The synergism of MCL1 and glycolysis on pediatric acute lymphoblastic leukemia cell survival and prednisolone resistance

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The synergism of MCL1 and glycolysis on pediatric acute lymphoblastic leukemia cell survival

and prednisolone resistance

Running Head: The synergism of MCL1 and glycolysis in leukemia

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Abstract

In vitro and in vivo resistance to prednisolone are predictive for an adverse prognosis in pediatric precursor-B acute lymphoblastic leukemia. Causes of resistance are still poorly understood. In this study, we observed that prednisolone exposure of prednisolone-sensitive patients’ leukemic cells decreased anti-apoptotic MCL1 protein levels by 2.9-fold, while MCL1 protein expression in prednisolone-resistant leukemic patients’ cells was unaffected (p<0.01). Locked nucleic acid oligonucleotides directed against MCL1 reduced MCL1 protein levels by 82±16% (p<0.05) in leukemic cells, decreased proliferation by 9-fold and sensitized to prednisolone up to 80.8-fold, compared to a non-silencing-control locked nucleic acid (p<0.05). Remarkably, we discovered that MCL1-silencing upregulated the glucose consumption of leukemic cells by 2.5-fold (p<0.05), suggesting a potential rescue mechanism mediated by glycolysis. Targeting glycolysis by 2-deoxyglucose synergistically inhibited leukemic survival by 23.2-fold in MCL1-silenced cells (p<0.05). Moreover, 2-deoxyglucose and MCL1 locked nucleic acid concomitantly sensitized leukemic cells to prednisolone compared to MCL1 locked nucleic acid or 2-deoxyglucose alone (p<0.05). In conclusion, these results indicate the need to target both MCL1 and glycolysis simultaneously to inhibit leukemic survival and sensitize acute leukemia patients towards prednisolone.

Key words: leukemia, prednisolone resistance, MCL1, glycolysis, LNA, 2-deoxyglucose.
Introduction

Acute lymphoblastic leukemia (ALL) is the most common cancer in children. Although cure rates have greatly improved over recent years, treatment is still ineffective in 20% of patients. Unsuccessful treatment can be ascribed to resistance of primary leukemic cells to antileukemic drugs. Poor prognosis is particularly associated with resistance to prednisolone in childhood ALL, both in vivo and in vitro. To increase current survival rates it is therefore necessary to overcome prednisolone resistance.

To identify therapeutic targets to overcome prednisolone resistance, we previously performed microarray analysis on primary BCP-ALL cells of pediatric patients. This indicated a role in prednisolone resistance for MCL1, an anti-apoptotic member of the BCL2 family that is frequently overexpressed in a variety of cancers and that contributes to cancer cell survival and apoptosis resistance. Functional studies revealed that silencing of MCL1 sensitized leukemic cells to prednisolone.

In addition, we and others discovered that glycolysis is increased in prednisolone-resistant leukemic cells. Microarray analysis on primary precursor-B acute lymphoblastic leukemia (BCP-ALL) cells of pediatric patients indicated an increased expression of several glycolytic enzymes and glucose transporters in prednisolone resistant patients. Furthermore, 2-deoxyglucose (2-DG), an inhibitor of glycolysis, sensitized both leukemic cell lines and patients’ ALL cells to prednisolone.

For three reasons, we now hypothesize that anti-apoptosis sustained by MCL1, and glycolysis are linked processes and concomitantly induce drug resistance in leukemia. 1) Cellular respiration and apoptosis are closely related survival pathways both associated with prednisolone resistance, and other targeted molecular leukemia therapies, such as imatinib. 2) Increased glucose metabolism has been directly linked to MCL1 stabilization and attenuation of apoptosis, and 3) BCL2 family members can, besides their apoptotic function, adjust oxidative phosphorylation.

In the present study, we show that silencing of MCL1 by specifically designed locked nucleic acid antisense oligonucleotides against MCL1 mRNA (MCL1 LNA) inhibited cell survival and sensitized to prednisolone in both BCP-ALL and T-ALL leukemic cells. Moreover, we discovered higher glucose consumption in ALL cells after MCL1 silencing by both shMCL1 and MCL1 LNAs. Most importantly, we demonstrate that 2-DG treatment of MCL1-silenced cells decreased glucose consumption and synergistically reduced leukemic survival. Moreover, MCL1 LNA and 2-DG concomitantly reversed prednisolone resistance.
in leukemic cells. These data provide evidence that MCL1 and glycolysis should be targeted simultaneously to effectively inhibit leukemic survival and to reverse prednisolone resistance in ALL.

**Methods**

**Cell culture and primary cells**
Leukemic cells from children with newly diagnosed ALL were isolated from bone marrow aspirates and prednisolone resistance was assessed, as previously described \(^1\). Informed consent was given by patients as approved by the local institutional review board. Only samples with ≥ 90% leukemic cells upon processing were used in the present study. Reh, 697, Sem, Jurkat, Loucy and HEK293T cells were obtained from DMSZ. The leukemic cell lines were cultured in RPMI+Glutamax (Gibco) and HEK293T cells in DMEM+Glutamax (Gibco) at 37°C in humidified air containing 5% CO\(_2\). Cell viability and cell count were determined by a trypan blue exclusion staining assay and analyzed by MACSQuant.

**LNA transfection and 2-deoxyglucose treatment**
Cell lines were cultured in the presence of either 10 μM locked nucleotide acid oligonucleotides directed against MCL1 (MCL1 LNA), i.e. SPC4120 (MCL1 LNA-a), SPC4342 (MCL1 LNA-b), SPC4343 (MCL1 LNA-c), or a non-silencing control oligonucleotide LNA, i.e. SPC3088. Twenty-four hours after LNA transfection, cells were cultured with and without 0.5 mM 2-deoxyglucose (Sigma). Supplemental Figure 1A-B illustrates that 0.5mM 2-deoxyglucose hampers cell count only modestly, while it has a quantifiable effect on glucose consumption. After 96 hours, culture medium was replaced by fresh medium containing fresh LNA +/- 2-DG.

**Apoptosis measurement**
AnnexinV/PI double positive and AnnexinV single positive cells were measured on the BD FACS Calibur flow cytometer.

**Quantitative RT-PCR**
*MCL1* mRNA levels were quantified by incorporation of SYBR Green (Thermo Scientific) by quantitative real-time PCR (Applied Biosystems 7900HT). Primers for *MCL1* were; 5’-GGAGGAGGAGAGTTGTAC-3’ (forward) and 5’-AAG GCA CCA AAA GAA ATG-3’ (reverse).
Reverse Phase Protein Array

Primary leukemic cells were cultured for 48h with 0 µg/ml, 1 µg/ml or 250 µg/ml prednisolone. Protein was isolated and lysates were spotted twice in triplicate on glass-backed nitrocellulose-coated array slides by the facility of Dr. E. F. Petricoin, George Mason University-Manassas USA. Antibodies used were: MCL1 antibody (Sigma HPA008455), Bcl-XL (Cell Signaling 2762), BCL-2 (Cell Signaling 2872) and p53 (Cell Signaling 9282).

Western Blot and Immunoblotting

Twenty micrograms of protein were used as input for western blot analysis using anti-MCL1 (Sigma HPA008455), anti BCL-2 (Cell Signaling 2872), anti-β-actin (Abcam ab6276), anti-Clathrin (Santa Cruz Sc12734), IRDye 800CW-labeled anti-rabbit and IRDye 680CW-labeled anti-mouse (Li-Cor IRDye). Protein levels were quantified using the Oddysey system.

In vitro MTT drug-resistance assay

Cytotoxicity of cells towards prednisolone (Bufa Pharmaceutical Products) were determined by the in vitro 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) drug-resistance assay, as described previously ¹.

Glucose-consumption assay

Glucose levels were measured with the glucose assay kit (Sigma, GAGO-20). To calculate glucose consumption, values were compared with plain RPMI glucose levels and corrected for cell growth.

Synergistic effect

Synergistic effects were calculated from equi-effective drug concentrations by the equitation postulated by Berenbaum ¹⁵.

Statistical analyses

Prednisolone exposure effects within either prednisolone resistant or sensitive patients was analyzed with a Kruskall-Wallis test. A Mann-Whitney U test was used to compare resistant to sensitive patients. The Mann-Whitney U test was also applied to analyze the effects of shMCL1. MCL1 LNA experiments were compared with a T test. p<0.05 was considered statistically significant.

Additional information regarding the methods section can be find in the supplements.
Results

Downregulation of MCL1 by prednisolone is impaired in prednisolone resistant leukemic cells of patients.

Glucocorticoids are known to induce apoptosis by downregulation of anti-apoptotic BCL-2 family members independent of p53 \(^{16,17}\). We analyzed protein expression of BCL-2 family members and p53 in three prednisolone sensitive and three resistant primary patient samples after exposure to prednisolone for 48h (Figure 1 and Supplemental Figure 2A-D). After \textit{in vitro} prednisolone exposure, the expression of MCL1 in leukemic cells of \textit{in vitro} prednisolone sensitive pediatric ALL patients significantly decreased by 2.9-fold (p<0.01). Whereas, in prednisolone resistant ALL patient cells, these levels did not change upon prednisolone exposure. Prednisolone did not affect the expression levels of BCL-XL, BCL2 nor p53 (Figure 1 and Supplemental Figure 2A-D). A similar decrease in MCL1 but not in BCL2 was seen using an extended dilution series of prednisolone (Supplemental Figure 2E).

\textbf{MCL1 is a potent target to inhibit leukemic survival and to sensitize to prednisolone in pediatric ALL.}

Three different newly-developed LNA oligonucleotides directed against MCL1 were up to 90% effective in silencing MCL1 in five distinct leukemic BCP-ALL and T-ALL cell lines, i.e. MLL-AF4\(^+\) BCP-ALL (SEM), ETV6-RUNX1\(^+\) BCP-ALL (REH), E2A-PBX1\(^+\) BCP-ALL (697), ETP-ALL (Loucy) and tetraploid T-ALL (Jurkat) cells (p<0.01 Supplemental Figure 3 and 4). MCL1 LNA-b provided the most potent and reproducible knockdown of these three MCL1 LNAs. Knockdown achieved by the three MCL1 LNAs was comparable to the knockdown obtained after stable lentiviral transduction of two short hairpin RNA directed against MCL1, i.e. shMCL1a and -b (p<0.05; Supplemental Figure 5). The MCL1 LNAs inhibited leukemic survival up to 90%, increased apoptosis up to 60% and sensitized to prednisolone up to 80.8-fold in five distinct leukemic cell lines, all compared to a non-silencing LNA control (p<0.05; Supplemental Figure 6, 7 and 8). These MCL1 LNA results were comparable to shMCL1 results (Supplemental Figure 9) and indicates that targeting MCL1 may be clinically important.

\textbf{MCL1-silenced cells upregulate glycolysis. Targeting glycolysis in MCL1 silenced cells synergistically inhibits leukemic survival and concomitantly reverses prednisolone resistance.}

To test our hypothesis that MCL1 and glycolysis cooperate in prednisolone resistance, we examined the consumption of glucose in five distinct MCL1 silenced cell lines. A significant increase in glucose consumption, on average 149%, was
observed upon MCL1 silencing by MCL1 LNA (p<0.01 MCL1 LNA-b Figure 2 and MCL1 LNA-a and LNA-c Supplemental Figure 10) and by shMCL1 (p<0.01 Supplemental Figure 11), compared to non-silencing LNA controls.

This finding suggested that MCL1 silenced cells might increase their glycolysis to rescue from apoptosis. This prompted us to investigate the effect of targeting both MCL1 and glycolysis on leukemic cell survival and prednisolone cytotoxicity. Co-exposure of MCL1 LNA and 2-DG significantly reduced glucose consumption (p<0.05 MCL1 LNA-b Figure 2, MCL1 LNA-a and MCL1 LNA-c Supplemental Figure 10). In all cell lines, cotreatment of cells with MCL1 LNA and 2-DG synergistically inhibited leukemic cell survival by 30%-75%, compared to MCL1 LNA or 2-DG alone, except in the tetraploid T-ALL (p<0.05, F_{syn} <1, Figure 3 Panel A and Supplemental Figure 12). Furthermore, the addition of prednisolone decreased leukemic survival even more (p<0.05 Figure 3 Panel B and Supplemental Figure 13). Incubation of leukemic cells with both MCL1 LNA and 2-DG concomitantly, albeit moderately, sensitized to prednisolone up to 1.48-fold compared to MCL1 LNA or 2-DG alone (p<0.05; Figure 3 Panel C and Supplemental Figure 14). The synergism of MCL1 LNA, 2-DG and prednisolone was best visible in the intermediate responsive cells, i.e. MLL-AF4^+ , ETV6-RUNX1^+ and ETP-ALL cell line, since the sensitive E2A-PBX1^+ cell line was already prone to die by monotherapy alone and the highly resistant tetraploid cell line will most likely need higher amounts of the drugs to demonstrate an effect. Overall, these data indicate that MCL1-silenced cells upregulate glycolysis and that targeting glycolysis in MCL1 silenced cells synergistically inhibits leukemic survival and concomitantly reverses prednisolone resistance.

Discussion

Prednisolone is the spearhead drug used in multi-drug treatment of ALL. Not only is the in vivo and in vitro response to prednisolone a strong prognostic factor for long-term clinical outcome \(^2\), relapsed ALL patients also acquire prednisolone resistance disproportionately to other anti-leukemic agents.\(^18\) To date, various mechanisms that sensitize leukemic cells in vitro to prednisolone have been described, including inhibition of the prednisolone interconverter 11β-HSD, knockdown of the glucocorticoid-dependent transcription regulator SMARCA4, inhibition of the voltage-dependent channel hERG1, which signals to ERK/PI3K/Akt survival pathways, knockdown of the calcium scavengers S100A8/S100A9, downregulation of anti-apoptotic MCL1, upregulation of pro-apoptotic BIM, and inhibition of glycolysis.\(^6,8,9,19-22\) Sensitizing cells to prednisolone may therefore require a multifactorial approach. In this study, we provide evidence that reduction of MCL1 levels and inhibition of glycolysis synergistically inhibits leukemic survival and concomitantly sensitizes to prednisolone in ALL cells.
Downregulation of anti-apoptotic BCL-2 family members is hampered in prednisolone resistant cells\(^6\). In the present study, we observed that prednisolone exposure decreased the expression of the anti-apoptotic MCL1 in leukemic cells of sensitive patients, whereas that of resistant cells remained unchanged. Prednisolone did not affect the expression of other BCL-2 family members (BCL-XL and BCL-2) nor p53. In line with this are microarray data on primary BCP-ALL, which identified higher anti-apoptotic \textit{MCL1} expression in prednisolone-resistant cells, but not higher Bcl-XL, BCL-2 or p53 expression\(^3\). We observed that silencing of MCL1 expression by two means, shMCL1 and MCL1 LNA, induced apoptosis and sensitized to prednisolone in BCP-ALL and T-ALL cell lines. This is consistent with previous findings in other leukemic cell lines that demonstrated prednisolone sensitization after direct knockdown of \textit{MCL1} by shMCL1 or indirect downregulation of MCL1 via inhibition of mTOR\(^6,7\). Another study reported that disruption of the complex between beclin-1 and MCL1 by obatoclax and exposure to dexamethasone activated autophagy-dependent cell-death in otherwise dexamethasone-resistant cells\(^23\). However, inhibition of autophagy only slightly induced resistance to dexamethasone, implying direct anti-apoptotic effects as well\(^23\). Overall, these data indicate MCL1 as a potent therapeutic target to convert glucocorticoid resistance. However, high expression of MCL1 is not predictive for an adverse clinical outcome, suggesting that additional mechanisms co-occur that induce prednisolone resistance in pediatric ALL\(^6\). Here, we observed that targeting MCL1 forces the glycolysis route thereby rescuing cells from prednisolone-induced apoptosis. It has been shown that BCL2 family members maintain the mitochondrial membrane potential by regulating the permeability transition pore and the ATP/ADP pump, both involved in oxidative phosphorylation\(^13,14\). Knockdown of MCL1 may therefore impair oxidative phosphorylation, forcing cells to produce ATP by glycolysis to ensure survival. This hypothesis is supported by our observation that treatment of leukemic cells with azide, a known inhibitor of oxidative phosphorylation, also increases glucose consumption (Supplemental Figure 15). Furthermore, an MCL1 amino-terminally truncated isoform was recently discovered that facilitates ATP production, respiration and maintenance of oligomeric ATP synthase in the mitochondria\(^24\). Our three MCL1 LNAs all target the 3’ UTR of MCL1 and therefore also diminish this truncated MCL1 isoform. Furthermore, the glycolysis and apoptotic pathways may be connected via activity of the BCL2 family member BAD. The phosphorylation of aminoacid S112 in the BH3 domain of BAD acts like a switch between the metabolic and pro-apoptotic functions ascribed to BAD. BAD resides in a mitochondrial complex together with glucokinase and contributes to the activity of this glucose-metabolizing enzyme\(^25\). Glucokinase mediates the first step in glycolysis by converting glucose into glucose-6-phosphate. Although it has been shown that MCL1 does not bind BAD directly\(^26\), silencing of MCL1 may indirectly induce BAD activity and/or may trigger glycolysis in a similar
way as the BAD/glucokinase complex. As a net result the glycolytic rate (and hence glucose consumption) will increase and this may provide a rescue mechanism against prednisolone-induced cell death. This speculative functional explanation yet awaits further studies. Our study showed that inhibition of both glycolysis and MCL1 synergistically inhibit leukemic cell survival and concomitantly sensitizes leukemic cells to prednisolone. These results indicate the need to target both pathways and suggest that targeting MCL1 as a single target may not yield the desired clinical effect.

We previously demonstrated that ALL cells increase glucose consumption to prevent prednisolone-induced apoptosis. Glucocorticoids inhibit intracellular glucose uptake by regulating the expression of glucose transmembrane transporter (GLUT) \(27-29\). Dexamethasone decreases GLUT-1 expression in ALL cells thereby decreasing glycolysis and inducing apoptosis. We observed higher expression of GLUT-1 in prednisolone resistant ALL patients. TXNIP, a negative regulator of glucose uptake is correlated to GLUT-1 expression and was also found upregulated after prednisolone treatment in sensitive patients. Further studies are needed to demonstrate whether TXNIP and GLUT-1 facilitate the higher glucose metabolism observed after MCL1 knockdown.

Our results suggest that treatment with MCL1 LNA antisense and 2-DG represent a promising approach to decrease leukemic cell survival and to sensitize ALL patients to prednisolone. LNA antisense may offer a more direct and specific way of silencing MCL1 than the current BCL-2 family inhibitors R-(-)-gossypol (AT101) and obatoclax (GX-15-070), which recently entered clinical phase I/II trials. Both inhibitors can block several members of the BCL-2 family, increasing the chance of side-effects in clinical practice. In contrast, LNA antisense specifically target the mRNA expression of one gene. We have shown in this study that MCL1 LNA effectively silences MCL1 mRNA and protein expression in ALL cells, comparable to knockdown with the more stable shMCL1. Notably, no delivery vehicles were necessary to ensure uptake of MCL1 LNA antisense molecules by the ALL cells. Moreover, LNA’s are conformationally structured to prevent most of the current hurdles in siRNA treatment, such as delivery, stability of the RNA molecules in circulation, strand bias and off-target effects. LNA antisense molecules are currently investigated in phase I early clinical trials for three different target genes: EZN-3042, an inhibitor of Survivin is being investigated in children with relapsed ALL (www. Clinicaltrials.gov, NCT01186328); EZN-4176, which targets the androgen receptor, is being studied in adults with castration-resistant prostate cancer (NCT01337518); and EZN-2968, an HIF-1A-inhibitor, is being examined in advanced solid tumours and lymphoma (NCT00466583). We here provide functional in vitro proof that MCL1 LNA antisense molecules are effective in inhibiting
leukemic cell survival and reversing prednisolone resistance and may offer merits to further investigate these MCL1-LNAs in clinical trials. However, as our data show that silencing of MCL1 increases glycolysis of cells, neither MCL1 LNA nor BH3 mimetics should be used as single agents. We have shown that this shift in energy metabolism can be exploited to concomitantly sensitize leukemic cells to prednisolone. 2DG may be a candidate agent since it has therapeutic potential and is proven to cause chemosensitisation in acute leukemia, breast cancer, and prostate cancer cells, and is now used in phase I clinical trials (www.ClinicalTrials.gov, NCT00096707).

In conclusion, MCL1 is a potent target to therapeutically inhibit leukemic survival and to reverse drug resistance in pediatric ALL. However, MCL1-silenced cells upregulate glycolysis, which may rescue cells from prednisolone-induced apoptosis. These data therefore provide evidence for concomitant causes of survival and resistance, and indicate that MCL1 and glycolysis should be targeted simultaneously to reduce leukemic cell survival and prednisolone resistance in ALL.

Authorship and Disclosures
I.M.A. designed and performed research, analyzed and interpreted data, and wrote the paper; B.R.H. and T.K. developed the LNA oligonucleotides; R.V.D. assisted in the LNA synergy experiments. W.E.E. discussed data and revised the paper; R.P. and M.L.DB. designed research, analyzed and interpreted data, and revised the paper.

B.R.H. and T.K. are employed at Santaris Pharma A/S, Hørsholm, Denmark. The other authors declare to have no conflicts of interests.
References


Figure Legends

Figure 1. Downregulation of MCL1 by prednisolone is impaired in prednisolone resistant leukemic cells of patients.

Leukemic cells of three in vitro prednisolone sensitive and three in vitro prednisolone resistant patients, were treated in vitro for 48 hours with 0 µg/ml, 1 µg/ml or 250 µg/ml prednisolone. Protein expression levels of MCL1, BCL-XL, BCL-2 and p53 were analyzed by reverse phase protein array. Bar indicates the mean plus SEM of three independent patient samples. (A Kruskal-Wallis test was used to compare 0, 1, 250 µg/ml data points indicated by and a Mann-Whitney U test was used to compare data between sensitive and resistant patients indicated by *p<0.05, **p<0.01). A.U. Arbitrary units.
Figure 2. MCL1-silenced cells upregulate glycolysis, which can be reduced by 2-DG.

Glucose consumption of MLL-AF4+ BCP-ALL, ETV6-RUNX1+ BCP-ALL, E2A-PBX1+ BCP-ALL, ETP-ALL and tetraploid T-ALL cell line after treatment with MCL1 LNA-b and/or a non-silencing control LNA (NSC) with or without 0.5mM 2-DG was examined with a glucose consumption assay. To calculate glucose consumption, values were compared with glucose levels in plain RPMI medium and corrected for cell growth. Data are presented as means plus SEM of three (ETV6-RUNX1+ BCP-ALL and Tetraploid T-ALL) or two independent experiments (*p<0.05, **p<0.01, ***p<0.001).

Figure 3. MCL1 silencing together with glycolysis inhibition synergistically inhibits leukemic cell survival and concomitantly sensitizes to prednisolone.

Panel A: Leukemic cell survival of a MLL-AF4+ BCP-ALL, ETV6-RUNX1+ BCP-ALL, E2A-PBX1+ BCP-ALL, ETP-ALL and tetraploid T-ALL cell line after treatment with MCL1 LNA-b and/or 0.5mM 2-DG for 168 hours, compared to non-silencing control LNA (NSC, set at 100%). Data are presented as means plus SEM of three (ETV6-RUNX1+ BCP-ALL and Tetraploid T-ALL) or two independent experiments (*p<0.05, **p<0.01, ***p<0.001). $F_{syn}$ represents the synergy factor, where <1 indicates synergy.

Panel B: Leukemic cell survival after prednisolone exposure for three days, i.e. 96 till 168 hours after start of MCL1 LNA-b and/or 0.5mM 2-DG in equivalent cell lines as in panel A. Data were compared to a non-silencing control LNA (NSC) without prednisolone (see also panel A, set to 100%), to visualize the total effect on cell survival after prednisolone. Data are presented as means plus SEM of three (ETV6-RUNX1+ BCP-ALL and Tetraploid T-ALL) or two independent experiments (*p<0.05, **p<0.01, ***p<0.001).

Panel C: Sensitivity of leukemic cells to prednisolone was measured in a 3-day MTT assay from t96 to t168 after treatment with MCL1 LNA-b and/or a non-silencing control LNA (NSC) with or without 0.5mM 2-DG. Cell survival depicted on the Y-axis was corrected for cell death induced by MCL1 knockdown and 0.5mM 2-DG itself in the absence of prednisolone, to visualize the absolute prednisolone effects. Data are presented as means plus SEM of three (ETV6-RUNX1+ BCP-ALL and Tetraploid T-ALL) or two independent experiments (*p<0.05, **p<0.01, ***p<0.001).
Figure 1

Protein expression (A.U.)

MCL1

Sensitive Patients  |  Resistant Patients

BCL-XL

Sensitive Patients  |  Resistant Patients

BCL-2

Sensitive Patients  |  Resistant Patients

P53

Sensitive Patients  |  Resistant Patients

Legend:
- 0 μg/ml Prednisolone
- 1 μg/ml Prednisolone
- 250 μg/ml Prednisolone

* p < 0.05
ns = not significant
Supplemental Data

The synergism of MCL1 and glycolysis on pediatric acute lymphoblastic leukemia cell survival and prednisolone resistance

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Additional information Methods:

**Cell culture and primary cells**

Medium was supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin (Gibco), 0.125 μg/ml fungizone (Gibco), and 10% Fetal Calf Serum (Integro). Reh, 697 and Sem are BCP-ALL cell lines with an $ETV6$-$RUNX1^+$, $E2A$-$PBX1^+$, $MLL$-$AF4^+$ translocation respectively. Jurkat is a mature tetraploid T-ALL cell line and Loucy is an immature early T-cell precursor (ETP-ALL) cell line. All cell lines were tested for their resistance to prednisolone. HEK293T, a human embryonal kidney cell line, was used for the production of viral particles.

**Lentiviral production and infection**

Lentiviral helper vectors pRSV-Rev (Addgene plasmid 12253), pMDLg/pRRE (Addgene plasmid 12251), pMD2.G (Addgene plasmid 12259) and psPAX2 (Addgene plasmid 12260) were provided by Prof. D. Trono (Geneva, Switzerland). pLKO.1 Mission short hairpin RNA (shRNA) vectors were purchased from Sigma-Aldrich, i.e. SHC005 against eGFP, and TRCN0000005518 and TRCN0000197024 against $MCL1$.

Infections were performed as follow; 70-80% confluent HEK293T cells were transfected with sh$MCL1$, pMD2.G and psPAX2 complemented with CaCl$_2$ and HEPES-buffered saline in the presence of 25 μM chloroquine (Sigma). Virus-containing supernatant was collected, filtered 0.45μm, and concentrated by ultracentrifugation at 32,000rpm, 1hr, 4°C. Viral titers were determined with a HIV-1 p24 Antigen ELISA kit according to the manufacturer’s protocol (ZeptoMetrix). Infection occurred during 45’ 1800 rpm spin-oculation of 0.5*10^6 cells/ml with 2.5 TU/cell viral particles and 5 μg/ml polybrene (Sigma-Aldrich). After 24h, infected cells were selected in 0.5 μg/ml in the case of 697 and Loucy, 1 μg/ml for Reh and Sem, and 2 μg/ml puromycin for Jurkat.

**Quantitative RT-PCR**

RNA was extracted using a Rneasy minikit (Qiagen) according to the manufacturer’s protocol. cDNA was synthesized from 1 μg RNA by 8 IU/μl MMLV (Promega), 20nM oligodT primers, 1μM random hexamer primers (Invitrogen), 200 μM dNTPs and 1 IU/μl RNAsin in MMLV-buffer (Promega). Primers used for the reference gene $RPS20$, were 5’- AAGGGCTGAGGATTTTG-3’ (forward) and 5’-CGTTGCGGCTTGTAG-3’ (reverse).

**Apoptosis measurement**

0.2*10^6 cells were incubated for 15 minutes in 200μl AnnexinV binding buffer (Molecular probes) containing 2 μg/ml propidium iodide (Molecular probes) and 1:1000 AnnexinV Alexa Fluor® 633 (Molecular probes).
Additional information Methods:

Reverse Phase Protein Array
Slides were stained with specific antibodies and incubated with a biotinylated secondary antibody. Slides were scanned using the NovaRay scanner and protein levels were calculated relative to the total amount of protein per sample using MicroVigene Software.

Western Blot and Immunoblotting
Cell pellets were lysed in lysis buffer supplemented with protease inhibitors, and protein concentration was quantified according to the BCA assay (Pierce).

Glucose-consumption assay
Briefly, the supernatant of cultured cells was diluted 25 times in milliQ, supplemented with assay solvent containing Glucose Oxidase, Peroxidase and o-Dianisidine and incubated for 30 minutes at 37°C. Hereafter, 12N sulfuric acid was added and levels of the spectrophotometric end-product oxidized o-Dianisidine were measured at 540nm using the Versamax (Molecular Devices).

Synergistic effect
Synergistic effects were calculated from equi-effective drug concentrations by the following equitation postulated by Berenbaum (15) \[ \text{Drug A}_{in\ combination\ with\ B} / \text{Drug A}_{alone} + \text{Drug B}_{in\ combination\ with\ A} / \text{Drug B}_{alone} \]. A synergy factor (Fsyn) <1 indicates synergy, whereas a Fsyn of 1 indicates additivity and a Fsyn >1 points to antagonism between two drugs.
Supplemental Figure 1. The effect of 2DG on glucose consumption and proliferation.

(A) Absolute glucose levels in the supernatant of ETV6-RUNX1+ BCP-ALL (REH) cells were measured with a glucose assay. RPMI control represents the amount of glucose present in culture medium incubated for 96 hours in the absence of 2DG. Cells cultured without 2DG have consumed >90% of the glucose present in RPMI culture medium after 96 hrs. Exposure to increasing concentrations of 2DG reduces the amount of glucose that is being consumed. In cells treated with 2mM 2DG the glucose levels are virtually unaffected compared to RPMI control medium, indicating that no glucose has been consumed. (B) ETV6-RUNX1+ BCP-ALL (REH) cells were counted by a trypan blue exclusion assay after exposure to 2DG for 96 hours. Glucose consumption (A) and cell viability (B) are correlated.
Supplemental Figure 2.

Downregulation of MCL1 by prednisolone is impaired in prednisolone resistant leukemic cells of patients. 

(A-D) Individual MCL1 and BCL-2 expression levels after prednisolone exposure of prednisolone sensitive and resistant patients' cells indicated in Figure 1. (E) MCL1 and BCL-2 western blot analysis of one sensitive patient and one resistant pediatric BCP-ALL patient’s cells after in vitro exposure for 48 hours with 0, 10, 100 or 250 µg/ml prednisolone. Clathrin was used as loading control.
Supplemental Figure 3.

LNA antisense directed against MCL1 efficiently silence the expression of MCL1 mRNA. MCL1 mRNA expression was measured after treatment with three different LNA antisense molecules against MCL1, i.e. MCL1 LNA-a, MCL1 LNA-b and MCL1 LNA-c in five distinct leukemic cell lines. Values were adjusted for expression of the housekeeping gene RPS20 and are relative to the non-silencing control (NSC). Data are presented as means plus SEM of three (ETV6-RUNX1+ BCP-ALL and tetraploid T-ALL) or two independent experiments ( *p<0.05, **p<0.01, ***p<0.001).
Supplemental Figure 4.

LNA antisense directed against MCL1 efficiently silence the expression of MCL1 protein. Protein expression of MCL1 was assessed at t168 with Western blot after LNA treatment. MCL1 protein expression was calculated with the Odyssey software, corrected for β-actin and is relative to the NSC. A representative Western blot for a BCP-ALL cell line, i.e. REH a ETV6-RUNX1+ BCP-ALL cell line and a T-ALL, i.e. Jurkat a Tetraploid T-ALL cell line is illustrated. Data are presented as mean plus SEM of three independent experiments (*p<0.05, **p<0.01).
Supplemental Figure 5.

Short hairpins directed against MCL1 efficiently silence the expression of MCL1. MCL1 mRNA expression in ETV6-RUNX1+ BCP-ALL cell line and Tetraploid T-ALL cell line was measured after lentiviral knockdown of MCL1 with two different constructs, i.e. shMCL1-a and shMCL1-b. Values were adjusted for expression of the housekeeping gene RPS20 and are relative to the non-silencing control (NSC). Protein expression of MCL1 was assessed with Western blot after lentiviral knockdown. MCL1 protein expression was calculated with the Odyssey software, corrected for β-actin and is relative to the NSC. A representative Western blot is illustrated. Data are presented as mean plus SEM of three independent experiments (*p<0.05, **p<0.01).
Knockdown of MCL1 by MCL1 LNA inhibits leukemic survival. The effect of MCL1 knockdown by MCL1 LNA on cell viability and cell count of five distinct leukemic cell lines was determined with a trypan blue exclusion assay and analyzed with the MACSQuant. Data are presented as mean plus SEM of three (ETV6-RUNX1+ BCP-ALL and Tetraploid T-ALL) or two independent experiments (*p<0.05, **p<0.01).
Supplemental Figure 7.

Knockdown of MCL1 by MCL1 LNA increases apoptosis. MCL1 knockdown-induced apoptosis in a representative BCP-ALL cell line, i.e. Reh an ETV6-RUNX1⁺ BCP-ALL cell line and a T-ALL cell line, i.e. Jurkat a Tetraploid T-ALL, was assessed on a flowcytometer using an AnnexinV/PI staining. The percentage of apoptotic cells was calculated using the quadrant method. Data are presented as means plus SEM of three independent experiments (*p<0.05).
Supplemental Figure 8.

Knockdown of MCL1 by MCL1 LNA sensitizes towards prednisolone. Sensitivity to prednisolone after MCL1 knockdown by MCL1 LNA was measured in a 3-day MTT assay. Sensitivity was corrected for cell death induced by MCL1 knockdown itself in the absence of prednisolone. Data are presented as mean plus SEM of three (ETV6-RUNX1+ and Tetraploid) or two independent experiments (*p<0.05, **p<0.01).

Legend:
- NSC
- MCL1 LNA-a
- MCL1 LNA-b
- MCL1 LNA-c
Supplemental Figure 9.

Knockdown of MCL1 by shMCL1 inhibits leukemic survival, increases apoptosis and sensitizes towards prednisolone. The effect of MCL1 knockdown by shMCL1 on cell viability and cell count of ETV6-RUNX1+ BCP-ALL cells and T-ALL cells was determined with a trypan blue exclusion assay and analyzed with the MACSQuant. MCL1 knockdown-induced apoptosis in a BCP-ALL and T-ALL cell line was assessed on a flowcytometer using an AnnexinV/PI staining. The percentage of apoptotic cells was calculated using the quadrant method. Sensitivity to prednisolone after MCL1 knockdown was measured in a 3-day MTT assay. Sensitivity was corrected for cell death induced by MCL1 knockdown itself in the absence of prednisolone. Data are presented as mean plus SEM of three independent experiments (*p<0.05, **p<0.01).
MCL1-silenced cells by MCL1 LNA upregulate glycolysis. Glucose consumption of five distinct leukemic cell lines after MCL1 knockdown by MCL1-LNAs was examined with a glucose assay. To calculate glucose consumption, values were compared with glucose levels in plain RPMI medium and corrected for cell growth. Data are presented as means plus SEM of three (ETV6-RUNX1* and Tetraploid) or two independent experiments (*p<0.05, **p<0.01, ***<0.001).

△ MCL1 LNA compared to NSC.
Supplemental Figure 11.

**MCL1-silenced cells by shMCL1 upregulate glycolysis.** Glucose consumption of a MCL1-silenced *ETV6-RUNX1*+ BCP-ALL cell line and a tetraploid T-ALL cell line by shMCL1 was examined with a glucose assay. To calculate glucose consumption, values were compared with glucose levels in plain RPMI medium and corrected for cell growth. Data are presented as means plus SEM of three independent experiments ( *p<0.05, **p<0.01). Off note, shMCL1-a is the only construct that targets exon 2 of MCL1, in contrast to shMCL1-b, LNA-a, LNA-b and LNA-c which are directed against exon 3. It may be that targeting exon 2 containing MCL1 transcripts does not affect glycolysis and/or that interference with exon 3 is more important for a functional effect on glycolysis.
Leukemic cell survival (%)

**SEM: MLL-AF4+ BCP-ALL**

MCL1 silencing and glycolysis inhibition synergistically inhibits leukemic cell survival. Leukemic cell survival of five distinct leukemic cell lines after treatment with either MCL1 LNA alone or in combination with 0.5 mM 2-DG was determined with a trypan blue exclusion assay and analyzed with the MACSQuant. Data are presented as means plus SEM of three (ETV6-RUNX1+ and Tetraploid) or two independent experiments (*p<0.05, ** p<0.01). Fsyn represents the synergy factor, where Fsyn<1 is synergistic.
Supplemental Figure 13.

**SEM:** *MLL-AF4*⁺ BCP-ALL

**Loucy:** ETP-ALL

**Jurkat:** Tetraploid T-ALL

Supplemental Figure 13.

**MCL1 knockdown, glycolysis inhibition and prednisolone treatment concomitantly inhibit leukemic cell survival.** Leukemic cell survival after 3-day, i.e. from t96 until t168, prednisolone exposure of *MLL-AF4*⁺ BCP-ALL, *E2A-PBX1*⁺ BCP-ALL, ETP-ALL and tetraploid T-ALL cells with either MCL1 LNA-b or 0.5mM 2-DG alone or in combination. Data were compared to NSC control without prednisolone, to visualize the total effect on cell survival of prednisolone, MCL LNA and 2-DG together. Data are presented as means plus SEM of three (*ETV6-RUNX1*⁺ and Tetraploid) or two independent experiments (*p<0.05, **p<0.01).
Supplemental Figure 14.

**MCL1 silencing together with glycolysis inhibition concomitantly reverses drug resistance.** Sensitivity of distinct leukemic cell lines to prednisolone after treatment with mock LNA control, MCL1 LNA-a and LNA-b and 0.5mM 2-DG alone or in combination was measured in a 3-day MTT assay. Sensitivity was corrected for cell death induced by MCL1 knockdown and 0.5mM 2-DG itself in the absence of prednisolone. Data are presented as means plus SEM of three or two independent experiments (*p<0.05, **p<0.01, ***p<0.001)
**Supplemental Figure 15.**

**Inhibition of oxidative phosphorylation by Azide augments glycolysis.**

Glucose consumption was calculated after treatment with a concentration range of Azide, an inhibitor of oxidative phosphorylation, relative to untreated *ETV6-RUNX1* BCP-ALL cells (REH), and corrected for cell growth. Data are presented as means plus SEM of two independent experiments (*p<0.05).*