Association of high-level MCL-1 expression with in vitro and in vivo prednisone resistance in MLL-rearranged infant acute lymphoblastic leukemia

Ronald W. Stam1, Monique L. Den Boer1, Pauline Schneider1, Jasper de Boer2, Jill Hagelstein1, Maria G. Valsecchi3, Paola de Lorenzo3, Stephen E. Sallan4,5, Hugh J.M. Brady2, Scott A. Armstrong4,5 and Rob Pieters1

1 Erasmus MC – Sophia Children’s Hospital, Department of Pediatric Oncology/Hematology, Rotterdam, The Netherlands.
2 Molecular Haematology and Cancer Biology Unit, Institute of Child Health and Great Ormond Street Hospital for Children, University College London, London, United Kingdom.
3 Department of Clinical Medicine, Prevention and Biotechnologies, Section of Medical Statistics, University of Milano-Bicocca, Monza, Italy.
4 Department of Pediatric Oncology, Dana-Farber Cancer Institute, Boston Massachusetts, USA.
5 Division of Pediatric Hematology / Oncology, Children’s Hospital, Boston Massachusetts, USA.

Correspondence:
Ronald W. Stam, Ph.D.
Erasmus MC - Sophia Children’s Hospital
Pediatric Oncology / Hematology
Room : Sp 2456
Dr. Molewaterplein 60
P.O. Box 2060
3000 CB Rotterdam, The Netherlands
Tel.: +31 10
Fax.: +31 10
E-mail : r.stam@erasusmc.nl

Running title: MCL-1 and glucocorticoid resistance in MLL-rearranged infant ALL

Financial support: This study was supported by a grant from the Sophia Foundation for Medical Research (SSWO grant 455). JB was sponsored by a Leukemia Research Fund.
Abstract

*MLL*-rearranged Acute Lymphoblastic Leukemia (ALL) represents an unfavorable type of leukemia that often is highly resistant to glucocorticoids like prednisone and dexamethasone. As the response to prednisone largely determines the clinical outcome of pediatric ALL patients, overcoming resistance to this drug may be an important step towards improving prognosis. Here we show how gene expression profiling identifies high-level *MCL-1* expression to be associated with prednisolone resistance in *MLL*-rearranged infant ALL, as well as in more favorable types of childhood ALL. To validate this observation, we determined *MCL-1* expression using quantitative RT-PCR in a cohort of *MLL*-rearranged infant ALL and pediatric non-infant ALL samples, and confirmed that high-level *MCL-1* expression is associated with prednisolone resistance *in vitro*. Also, *MCL-1* expression appeared to be significantly higher in *MLL*-rearranged infant patients who showed a poor response to prednisone *in vivo* as compared with prednisone good responders. Finally, down-regulation of MCL-1 in prednisolone resistant *MLL*-rearranged leukemia cells by RNA interference, to some extent led to prednisolone sensitization. Collectively, our findings suggest a potential role for MCL-1 in glucocorticoid resistance in *MLL*-rearranged infant ALL, but at the same time strongly imply that high-level *MCL-1* expression is not the sole mechanism providing resistance to these drugs.
Introduction

Acute Lymphoblastic Leukemia (ALL) in infants (i.e. children less than 1 year of age) is characterized by an exceptionally high incidence (~80% of the cases) of chromosomal translocations affecting the MLL gene. These chromosomal abnormalities, believed to be initiating events in leukemogenesis, usually involve reciprocal translocations fusing the N-terminal portion of the MLL gene to the C-terminal region of one of its translocation partner genes, of which over 50 have been described. The presence of MLL rearrangements represents the most important independent predictor of an adverse outcome. While current therapies for pediatric ALL in general result in long-term survival in >80% of the cases, these therapies fail in >50% of infants diagnosed with MLL-rearranged ALL.

A major obstacle hampering successful treatment results in MLL-rearranged infant ALL is cellular resistance to several drugs used in the treatment of ALL. In vitro studies showed that MLL-rearranged infant ALL cells are particularly highly resistant to prednisolone, the spearhead drug of ALL treatment regimes. Moreover, ~30% of the MLL-rearranged infant ALL patients show a poor prednisone response in vivo, defined as the presence of ≥1000 leukemic blasts/μL after a 7-day window of prednisone mono-therapy. In contrast, among children older than 1 year of age (non-infants) diagnosed with ALL, less than 10% of the patients show a poor in vivo response to prednisone. Both in vitro prednisolone sensitivity as well as the in vivo prednisone response are predictive for clinical outcome in pediatric ALL in general. Thus, the poor prognosis for MLL-rearranged ALL may to a large extent be associated with prednisone resistance. Therefore, overcoming resistance to this drug may be an important step towards a more favorable prognosis for MLL-rearranged leukemias.

Comparing gene expression profiles of pediatric ALL patients (above the age of 1 year) either in vitro sensitive or resistant to prednisolone, we recently found increased expression of the anti-apoptotic BCL-2 family member MCL-1 in prednisolone resistant patients samples. MCL-1 is considered an important cell survival factor in peripheral blood B-lymphocytes and is often overexpressed in hematopoietic malignancies including B-cell Chronic Lymphoblastic Leukemia (CLL) and Multiple Myeloma (MM). Here we show that increased MCL-1 expression is not only associated with in vitro prednisolone resistance in pediatric ALL but also prominently in MLL-rearranged infant ALL. MCL-1 expression appears to be one of the few overlapping genes that commonly discriminates between prednisolone resistance and sensitivity when gene expression signatures associated with
prednisolone resistance for both of these patient groups are compared. Furthermore we demonstrate that among MLL-rearranged infant ALL patients elevated MCL-1 expression is predictive for both the in vitro prednisolone as well as to some extent for the in vivo prednisone response. Finally we demonstrate that reducing MCL-1 protein expression moderately sensitizes prednisolone-resistant MLL-rearranged leukemia cells to this drug in vitro.
Materials and Methods

Patient samples.

Untreated primary bone marrow and/or peripheral blood samples from infants (<1 year of age) diagnosed with ALL were collected at the Erasmus MC - Sophia Children’s Hospital and other hospitals participating in the INTERFANT-99 treatment study. Samples from pediatric ALL patients older than 1 year of age (non-infants) were obtained from our cell bank at the Erasmus MC - Sophia Children’s Hospital. Approval for these studies was obtained from the Erasmus MC Institutional Review Board. Informed consent was obtained according to the Declaration of Helsinki.

Within 24 hours after sampling, mononuclear cells were isolated by density gradient centrifugation using Lymphoprep (density 1.077 g/ml; Nycomed Pharma, Oslo, Norway), and resuspended in RPMI 1640 medium (Dutch modification without L-glutamine; Invitrogen life technologies, Breda The Netherlands) supplemented with 20% fetal calf serum (FCS; Integro, Zaandam, The Netherlands), 2 mM L-glutamine (Invitrogen) 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml sodium selenite (ITS media supplement; Sigma, St Louis MO, USA), 100 IU/ml penicillin, 100 μg/ml streptomycin, 0.125 μg/ml fungizone and 0.2 mg/ml gentamycin (Invitrogen). Contaminating non-leukemic cells were removed using immunomagnetic beads as described elsewhere. All samples used contained >90% leukemic cells, as determined morphologically on May-Grünwald-Giemsa (Merck, Darmstadt, Germany) stained cytospins.

As part of the INTERFANT-99 study, the infant ALL samples were screened for the presence of MLL rearrangements by FISH analysis, and the type of translocation was determined using PCR analysis. An overview of available patient characteristics and clinical parameters are listed in Supplemental table 1.

In vitro prednisolone cytotoxicity and in vivo prednisone response.

In vitro prednisolone (Bufa, Uitgeest, The Netherlands) cytotoxicity was determined using the MTT assay as extensively described before. The in vivo response to prednisone in infant ALL patients was determined after a 7-day window of prednisone mono-therapy (prior to combination chemotherapy) as part of the INTERFANT-99 treatment protocol. Patients are defined as prednisone poor responders (PPRs) when ≥1000 leukemic blasts/μL peripheral blood remain present after the 7-
day prednisone window. In cases where the amount of leukemic blasts drops below 1000/μL, the patients are defined as prednisone good responders (PGRs).

**Microarray analysis of prednisone-sensitive and prednisone-resistant ALL samples**

Patient samples were characterized as *in vitro* sensitive or resistant to prednisolone based on the LC50 value (i.e. the concentration of prednisolone lethal to 50% of the leukemic cells), as obtained from MTT assays (described above). Samples were defined as prednisolone-sensitive at LC50 values <0.1 ug/mL prednisolone, and prednisolone-resistant at LC50 values >150 ug/mL prednisolone. Gene expression data (Affymetrix HU133A microarrays) were normalized using dChip,17 and filtered with a max-min=100, and max/min=4. The probe sets that correlated with sensitive/resistant distinction were determined using a signal-to-noise statistic and permutation testing. The accuracy of the gene expression signatures were assessed using a cross-validation approach as described previously.18 Briefly, for both the infant and non-infant ALL signatures separately, one sample was withheld and the specified number of probe sets that best correlated with the prednisolone sensitive/resistant class distinction were identified. Next, the class of the withheld sample was determined based on the gene expression pattern of the probe sets identified in this manner. This process was repeated for all samples and the error rate in class prediction was determined and plotted against the number of probe sets used to build the model. The models were generated using 1 to 65 probe sets. The raw microarray data are listed in Supplemental Table 2. All microarray data can be found in the GEO public database under accession number GSE19143.

**Quantitative real-time PCR (TaqMan) analysis**

Total RNA was extracted from a minimum of 5 x10⁶ leukemic cells using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions, and quantified on a spectrophotometer. The integrity of the extracted RNA was assessed on 1% agarose gels. Extracted RNA was reverse transcribed and the obtained cDNA was used to quantify *MCL-1* mRNA expression relative to the endogenous housekeeping gene RNaseP, using quantitative real-time PCR (TaqMan©) as described previously.19 Primer combinations used to amplify *MCL-1* were as follows: *forward*: 5’-CGC CAA GGA CAC AAA G-3’, and *reverse*: 5’-AAG GCA CCA AAA GAA ATG-3’, for *RNaseP* the primers were: *forward*: 5’-TTG GGA AGG TCT GAG ACT A-3’, and *reverse*: 5’-TCA GCC ATT GAA CTC ACT T-3’. The dual-labeled
TaqMan probed used to detect amplified sequences were: \textit{MCL-1}: 5’-(FAM)-TTT CAG CGA CGG CGT AACA-(TAMRA)-3’ and for \textit{RNaseP}: 5’-(FAM)-AGG TCA GAC TGG GCA GGA GAT-(TAMRA)-3’.

\textit{Cell line culture}

Both SEMK2 and MV4-11 cells were maintained in RPMI 1640 with L-Alanyl-L-Glutamine (Invitrogen) supplemented with 10% FCS (Integro), 100 IU/ml penicillin, 100 μg/ml streptomycin and 0.125 μg/ml fungizone (Invitrogen) and grown as suspension cultures at 37°C in humidified air containing 5% CO₂. The SEMK2 represents a B-lineage \textit{MLL}-rearranged ALL cell line\textsuperscript{20}, and MV4-11 is an \textit{MLL}-rearranged Acute Myeloid Leukemia (AML) cell line. Both cell lines harbor \textit{MLL} translocation t(4;11) and are highly resistant to prednisolone \textit{in vitro}.

\textit{RNA interference}

MCL-1 knock-down in the prednisone-resistant \textit{MLL}-rearranged ALL and AML cell lines SEMK2 and MV4-11 were performed essentially as described before\textsuperscript{21}. Briefly, the RNA interference sequence for human MCL-1, GGACTGGCTAGTTAAACAAAG, as well as a validated non-silencing control (RHS1707; Openbiosystems) were cloned in to the pPRIME-SFFV-GFP lentiviral vector which enables the efficient expression of shRNAs in hematopoietic cells from the RNA Polymerase II promoter SFFV. The vector contains a GFP that allows for marking of lentiviral integration. Virus was produced by transient transfection of 293FT with a 3:1:4 mixture of psPAX-2, pMD2G-VSVG (kind gifts of Dr. D. Trono, University of Geneva) and pPRIME-SFFV-GFP. After 24 hours medium was refreshed and virus-containing medium was harvested 48 hours after transfection. Upon filtration through a 0.45 μm cellulose acetate filter, the virus stock was used to infect SEMK2 or MV4-11 cells. Infected cells were analyzed for MCL-1 expression by immunoblotting, and the \textit{in vitro} prednisolone sensitivity was determined by 2-day MTT assays performed in triplicate. These procedures were then repeated in order to establish reproducibility among independent experiments.

\textit{Immunoblotting}

SDS-PAGE and immunoblotting were performed essentially as described earlier\textsuperscript{21}. Total cellular protein lysates were resolved on polyacrylamide gels and transferred to a nitrocellulose membrane
(Amersham Biosciences). The membranes were probed with a rabbit anti-Mcl-1 polyclonal antibody. After incubating with respective secondary antibodies conjugated with horseradish peroxidase, MCL-1 was visualized using the ECL Kit (Amersham Biosciences).
Results

Microarray analysis of prednisone-sensitive and prednisone-resistant ALL samples.

Comparing gene expression profiles between prednisolone-resistant and prednisolone-sensitive pediatric ALL patients revealed gene expression signatures associated with prednisolone resistance for both non-infant (>1 year of age) or infant (<1 year of age) ALL (Figure 1A). However, assessment of the accuracy of these signatures to predict prednisolone resistance or sensitivity using a cross-validation approach showed that, in particular in the infant ALL signature, the accuracy was rather low (Figure 1B). In other words, the error rate of re-assigning infant ALL samples to their original class (i.e. prednisolone sensitive or resistant) after being withheld from the analysis, tended to be ~30% (Figure 1B), presumably due to the relatively low numbers of patient samples used to build these signatures. Therefore, we decided to merge both signatures in search for overlapping genes associated with prednisolone resistance in both patient groups. As shown in Figure 1C, elevated expression of Solute carrier 2 (SLC2), Fos-like antigen 2 (FOSL2), and MCL-1 appeared to be characteristic for both prednisolone-resistant infant as well as prednisolone-resistant non-infant ALL samples. Whereas both MCL-1 and SLC2 occurred in the top50 of up-regulated genes associated with prednisolone resistance in both the infant and non-infant ALL signatures (Figure 1A), this was not the case for FOSL2. Our approach to merge both signatures in search of genes that are associated with prednisolone resistance in both infant and non-infant ALL cases was not limited to the most significantly differentially expressed genes in both patient groups, but included the entire datasets. MCL-1, SLC2 and FOSL2 may therefore not be the most significantly up-regulated genes in either the infant or non-infant ALL signatures when evaluated separately, but do represent the few genes associated with prednisolone resistance in both patient groups.

Correlation between high-level MCL-1 expression and in vitro resistance to prednisolone.

To validate our microarray data, we determined MCL-1 mRNA expression in MLL-rearranged infant ALL (n=23) and non-infant ALL (n=20) samples using quantitative real-time PCR (TaqMan). For the sake of uniformity, infant ALL samples not carrying translocations of the MLL gene were intentionally excluded from these experiments, as the prognosis (and therapy response) of these patients are significantly more favorable as compared to MLL-rearranged infant ALL patients. Among both patient
groups, we observed a wide and continuous range of MCL-1 expression levels (Figure 2A and 2C). Using the median or mean MCL-1 expression as a cut-off, we divided the samples into two groups either expressing “high” or “low” MCL-1 levels. For the MLL-rearranged infant ALL patients the median expression coincided with the mean expression value. Next, we compared the in vitro prednisolone response between patients displaying high or low MCL-1 expression among both patient groups separately. As expected, MLL-rearranged infant ALL and pediatric non-infant ALL samples displaying high MCL-1 expression were significantly (p<0.05) more resistant to prednisolone as compared to patients expressing lower levels of MCL-1 (Figure 2B and 2D). Interestingly, high level MCL-1 expression was also associated with the glucocorticoid dexamethasone (closely related to prednisolone), but not with increased resistance to unrelated drugs like L-asparaginase, vincristine, daunorubicin and cytarabine (Ara-C) (Supplemental Figure 1).

Correlation between high-level MCL-1 expression and in vivo prednisone resistance.

The 7-day window of prednisone monotherapy (prior to the combination chemotherapy) implemented in the INTERFANT-99 treatment protocol and used to ascribe infant ALL patients to either the standard-risk (GPR; good prednisone response) or high risk (PPR; poor prednisone response) arm of the protocol, provided an opportunity to correlate MCL-1 expression levels with the in vivo prednisone response in MLL-rearranged infant ALL patients. This revealed that the MCL-1 expression is significantly (p=0.031) higher in MLL-rearranged infant ALL patients with a poor prednisone response in vivo (Figure 3A). Of note is that the three patients with the highest MCL-1 expression appeared to have shown a good prednisone response in vivo (Figure 3A). However, despite their favorable in vivo prednisone response, these patients all relapsed, two of which within the first year upon diagnosis. Nevertheless, a clear relationship between the level of MCL-1 expression and clinical outcome or risk of relapse was not found, possibly due to the low numbers of patient samples for which these data was available. Moreover, the correlation between in the in vitro prednisolone and in vivo prednisone response was definitely not absolute, as some patients showing a poor prednisone response in vivo appeared sensitive to prednisolone in vitro, and vice versa. Figure 3B shows the differences in MCL-1 expression in MLL-rearranged infant ALL patients that are sensitive to prednisone both in vitro and in vivo, compared with patients resistant to prednisone either in vitro and/or in vivo. This comparison underlines the obscure relationship between the prednisolone response in vitro and the prednisone
response \textit{in vivo}, but also demonstrate that the \textit{MCL-1} expression in \textit{MLL}-rearranged infant ALL patients that are sensitive to prednisolone \textit{in vitro} and respond well to prednisone \textit{in vivo}, is markedly reduced as compared to more resistant patients.

\textit{Down-regulation of MCL-1 induces sensitivity to prednisolone.}

To determine whether MCL-1 protein down-regulation in prednisolone resistant leukemia cells reverses the resistant phenotype and induces prednisolone sensitivity, we knocked-down MCL-1 expression in the \textit{MLL}-rearranged ALL and AML cell lines SEMK2 and MV4-11 using RNA interference (RNAi). As shown in Figure 4A, the level of MCL-1 protein expression in SEMK2 cells was successfully decreased (but not completely diminished) by transfecting these cells with vectors encoding short-hairpin RNAs (shRNAs) against human MCL-1 by viral infection (Figure 4A). Compared to SEMK2, MV4-11 expressed ~3-fold higher MCL-1 protein levels, and the maximally achieved reduction in MCL-1 expression was ~50%. Control cells were either SEMK2 or MV4-11 cells virally infected with empty vectors only. Compared to the control cells, SEMK2 cells in which MCL-1 expression was reduced became more sensitive to prednisolone. MV4-11 cells, in which MCL-1 knock-down was less pronounced, only showed moderate sensitization to prednisolone (Figure 4C). \textit{In vitro} prednisolone sensitivity was assessed by 2-day prednisolone cytotoxicity assay (MTT-assay) performed in triplicate. Data shown in Figure 4B and 4C are MTT-assay results derived from two independent RNAi experiments.
Discussion

*MLL*-rearranged ALL represents an aggressive and difficult to treat type of leukemia commonly diagnosed in children less than 1 year of age (i.e. infants). Despite various attempts to intensify therapy in order to improve prognosis for these very young children, survival rates remain at best ~50%.\(^1\) Significantly contributing to this poor prognosis is cellular drug resistance. Leukemic cells from *MLL*-rearranged infant ALL patients are highly resistant, both *in vitro* and *in vivo*, to glucocorticoids (like prednisone and dexamethason) which form the backbone of ALL treatment protocols. Since it has been established that both the *in vitro* prednisolone and *in vivo* prednisone response are highly predictive for clinical outcome among ALL patients\(^7,9,10\), overcoming glucocorticoid resistance may be an important step towards an improved outcome for *MLL*-rearranged ALL. For this the biological mechanism(s) that maintain resistance to these drugs should be elucidated and understood in order to be able to modulate glucocorticoid resistance.

Here we show that high-level expression of the anti-apoptotic BCL-2 family member *MCL-1*, is associated with *in vitro* prednisolone resistance in both *MLL*-rearranged infant ALL, as well as in non-infant pediatric ALL. The fact that high-level *MCL-1* expression appeared to be associated with *in vitro* prednisolone resistance in both of these patient groups indicates that this may involve a general resistance mechanism protecting ALL cells from glucocorticoid induced apoptosis. In subsequent experiments focusing on *MLL*-rearranged infant ALL samples, we confirmed that patients expressing high levels of *MCL-1* not only are significantly more resistant *in vitro* to prednisolone, but also to the closely related glucocorticoid dexamethasone (Supplemental Figure 1). Furthermore, we demonstrate that, to some extent, high-level *MCL-1* expression was associated with a poor response to prednisone *in vivo*. However, the range of *MCL-1* expression in both the prednisone good and poor responders is pronounced, and the three patients expressing the highest levels of MCL-1 appeared to have experienced a good *in vivo* response to prednisone. This indicates that, despite of the tendency of patients with a poor *in vivo* prednisone response expressing higher levels of *MCL-1*, the correlation is not entirely conclusive, suggesting that high-level *MCL-1* expression may not be the sole mechanism driving prednisone resistance *in vivo*. In support of this, we did not find a convincing correlation between the level of *MCL-1* expression and clinical outcome (for clinical parameters see:
Supplemental table 2). An alternative interpretation of this observation could be that modulation of prednisolone resistance alone may not be sufficient to significantly improve prognosis.

To validate the role of MCL-1 expression in maintaining prednisolone resistance in vitro, we conducted MCL-1 knock-down experiments, demonstrating that prednisolone resistant MLL-rearranged ALL (SEMK2) cells become more sensitive to this drug when MCL-1 protein expression is markedly reduced. In MLL-rearranged AML (MV4-11) cells however, MCL-1 protein expression was markedly higher, and MCL-1 protein expression could only be reduced for about 50%. Consequently, the prednisolone sensitizing effects in these cells were only moderate. Collectively, these results clearly demonstrate that merely reducing the levels of MCL-1 expression is sufficient to completely reverse the prednisolone-resistant phenotype. Again, these observations emphasize that up-regulated MCL-1 expression presumably is not the only mechanism maintaining prednisolone resistance in MLL-rearranged infant ALL (see below).

As a member of the anti-apoptotic BCL-2 protein family, MCL-1 may be expected to raise resistance to apoptosis induced by various chemotherapeutic drugs. However, we observed that elevated MCL-1 expression is only associated with resistance to the glucocorticoids prednisolone and dexamethasone, but not with resistance to other drugs used in the treatment of ALL like vincristine, L-asparaginase, daunorubicin and Ara-C (cytarabine) (Supplemental Figure 1). A possible explanation for this remarkable phenomenon may lie in both the mechanism by which MCL-1 exerts its anti-apoptotic function and the way in which glucocorticoids induce apoptosis. Glucocorticoids specifically trigger apoptosis by inducing Ca\(^{2+}\) release from the endoplasmic reticulum (ER) and thereby increase the intracellular Ca\(^{2+}\) levels.\(^{22,23}\) These cytosolic Ca\(^{2+}\) signals are then transmitted to the mitochondria. Repetitive increases in mitochondrial Ca\(^{2+}\) levels eventually result in the release of cytochrome c, leading to apoptosis. MCL-1 is localized to the mitochondria and is able to inhibit mitochondrial Ca\(^{2+}\) signals, preventing cytochrome c release and apoptosis.\(^{24}\) Thus, increased MCL-1 expression in leukemic cells may result in resistance to glucocorticoids in ALL cells due to inhibition of the mitochondrial Ca\(^{2+}\) signals induced by these drugs. If so, the absent correlation between high-level MCL-1 expression and increased resistance to the other drugs tested would imply that apoptosis induced by these other drugs are less dependent on increasing cytosolic and mitochondrial Ca\(^{2+}\) levels. In support of this, we recently showed that in ALL cells vincristine and daunorubicine induced apoptosis do not require caspase-3 activation by the apoptosome (consisting of cytochrome c, Apaf-1
(apoptotic protease-activating factor-1), and pro-caspase 9)\textsuperscript{25,26}, which can only be formed when cytochrome c is released from the mitochondria. Possibly these agents initiate apoptosis in ALL cells utilizing alternative mechanisms that are not affected by increased inhibition of mitochondrial Ca\textsuperscript{2+} signals established by elevated levels of MCL-1. However, suppression of mitochondrial Ca\textsuperscript{2+} signals most certainly is not the only mechanism by which MCL-1 may inhibit glucocorticoid-induced apoptosis. Erlacher \textit{et al.}, demonstrated that the pro-apoptotic BH3-only proteins PUMA and BIM are required and rate-limiting for glucocorticoid-induced apoptosis in lymphoid cells \textit{in vivo}.\textsuperscript{27} In line with this, we recently showed that enforced over-expression of MCL-1 sequesters BIM in primary lymphocytes, preventing it from activating pro-apoptotic BAX/BAK, and thereby rendering resistance to glucocorticoid-induced apoptosis.\textsuperscript{28} Thus, the ability of MCL-1 to suppress mitochondrial Ca\textsuperscript{2+} signals and inhibit glucocorticoid-induced apoptosis not only depends on the MCL-1 expression level, but presumably is also influenced by the BIM and/or PUMA expression levels. Although our data did not identify significant up-regulated expression of BIM or PUMA to be a common feature among prednisolone sensitive pediatric ALL samples, it remains possible that some patients express sufficient amounts of either BIM or PUMA to trigger glucocorticoid-induced apoptosis despite of high MCL-1 expression. If so, this would further explain how some \textit{MLL}-rearranged infant ALL patients show a good \textit{in vivo} response to prednisone, while at the same time displaying high-level \textit{MCL-1} expression.

We showed that down-regulation of MCL-1 expression in prednisolone resistant \textit{MLL}-rearranged ALL (SEMK2) cells to some extent induces prednisolone sensitivity. Although the prednisolone sensitizing effects of MCL-1 down-regulation appeared moderate, the cytotoxic response tended to resemble that of prednisolone sensitive \textit{MLL}-rearranged infant ALL cells. Therefore, finding therapeutically applicable agents that are able to reduce MCL-1 expression in ALL cells during glucocorticoid treatment, may increase the efficacy of these drugs and as such may contribute to the prevention of early relapse of the disease. Recently we showed that the mTOR inhibitor rapamycin could represent such a glucocorticoid sensitizing agent. Comparing the same gene expression signature of prednisolone resistance/sensitivity in non-infant ALL patients as presented in Figure 1A, to a database of drug-associated gene expression profiles (connectivity mapping), revealed that rapamycin induces a gene signature that matches that of prednisone sensitive ALL patients.\textsuperscript{28} This study further demonstrated that rapamycin could suppress MCL-1 expression, thereby sensitizing malignant lymphoid cells to glucocorticoids. Moreover, the glucocorticoid sensitizing effects of
Rapamycin appeared to rely on activation of the AKT/mTOR pathway, and rapamycin is currently used in clinical trials for malignancies that depend AKT/mTOR activation. Unfortunately rapamycin did not result in MCL-1 down-regulation nor to glucocorticoid sensitivity in primary MLL-rearranged infant ALL cells (unpublished data), suggesting that the AKT/mTOR pathway may not play a significant role in this type of leukemia. Nevertheless, our data emphasize that sustained high-level MCL-1 expression represents a general mechanism of glucocorticoid resistance among ALL patients, including MLL-rearranged infant ALL cases. For this latter group of patients the challenge now is to identify compounds that do suppress MCL-1 expression in these cells. Several studies have reported compounds that induce apoptosis by targeting MCL-1. MCL-1 plays an important role in the survival of B-cell chronic lymphoblastic leukemia (B-CLL), as well as in multiple myeloma (MM) cells, in which, like in MLL-rearranged ALL, abundant MCL-1 expression is also frequently observed.\textsuperscript{14,29} Recently, honokiol (a natural phenolic compound extracted from the root and stem bark of several species of Magnolia) was found to induce leukemic cell death in primary B-CLL cells by down-regulating MCL-1 expression.\textsuperscript{30} Similarly, two synthetic compounds, i.e. seliciclib (CYC202 or R-roscovitine) and R-etodolac (SDX-101) induced apoptosis in MM cells by directly targeting MCL-1.\textsuperscript{31,32} However, despite numerous attempts in our laboratory we observed that none of these compounds was able to reduce MCL-1 expression and induce glucocorticoid sensitivity in MLL-rearranged ALL cells (unpublished data).

Taken together, our study demonstrates an important role of MCL-1 in glucocorticoid resistance in MLL-rearranged infant ALL, warranting further studies to identify drugs that effectively down-regulate MCL-1 expression, to be co-administered during glucocorticoid-based treatment regimes. Our observations also strongly suggest that high-level MCL-1 expression presumably is not the sole mechanism maintaining glucocorticoid resistance, and additional studies in search for alternative or co-operating mechanisms remain important to fully understand and eventually circumvent or overcome glucocorticoid resistance in patients.
Acknowledgements

The authors wish to express their gratitude to the members and participating hospitals of the INTERFANT-99 for supporting this study by providing leukemic samples. Members of INTERFANT-99 are: Campbell, M. (PINDA), Felice, M. (Argentina), Ferster, A. (CLCG), Hann, I. and Vora, A. (UKCCSG), Hovi, L. (NOPHO), Janka-Schaub, G. (COALL), Li, CK. (Hong Kong), Mann, G. (BFM-A), Mechinaud, F. (FRALLE), Pieters, R. (DCOG), de Rossi, G. and Biondi, A. (AIEOP), Rubnitz J. (SJCRH), Schrappe, M. (BFM-G), Silverman, L. (DFCI), Stary, J. (CPH), Suppiah, R. (ANZCHO), Szczepanski, T. (PPLLSG), Valscechi, M. and de Lorzenzo, P. (CORS).

Author Contribution

R.W. Stam : Designed and performed research, wrote manuscript.

M.L. den Boer : Reviewed manuscript.

P. Schneider : Performed research.

J. de Boer : Performed research.

J. Hagelstein : Performed research.

M.G. Valsecchi : Patient information collection and statistics.

P. de Lorezno : Patient information collection and statistics.

S.E. Sallen : Reviewed manuscript.

H.J.M. Brady : Reviewed manuscript, performed research.

S.A. Armstrong : Performed research, reviewed manuscript.

R. Pieters : Designed research, reviewed manuscript.

The authors have no conflict of interest to disclose.
References


Legends to the Figure

Figure 1. Gene expression profiling identifies high-level MCL-1 expression as a common marker for prednisone resistance among childhood ALL patients.

A. Shows the top 50 of up-regulated probe sets correlating to either prednisone resistance or sensitivity in non-infant (>1 year of age) (n=27) and infant (< 1 year of age) (n=25) pediatric ALL patients. Raw microarray data can be found in Supplemental Table 1. B. Classification accuracy of prednisolone sensitive or resistant infant ALL or non-infant ALL samples on the basis of their gene expression profile. The error rate in class prediction is plotted against the number of probe sets used to build the model. C. Shows the overlapping genes that are associated with prednisone resistance in both the infant and non-infant ALL signatures. Merging the signatures reveals that high-level MCL-1 expression is common to both non-infant and infant ALL patients that are resistant to prednisone in vitro.

Figure 2. High-level MCL-1 expression is associated with in vitro prednisolone resistance in both MLL rearranged infant ALL and non-infant pediatric precursor B ALL.

Distribution of the relative MCL-1 expression as determined by quantitative real-time RT-PCR (TaqMan) analyses among A. MLL rearranged infant ALL and B. non-infant (children >1 year of age) B-ALL patients. Dotted lines either indicate the mean or median expression among both patient groups. Among the MLL rearranged infant ALL cases the median and mean MCL-1 expression coincided. The median or mean MCL-1 expression level was used as the cut-off between patients, dividing the patients into groups displaying “high” or “low” MCL-1 expression. Mean cytotoxic dose-response curves for prednisolone in patients characterized by high or low MCL-1 expression are shown for C. MLL rearranged infant ALL patients and D. non-infant ALL patients, respectively. Differences in mean cytotoxicity responses between patient groups were statistically analyzed using the 2-tailed Student t test. Error bars represent the standard error of the mean (SEM). * Indicate significant differences between the means of the groups at p<0.05.
Figure 3. High-level MCL-1 expression is associated with in vivo prednisone resistance in MLL rearranged infant ALL.

A. Based on a 7-day window of prednisone mono-therapy, MLL rearranged infant ALL patients are classified as prednisone good responders (PGRs; <1000 leukemic blasts/μL) or prednisone poor responders (PPRs; ≥1000 leukemic blasts/μL). The graph shows the relative MCL-1 expression as determined by quantitative real-time RT-PCR (TaqMan), in both patient groups. B. Shows the differences in relative MCL-1 expression in infant ALL patients that are PGRs and are sensitive to prednisolone in vitro, compared with patients that are resistant either in vivo (PPRs) and/or in vitro. Dots represent individual patients, lines indicate the median MCL-1 expression level in each group. The difference between patient groups was statistically analyzed using the Mann-Whitney U test.

Figure 4. Down-regulation of MCL-1 sensitizes prednisone resistant MLL rearranged ALL cells.

A. RNA interference (RNAi) experiments delivering short hairpin RNA (shRNA) molecules directed against human MCL-1 into prednisolone resistant SEMK2 (MLL-rearranged ALL) or MV4-11 (MLL-rearranged AML) cells by viral infections, show severe suppression in MCL-1 protein expression as compared to control cells (infected with empty vectors). The indicated ratios are normalized against β-tubulin, and represent MCL-1 intensities relative to SEMK2 control cells (first lane) The effects of MCL-1 down-regulation on the in vitro prednisolone response in B. SEMK2 or C. MV4-11 cells were assessed by 2-day MTT cytotoxicity assays, performed in triplicate. The graphs show the mean prednisolone response curves in cells infected with either empty vectors (C: controls) or vectors encoding shRNAs against human MCL-1 (KD: knock-down), derived from two independent RNAi experiments.
Figure 1

A. 

<table>
<thead>
<tr>
<th>Infant ALL:</th>
<th>Non-infant ALL:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resistant</td>
<td>Sensitive</td>
</tr>
</tbody>
</table>

B. 

![Graph showing error rate vs. # probe sets]

Infant signature: Shown by red line.
Non-infant signature: Shown by black line.

C. 

Infant ALL:

<table>
<thead>
<tr>
<th>Sensitive</th>
<th>Resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCL-1</td>
<td>Solute Carrier 2</td>
</tr>
<tr>
<td></td>
<td>Fos-like Antigen 2</td>
</tr>
</tbody>
</table>

Non-infant ALL:

<table>
<thead>
<tr>
<th>Sensitive</th>
<th>Resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solute Carrier 2</td>
<td>MCL-1</td>
</tr>
<tr>
<td>Fos-like Antigen 2</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3

A. MCL-1 expression relative to RNaseP (%)

B. MCL-1 expression relative to RNaseP (%)

- PGRs (in vivo sensitive)
- PPRs (in vivo resistant)

- PGRs and in vitro sensitive
- PPRs or in vitro resistant

p < 0.01
Association of high-level MCL-1 expression with in vitro and in vivo prednisone resistance in MLL-rearranged infant acute lymphoblastic leukemia

Ronald W. Stam, Monique L. Den Boer, Pauline Schneider, Jasper de Boer, Jill Hagelstein, Maria G. Valsecchi, Paola de Lorenzo, Stephen E. Sallan, Hugh J.M. Brady, Scott A. Armstrong and Rob Pieters