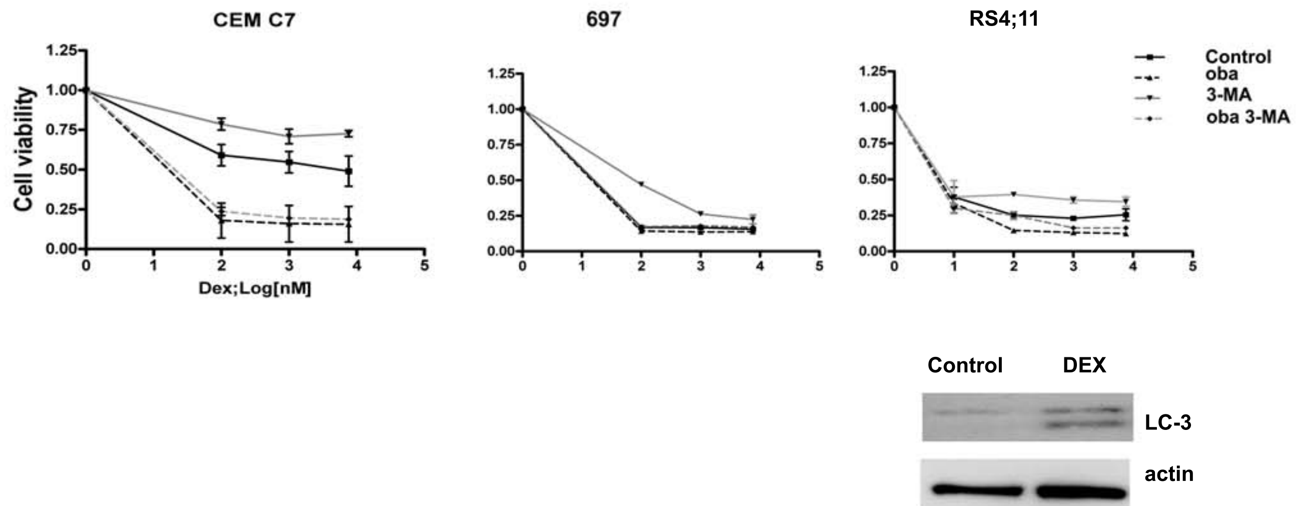
Supplementary Figure S4.
(**A**) Caspase-3 activation in ALL cells was measured by flow cytometry using the CaspGlow Red staining kit. In GC-resistant ALL cells, caspase-3 was only marginally activated after treatment with either dexamethasone, obatoclax or the combination (upper panel). In contrast, in GC-sensitive cells, dexamethasone treatment lead to caspase-activation, which was not further enhanced by co-treatment with obatoclax (lower panel). (**B**) Wild-type Jurkat cells were treated for 2, 8 and 30h as indicated. For control, STS was used. Activation of caspase- 9 was assessed by Western blotting. (**C**) Wild-type Jurkat cells were treated for 2, 8 and 30h as indicated. Activation of caspase-3 as well as levels of BIM and BID were assessed by Western blotting.

Figure S5



Supplementary Figure S5.
The effect of inhibition of autophagy by either 3-MA or downregulation of Beclin-1 was assessed in an independent cell line, CEM-C1. Cells were incubated for 72h with indicated concentrations of compounds and cell viability was assessed with the MTT assay (upper panel). Likewise, downregulation of beclin-1 conferred resistance to treatment with obatoclax and DEX, as assessed with the MTT assay (lower panel).

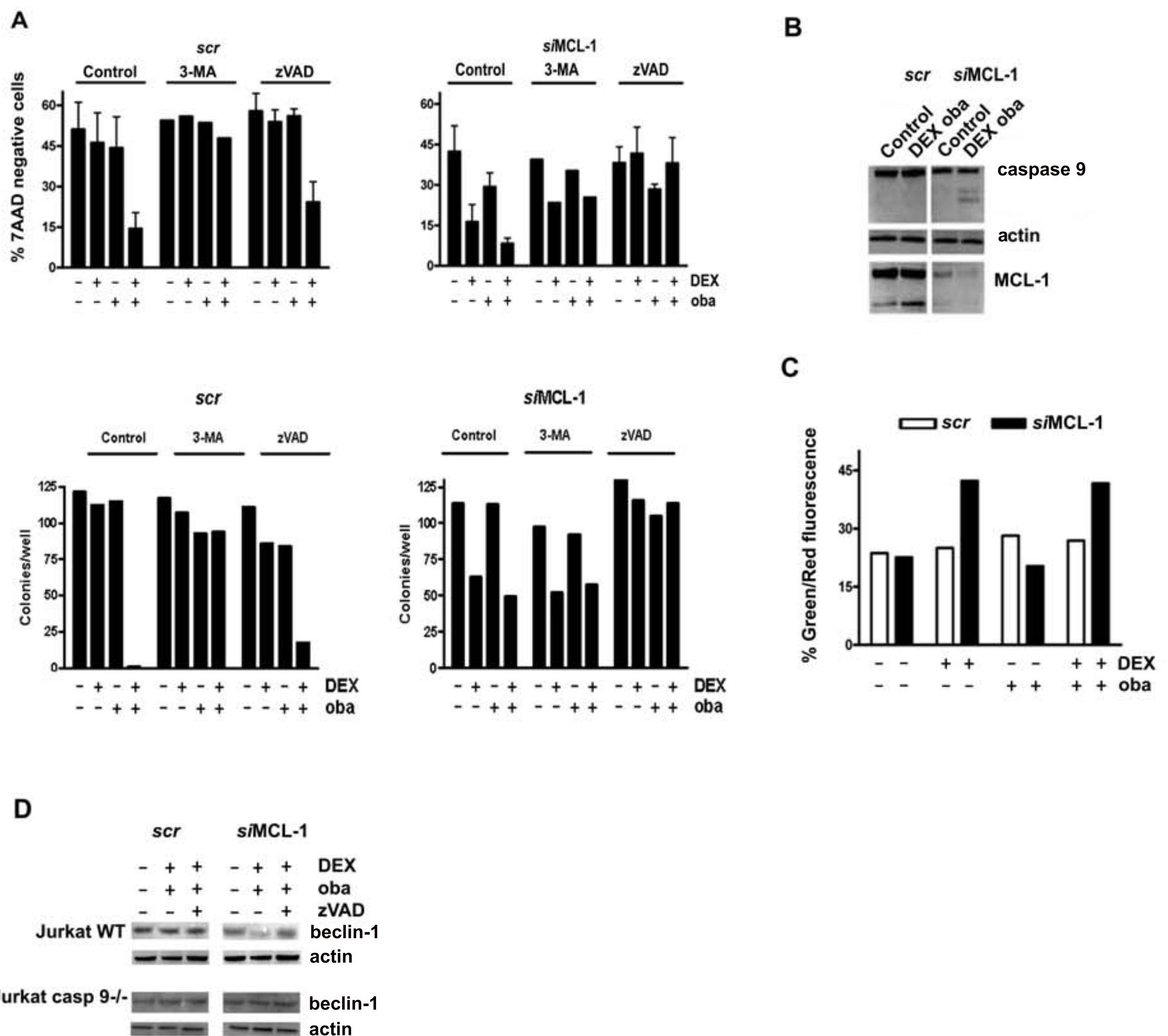
Figure S6



Supplementary Figure S6.

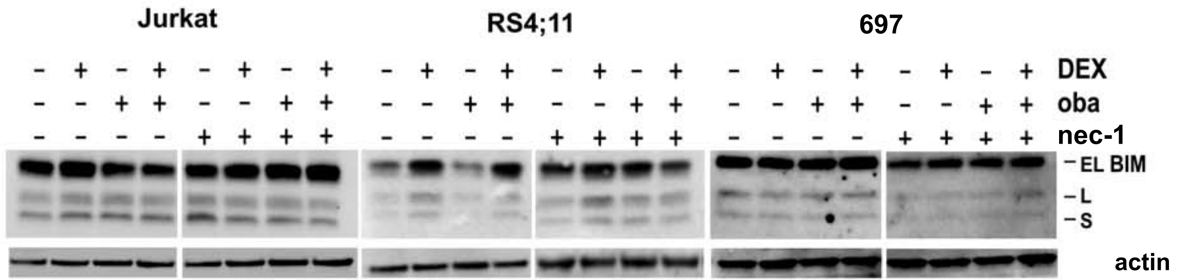
Inhibition of autophagy using 3-MA only partially restored steroid resistance in steroid-sensitive CEM-C7. In the steroid-sensitive lines RS4;11 and 697 cells, no rescue occurred after 3-MA treatment (upper panel). However, LC3-II generation still occurred in RS4;11 cells after treatment with DEX (lower panel).

Figure S7



Supplementary Figure S7. Downregulation of MCL-1 facilitates induction of apoptosis after treatment with DEX.
(**A**) Effect of siRNA-mediated knock-down of MCL-1 on the response to DEX and / or obatoclax was assessed by flow cytometry using 7AAD stainings with or without treatment with indicated agents for 72h. Downregulation of MCL-1 lead to partial sensitization to DEX. (**B**) Activation of caspase-9 after downregulation of MCL-1 and incubation with DEX and obatoclax.
(**C**) Percentage of cells with JC-1 monomers corresponding to cells with a lossof the mitochondrial potential is shown after downregulation of MCL-1 and treatment with vehicle, DEX, obatoclax or the combination. (**D**) Beclin-1 was decreased after downregulation of MCL-1 and treatment with DEX and obatoclax in Jurkat wild type cells (upper panel). Decrease of beclin-1 did not occur in the presence of zVAD.fmk or in Jurkat cells devoid of caspase-9 (lower panel).

Figure S8



Supplementary Figure S8.
Nec-1 inhibits BIM induction in steroid-sensitive ALL cells. Jurkat or RS4;11 cells were incubated with indicated agents for 16h, and BIM induction was assessed by western blotting. In steroid-resistant ALL cells (Jurkat), BIM induction did not occur, and no effect of nec-1 could be observed. In steroid-sensitive RS4;11 cells, BIM induction by dexamethasone was inhibited by nec-1.