Early and Very Early GRIM19 and MCL1 Expression Are Correlated to Late Acquired Prednisolone Effects in a T-Cell Acute Leukemia Cell Line

Lambrou G, Maria Adamaki, Kyriaki Hatziagapiou, Styliani A. Geronikolou, Athanasios N. Tsartsalis, and Spiros Vlahopoulos

Abstract

Glucocorticoids (GCs) are still first-line drugs for the treatment of childhood acute lymphoblastic leukemia (ALL). Prednisolone is a corticosteroid and one of the most important agents in the treatment of ALL. We report here a study of Prednisolone treatment using as a model a leukemia cell line with subsequent investigation of resistance-related gene expression. Gene silencing has been used in order to identify significant targets of resistance to GC-induced apoptosis in ALL cells. We analyzed effects of increasing doses of Prednisolone on ALL cell survival and growth, and we monitored immediate effects on gene expression through gene expression assays. We determined Prednisolone cytotoxicity and cell cycle distribution as well as DNA content. Upon treatment with escalating Prednisolone concentration, we observed a gradual decline in cell survival. MCL1 and GRIM19 were investigated as possible genes for the intrinsic capacity of this cell line to respond to corticosteroid and a snapshot of *early* changes was examined. Early *MCL1* and *GRIM19* expression correlated significantly to late GC-induced apoptosis. Prednisolone competitively induces MCL1 expression. Consistently with previous studies on primary leukemia blasts, cells are sensitive to proteasome inhibitor MG132; no interference of Prednisolone with MG132 effects on this cell line was noted. The inherent plasticity of clinically evolving cancer justifies approaches to characterize and prevent undesirable activation of early oncogenic pathways. Study of the pattern of intracellular signal pathway activation by anticancer drugs can lead to development of efficient treatment strategies by reducing detrimental secondary effects.

Lambrou. G (✉) · M. Adamaki · K. Hatziagapiou
S. Vlahopoulos
National and Kapodistrian University of Athens, First Department of Pediatrics, Choremeio Research Laboratory, Athens, Greece
e-mail: glamprou@med.uoa.gr; madamaki@med.uoa.gr; khatziag@med.uoa.gr; sblachop@med.uoa.gr

S. A. Geronikolou
Biomedical Research Foundation of Academy of Athens, Clinical, Translational, Experimental Surgery Research Centerment of Pediatrics, Athens, Greece

A. N. Tsartsalis
Naval Hospital of Athens, Department of Endocrinology Diabetes and Metabolism, Athens, Greece

1   Introduction

Childhood leukemia is the commonest form of
childhood cancer and represents clonal prolifera-
tion of transformed hematopoietic cells as a
result of genomic and proteomic changes [1–3].
Treatment of childhood leukemia has been exten-
sively successive during the recent years. Patient
complete remission has reached 80–85%, yet a
remaining 15–20% still relapses. In the treatment
of childhood leukemia, glucocorticoids (GC) still
remain as first-line drugs as well as response to
GC treatment still remains a significant prognos-
tic factor [4–7]. Glucocorticoids enter the cell
passively and bind to a 97 KiloDalton protein, the
glucocorticoid receptor (GR) [8–11], causing
through allosteric effects exposure of nuclear
localization signals on the surface of GR. GR
next enters the nucleus, binding to the glucocorti-
coid response element (GRE) followed by acti-
vated transcription of target genes caused by the
effect of the receptor on the genome. The action
of the GR depends solely on the type of tissue or
cell that it affects. In the case of neoplasmatic
lymphocytes (e.g., ALL), it is known that it sets
the cell on its programmed death (apoptosis)
pathway. Therefore, Prednisolone is widely used
for leukemia treatment [11]. To reduce the com-
plexity due to feedback mechanisms affecting
gene expression, a snapshot of *early* direct effects
on gene expression is necessary to be obtained at
an early stage under glucocorticoid treatment.
Such an analysis is expected to include key
genetic regulators and initiators of further down-
stream pathways.

The significance of Prednisolone resistance in
acute lymphoblastic leukemia (ALL) is thor-
oughly discussed in the literature. Prednisolone
is a glucocorticoid and one of the most important
agents in the treatment of ALL, especially in chil-
dren, a very sensitive group of patients [2].
Resistance to Prednisolone is considered to be of
crucial importance for disease prognosis [2].
Toward the identification of resistance mecha-
nisms, several tools and approaches can be
deployed. Gene expression and gene regulatory
mechanisms are considered crucial for the study
of diseases and, in particular, ALL. Gene
expression analysis of GC-treated ALL cells may
allow discovery of drugs which in combination
with glucocorticoids may increase the effective-
ness of anti-leukemia therapies. The use of high-
throughput technologies provided new insights
into cancer, in general, and to leukemia specifi-
cally as far as, diagnosis, prognosis, treatment
response, and new therapeutic targets is
concerned.

Previous studies have indicated that the cell
line model used manifested higher proliferation
rates at very low Prednisolone concentrations [1,
12]. These findings indicated that the two lowest
concentrations of Prednisolone used (10 nM and
1 μM) had a mitogenic effect. Interestingly, it has
been also found that total cell death after expo-
sure to 1 μM Prednisolone is similar to that
observed for higher doses (>100 μM). However,
cells treated with 1 μM Prednisolone grew faster
than untreated cells and cells treated with higher
doses. Cells exposed to >1 μM Prednisolone
manifested the same growth potential as untreated
cells [1, 12]. Previous cytotoxicity data indicated
that the CCRF-CEM cell line exhibited resis-
tance to corticosteroid-induced apoptosis, 72 h
after treatment. The highest dosage (700 μM,
which was ~8-fold higher from the average
in vivo dosage, which was calculated to be
100 μM) manifested a combined apoptotic and
necrotic effect. This was in contrast to the case of
GC-sensitive cells where up to 80% total cell
death has been reported after treatment with only
20 nM dexamethasone. In the same cell model, it
appeared that the first 48 h showed no dose-
dependent cumulative cytotoxic effect of
Prednisolone. Upon 48 h of incubation, a dose-
dependent escalation of cytotoxicity started to
become evident, which was manifested clearly as
a dose-dependent effect after 72 h of Prednisolone
stimulation. Specifically, upon 72 h, apoptosis
exhibited a dose-dependent effect while necrosis

peaked at 1 μM, dropped sharply between 1 μM and 40 μM to increase dose-dependently again. Interestingly, bioinformatics analysis has shown that CRE-BP1/c-Jun (AP-1) heterodimer transcription factor was overexpressed transcription factors of differentially expressed genes under Prednisolone treatment, where c-Jun is induced by GCs at the transcriptional level. C-Jun expression it has been shown that it is important in the GC-induced apoptosis. However, at protein level there is an inhibition of c-Jun causing a delay in its expression. Another exhibited effect of Prednisolone on the leukemic cell system CCRF-CEM was the observation of high proliferation levels. Exhibited proliferation levels were in contrast with the significant total cell death observed at low concentrations (<1 μM) in a previous study [1, 12]. Even at high Prednisolone concentrations (>100 μM, the proliferation rates were similar to those observed for untreated cells. Apoptotic death is at lower levels than that of untreated cells. The small peak in total cell death at 1 μM of Prednisolone concentration was mainly an effect of a peak in necrosis for the same concentration. Despite the increased necrosis, cell growth exceeded that corresponding to the control and higher doses due to the mitogenic effect. At higher doses (>1 μM), Prednisolone operated as a dose-dependent cytotoxic agent but still proliferation remained at the same levels as that of untreated cells. The dose dependency of mitogenic behavior was similar to that reported in several in vitro and in vivo studies for different cell types [13–15].

It has been originally reported that the NF-κB(p65) regulation is altered due to GR-mediated I-κB regulation [16]. NF-κB nuclear translocation, when stimulated, is expected to take place within the first 30–60 min after stimulation [17]. At the same time it has been also reported that NF-κB(p65) manifests a constitutive presence in the nucleus of lymphoblast cells. Possible reasons for the constitutive nuclear presence of NF-κB RelA might be a) the inability of the GR to inhibit NF-κB from entering the nucleus and b) the absence of the I-κBα protein in this cell system. It has been reported that the Iκ-Bα protein is degraded through the

ubiquitin-proteasome pathway [18, 19]. This led to the hypothesis that proteasome plays a role in the phenotype of this cell line. Proteasome inhibition was previously reported as selectively lethal to leukemia stem cells causing a dose-dependent growth inhibition of CCRF-CEM cells [1, 12, 20]. From a previous work, it has become evident that Prednisolone works in a dual mechanism activating different GR transactivation or transrepression pathways, which hinted about a potential role of NF-κB in the CCRF-CEM cells. Bioinformatics analysis has shown that NF-κB was prevalent as a transcription factor binding motif (TFBM) in genes regulated by Prednisolone [1, 12]. There is an integrated circuit between GR, Iκ-Bα, and NF-κB reported previously as a dual mechanism of action of GCs. This means that the GR induces Iκ-Bα expression, which inhibits NF-κB transactivation, while GR inhibits NF-κB transactivation via physical interaction. Another piece of evidence is the prevalence of GR/NF-κB-related TFBMs in the analysis of the microarray data. Protein expression analysis showed that the glucocorticoid receptor (GR) did not immediately inhibit the NF-κB entry in the nucleus, directly or through the inhibitory protein I-κB [1, 12]. From previous reports we have highlighted the role of specific genes in acute lymphoblastic leukemia. In particular, *AML1* [12, 21], *IRF4* [12, 22], *MEIS1* [12, 23], *HOXA9* [12, 23], and *GRIM19* [1, 12, 20] were found to play a significant role in ALL pathogenesis. We have further examined the role of these genes for their participation in resistance to GC-induced apoptosis.

2 Materials and Methods

2.1 Cell Culture

The CCRF-CEM cell line was used as the model, obtained from the European Collection of Authenticated Cell Cultures (ECACC). This cell line is a T lymphoblast leukemia cell line, known to be resistant to glucocorticoids (GC). CCRF-CEM is a human T-cell line originally isolated from a child with acute lymphoblastic leukemia

[24]. Cell culture conditions have been extensively discussed previously [1, 20–23]. Twenty-four hours (24 h) before the application of Prednisolone (which is called −24 h time in the present study) cells were harvested by centrifugation at 1000 rpm for 10 min on a KUBOTA centrifuge. Cells were seeded at an initial concentration of 1.0×10^3 to 1.3×10^3 cells/µl in a final medium volume of 10 ml. Cell population counts were determined with the use of a Nihon Kohden CellTac-α hematology analyzer.

2.2   Prednisolone Treatment

Concentrations of Prednisolone treatment were selected on the basis of the average in vivo dosage administrated intravenously to children at ages between 1 month and 12 years old as previously reported [1]. Finally, the concentrations, control (0 µM), 100 nM, 100 µM, and 700 µM were chosen for further cell treatment, where 100 µM is estimated to be the mean in vivo concentration in pediatric patients between 1 month and 12 years old. Cell number was then determined at −24 h, 0 h, 1 h, 4 h, and subsequently every 24 h.

2.3   Experimental Setup

Experiments were performed in 10 cm^2 cell culture flasks. Experimental setup included cell culture flasks, where (a) cells were grown and no Prednisolone was added (control or reference experiment), (b) cells were grown under 10 nM Prednisolone, (c) cells were grown under 100 µM Prednisolone, and (d) cells were grown under 700 µM Prednisolone. This experimental setup was used for the investigation of gene expression of specific genes. Another exactly similar setup was used for the implementation of the gene silencing experiments.

Cell cycle distribution and DNA content was determined with standard PI (propidium iodide, Invitrogen Inc.) staining as described previously on a Beckman Coulter flow cytometer Flow-Count XL [1, 20]. All concentrations and time

point experiments consist at least of triplicate experiments.

RNA was isolated with TRIzol (Invitrogen Inc.) as described from the manufacturer. Gene expression was evaluated with qRT-PCR. Genes examined included the *GRIM19* (*NDUFA13*) (Refseq NM_015965), *AML1* transcript variant 1 (Refseq NM_001754.4), *AML1* transcript variant 2 (Refseq NM_001001890.2), *IRF4* (Refseq NM_001195286.2), *MCL1* (Refseq NM_021960.5), *MEIS1* (Refseq NM_002398.3), *HOXA9* (Refseq NM_152739), *GAPDH* (Refseq NM_002046), and *b-actin* (Refseq X00351). Investigated genes were tested for three samples; control, 10 nM, 100 µM, and 700 µM Prednisolone at 1 h, 4 h, and 72 h treatment, using the one-step Plexor™ qRT-PCR kit (Promega Inc.) [25, 26]. Primer sequences are summarized in Table 1. The qRT-PCR conditions used have been described previously [1, 20–23].

Standard curves were created in order to check for the method consistency gene expression accuracy. Two experiments were performed: one with the *GRIM19* (Accession NM_015965) primers, using a dilution series, for the standard curve, from the control RNA (0 µM Prednisolone at 4 h) comprising six points, each in a duplicate (2×50 ng, 2×5 ng, 2×0.5 ng, 2×0.05 ng, 2×5 pg, 2×0.5 pg), and a second experiment comprising both sets of primers for *GRIM19* (Accession NM_015965), *b-actin* (Accession X00351), and *GAPDH* (Accession NM_002046). Experiments were performed either in multiplexing between GRIM19 and b-actin or GAPDH or without any multiplexing. The results of the standard curves are presented in Fig. 1.

2.4   MCL1 Silencing (siRNA Experimentation)

Cells under Prednisolone treatment were further processed for MCL1 silencing. Cells were cultured in 10 cm^2 flasks, up to a volume of 5 ml, and were transfected according to the instructions of the Lipofectamine®3000 (ThermoScientific, New York, CA). Thereafter, the treated cells were assigned into the following

Table 1 Primer sequences of investigated genes

| Gene name	Accession	Primer sequence	
GRIM19 (*NDUFA13*)	NM_015965	Forward	ACCGGAAGTGTGGGATACTG
		Reverse	GCTCACGGTTCCACTTCATT
AML1, isoform *AML1c*	NM_001754.4	Forward	GTGGGTACGAAGGAAATGACTCAAA
		Reverse	GCAGCGTGGTAAAAGAAATCATTGAG
IRF4	NM_001195286.2	Forward	AGCGCATTTCAGTAAATGTAAACACA
		Reverse	TCTTGTGTTCTGTAGACTGCCATCA
MCL1	NM_021960.5	Forward	CTTTTGGCTACGGAGAAGGAG
		Reverse	GTCACAATCCTGCCCCAGTT
MEIS1	NM_002398.3	Forward	AATCCCTTAACGTCTCCAGCAAC
		Reverse	TCTTGGAAACGGAGCGCTTTTAT
HOXA9	NM_152739	Forward	CCGTTACAATCAGCATTCATTTCCT
		Reverse	AACAGTGAGGAAATTCGGAGCTATAC
GAPDH	NM_002046	Forward	TGAGCACAGGGTACTTTATTGATGGT
		Reverse	GTTGCCATGTAGACCCCTTGAAGA
b-actin	X00351	Forward	GTAGATGGGCACAGTGTGGGTGA
		Reverse	TGTGCTATCCCTGTACGCCTC
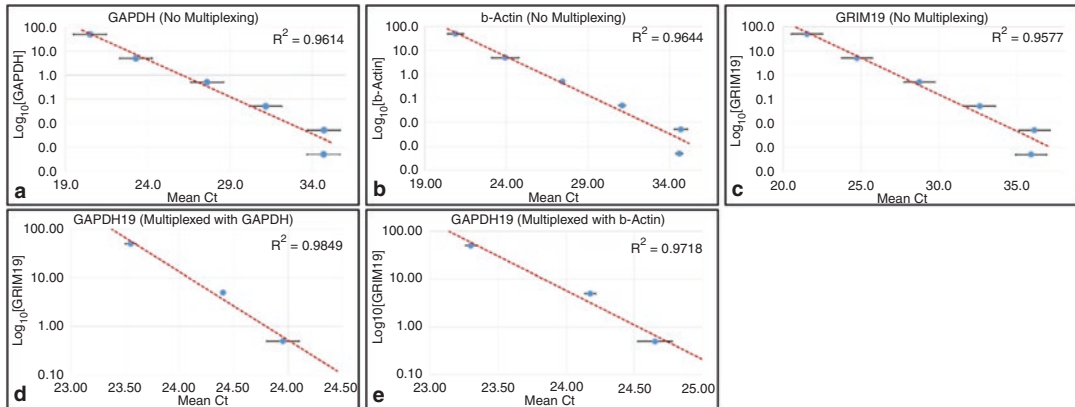
Fig. 1 Standard curves of GRIM19, GAPDH, and b-actin genes in multiplexing or not mode. In particular, standard curve experiments included a six-point standard curve for un-multiplexed GAPDH (R^2 = 0.96) (**a**), a six-point standard curve of b-actin (R^2 = 0.96) (**b**), a six-point standard curve of un-multiplexed GRIM19 (R^2 = 0.96) (**c**), three-point GRIM19 multiplexed with GAPDH (R^2 = 0.98) (**d**), and multiplexed GRIM19 with b-actin (R^2 = 0.97) (**e**)

categories: Negative Control 1 (NC1) (no siRNA, no transfection reagent), Negative Control 2 (NC2) (no siRNA, with transfection reagent), Control 1 (C1) (cells transfected with MCL1 silencing sequence only), cells treated with MCL1-siRNA and 10 nM Prednisolone (Prednisolone Treatment 1 (Treat1)), cells treated with MCL1-siRNA and 100 μM Prednisolone (Prednisolone Treatment 2 (Treat2)), cells treated with MCL1-siRNA treated and 700 μM Prednisolone (Prednisolone Treatment 3 (Treat3)), and cells treated with 700 μM Prednisolone without any MCL1-siRNA (Prednisolone Treatment 4 (Predni)). The transfected cells were cultured in a 5% CO2 incubator at 37 °C. Following incubation for 6–8 h, the original medium was replaced by complete medium for another 24 h incubation for further experimentation. Relative gene expression was considered with respect to the NC1 and NC2 experiments.

2.5   Bioinformatics Analyses

Gene network was formed by interactions identified using the Coremine web tool[1]. *GRIM19*, *AML1*, *MEIS1*, *IRF4*, *HOXA9*, *MCL1,* and Prednisolone were incorporated into the web tool and known interactions were identified. Genes were inserted as a model in the SimBiology Toolbox of the MATLAB® Computational Environment (the MathWorks, Inc.). Gene ontology and pathway annotation were performed with the WebGestalt web tool [27–29].

2.6   Statistical Analysis

Continuous data are presented as mean ± standard deviation (SD) unless otherwise stated. Flow cytometry and cell cycle data were analyzed using the algorithms proposed by Watson et al. and Ormerod et al. [30, 31]. Statistical analysis was performed using the *T-test* for the proliferation, cytotoxic, cell cycle, and gene expression data. Relative gene expression was estimated with the 2^{-Ct} with respect to the expression of GAPDH and actin genes. Data pre-processing was performed with Microsoft® Excel. Statistical and data analyses have been performed using the MATLAB computational environment (the MathWorks, Inc.). All differences were considered statistically significant if they obtained a *p-value* of $p < 0.05$ unless otherwise defined. Post hoc comparisons (adjusted with *Bonferroni* criterion) were also performed when significant differences ($p < 0.05$) of the gene expression data.

2.7   Ethics Statement

No human or animal samples and/or subjects were used in the present study.

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[1]https://coremine.com/medical

3   Results

3.1   Cell Proliferation and Apoptosis

Cell proliferation showed that cells are resistant to Prednisolone since they significantly continued to grow after 72 h as compared to 0 h and 4 h treatment (Fig. 2a). At the same time, cells manifested relatively low levels of apoptosis, yet cells under 700 μM and 100 μM manifested a late apoptotic effect at 48 h, which increased at 72 h (Fig. 2b). Apoptotic effect was significant at 48 h and 72 h as compared to 0 h of treatment.

3.2   Functional Annotation of Genes Under Investigation

Our first attempt included the investigation of our genes with respect to their functional annotation that is for their known functions as well as known participation in cellular signaling pathways. Known connections were investigated with the *Coremine* web tool, and we have found that examined genes, Prednisolone, and leukemia did manifest a connection (Fig. 3a). Additionally, gene ontology (GO) annotation revealed that genes participate in hematopoetic processes, as well as MCL1 and GRIM19 participate in apoptotic and metabolic processes (Fig. 3b). Similarly, pathway annotation analysis revealed that MCL1 and GRIM19 do also participate in apoptotic and metabolic pathways (Fig. 3c). These findings constituted the first hint that MCL1 and GRIM19 were of two genes of interest probably participating in resistance to GC-induced apoptosis.

3.3   Gene Expression

Cells under Prednisolone treatment were examined for the gene expression profiles of *GRIM19* (Fig. 4a), *AML1* (Fig. 4b), *IRF4* (Fig. 4c), *MCL1* (Fig. 4d), *HOXA9* (Fig. 4e), and *MEIS1* (Fig. 4f) at 1 h of treatment. Significant differences were manifested between concentrations of Prednisolone for all genes, except for *MCL1*.
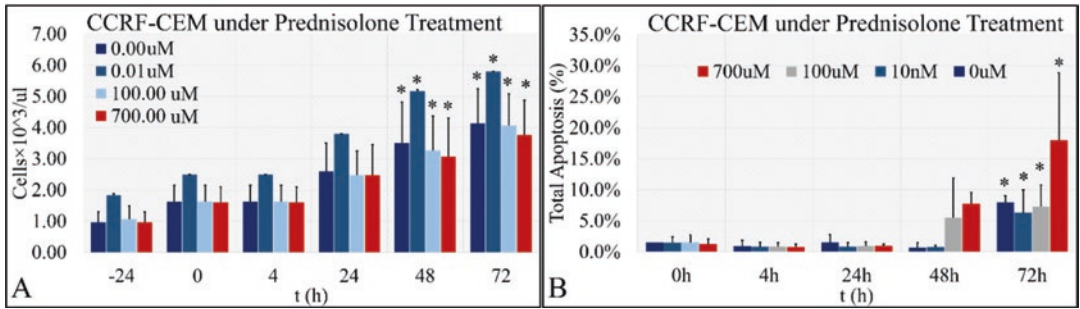
Fig. 2 Cell proliferation and apoptosis of CCRF-CEM cells. Cell proliferation has been measured under Prednisolone treatment (**a**). In addition, apoptosis has been estimated and it has been found that Prednisolone manifests a late apoptotic effect at 48 h and 72 h (**b**) (asterisks denote a significance at the $p < 0.05$ level between estimated factors at 48 h and 72 h and 0 h)
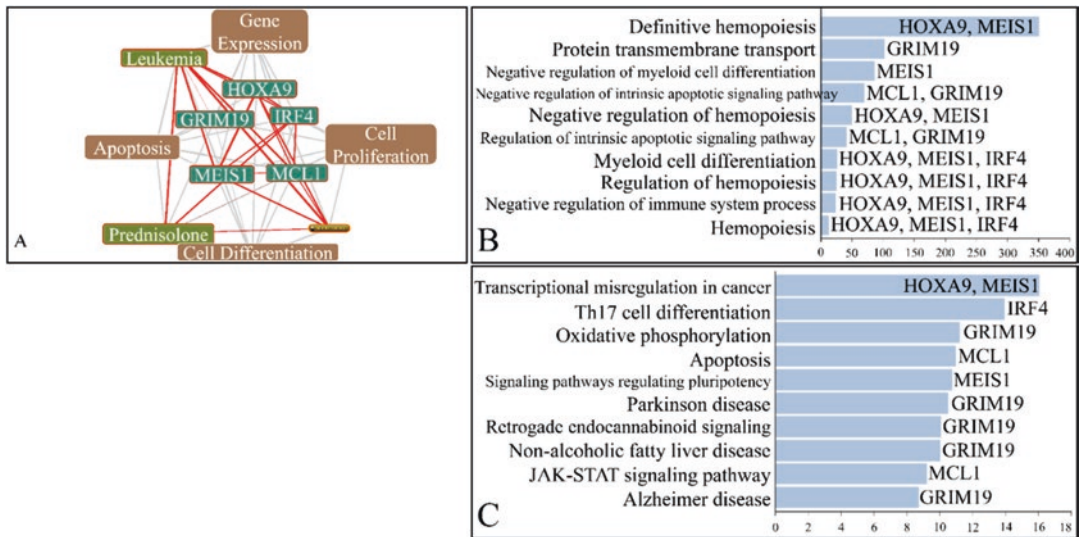

Fig. 3 Functional annotation of the genes under investigation. Results from Coremine (**a**), WebGestalt gene ontology (**b**), and WebGestalt pathway analysis (**c**) are presented

Almost all genes manifested significantly different expression levels within the same Prednisolone treatment (Fig. 4g).

Interestingly, there was no significant difference in the expression levels between *GRIM19* and *MCL1* genes, indicating similar expression levels for these two genes. This hinted us to a possible common function between the two genes and we investigated their expression levels at 4 h which is presented in Fig. 5. It appeared that *GRIM19* and *MCL1* manifested very similar expression patterns, except for a marginal difference between *MCL1* and *GRIM19* at 100 μM and 700 μM

(Fig. 4a), with respect to Prednisolone concentration at 4 h. Interestingly, *GRIM19* expression manifested a linear behavior ($R^2 = 0.91$) (Fig. 5a), and *MCL1* manifested a bell-shaped regression ($R^2 = 0.95$) indicating their biological role in GC regulation. In order to further investigate this finding, correlation analysis has shown that *GRIM19* and *MCL1* manifested the highest correlation coefficient with respect to each other, but also with respect to apoptosis at 72 h as compared to the other genes (Table 2). In addition, *GRIM19* levels manifested significant correlation with respect to cell proliferation at 72 h ($r = 0.64$).
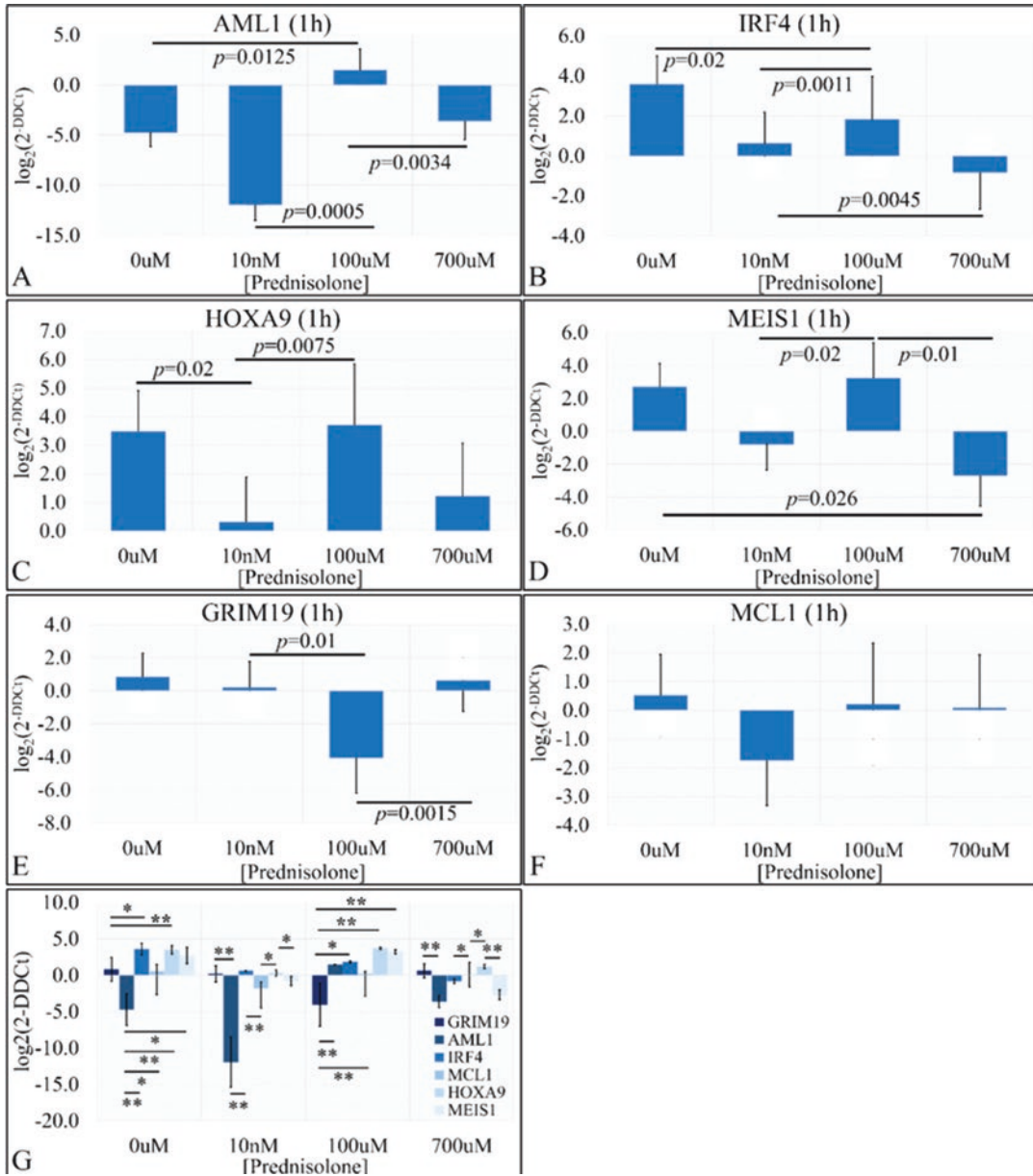
Fig. 4 Expression levels of *AML1* (**a**), *IRF4* (**b**), *HOXA9* (**c**), *MEIS1* (**d**), *GRIM19* (**e**), and *MCL1* (**f**) were estimated for the CCRF-CEM cells under Prednisolone treatment at 1 1 h. Interestingly, significant differences were manifested between all genes at different concentrations except for *MCL1* (**f**). Similarly, significant differences were manifested between gene expression in each concentration (**g**) (*asterisk denotes a significance at the $p < 0.05$ level and **denote a significance at the $p < 0.01$ level)

We have further examined the gene expression profiles by attempting to sort gene expression levels using a hierarchical clustering (HCL) approach. In particular, we have found that apoptosis levels at 72 h were strongly correlated with *MCL1* expression levels at 4 h and in addition with *GRIM19* also at 4 h (Fig. 6), reinforcing our previous observation of regression results (Fig. 5a). Based on our observations we have come up with the hypothesis that *MCL1* could
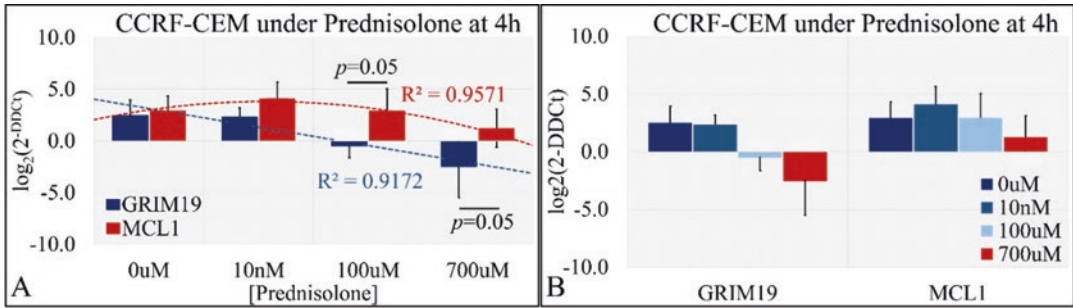
Fig. 5 Gene expression of GRIM19 and MCL1 in cells under Prednisolone treatment at 4 h with respect to the Prednisolone concentration (**a**) and the genes (**b**)

Table 2 Correlation analysis of GRIM19 and MCL1 with respect to each other's expression as well as with respect to apoptosis at 4 h and 72 h (correlation coefficient (*r*) in bold depicts significant correlation between variables)

	GRIM19 (4 h)	*MLC1* (4 h)	Apoptosis (1 h)	Apoptosis (4 h)	Apoptosis (72 h)
GRIM19 (4 h)	1.00	**0.84**	0.81	0.54	**−0.82**
MLC1 (4 h)		1.00	0.87	0.23	**−0.93**
Apoptosis (1 h)			1.00	0.65	−0.99
Apoptosis (4 h)				1.00	−0.53
Apoptosis (72 h)					1.00

probably play a significant role in resistance to GC-induced apoptosis, or MCL1 could be a gene directly regulated by Prednisolone, i.e., the GR. Examining the expression levels of *GRIM19* and *MCL1* at 72 h, we have found that both genes manifested similar patterns as in previous time points (Fig. 7).

In particular, *GRIM19* at 72 h did manifest significant correlation with respect to *MCL1* ($r = 0.77$) and apoptosis at 72 h ($r = 0.72$) (Table 3), yet *MCL1* expression levels at 72 h did not manifest a significant correlation to apoptosis at 72 h ($r = 0.2$) (Table 3). However, when accounting for the *GRIM19* and *MCL1* expression levels of cells under Prednisolone treatment, excluding control samples, we have found that both *GRIM19* and *MCL1* manifested significant correlation to apoptosis at 72 h ($r = 0.93$, $r = 0.99$, respectively) (Table 3). This interesting finding was probably due to the observed anti-apoptosis manifested by the cells under Prednisolone treat-

ment. Finally, it also appeared that *GRIM19* expression levels at 72 h manifested a significant negative correlation with cell proliferation also at 72 h ($r = −0.88$).

Our previous observations have led us to the hypothesis that *MCL1* could probably play a significant role in resistance to GC-induced apoptosis and especially under Prednisolone treatment. For that reason we have attempted to silence the *MCL1* gene by transfection, and we have found that indeed *MCL1* is regulated by Prednisolone in a dose-dependent manner. Cells were transfected at 48 h and *MCL1* levels were evaluated 24 h later.

In particular, it appeared that silenced cells for the *MCL1* gene manifested a gradual decrease in *MCL1* expression with respect to Prednisolone from 10 nM to 700 μM (Fig. 8a). Non-transfected cells manifested significant difference with respect to the expression levels of MCL1 in cells under 10 nM and 100 μM but not under 700 μM
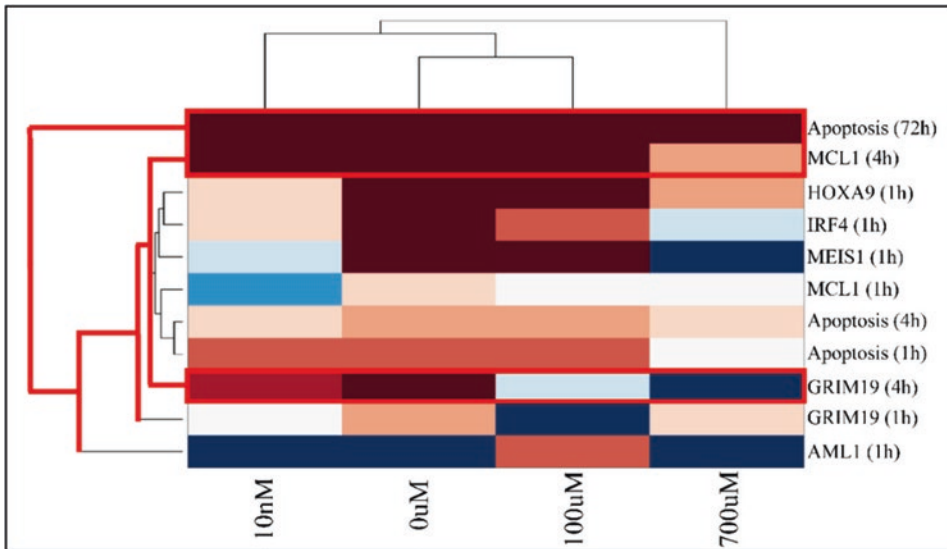
Fig. 6 Hierarchical clustering (HCL) of investigated genes. A correlation between apoptosis at 72 h and *MCL1* and *GRIM19* at 4 h was observed
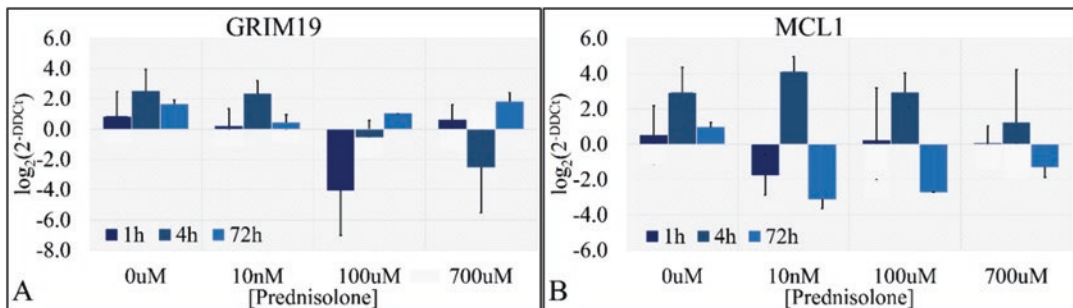

Fig. 7 Gene expression of GRIM19 (**a**) and MCL1 (**b**) in cells under Prednisolone treatment at 1 h, 4 h, and 72 h

Table 3 Correlation analysis of GRIM19 and MCL1 with respect to each other's expression at 72 h as well as with respect to apoptosis at 1 h, 4 h, and 72 h (correlation coefficient (*r*) in bold depicts significant correlation between variables, as well as *depicts gene expression after excluding the control expression values for those genes)

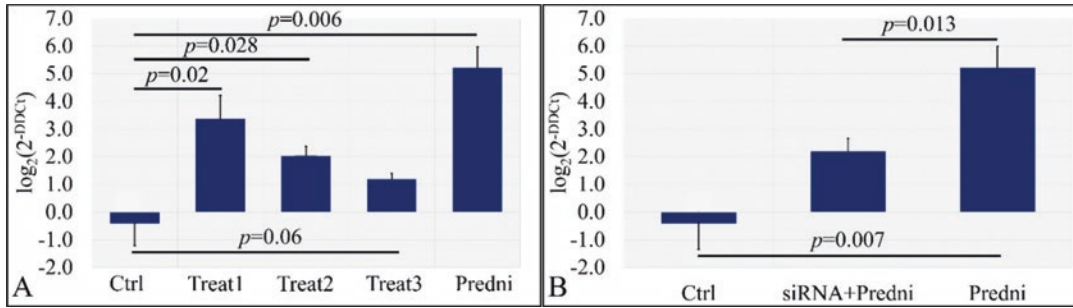|	GRIM19 (72 h)	MCL1 (72th)	GRIM19 (72 h)*	MCL1 (72 h)*	Apoptosis (1 h)	Apoptosis (4 h)	Apoptosis (72 h)
GRIM19 (72 h)	1.00	**0.77**	NaN	NaN	-0.60	0.22	**0.72**
MCL1 (72 h)		1.00	NaN	NaN	-0.08	0.64	0.20
GRIM19 (72 h)*			1.00	**0.97**	**-0.89**	-0.31	**0.93**
MCL1 (72 h)*				1.00	**-0.97**	-0.53	**0.99**

Fig. 8 Gene expression of MCL1 after silencing (**a**) and the mean expression of MCL1 with respect to the silenced and not silenced experiments (**b**) at 72 h (Legend: Ctrl, Control experiment, which included cells treated with the silencing MCL1 sequence and no Prednisolone; Treat1, Cells treated with 10 nM Prednisolone and the MCL1 silencing sequence; Treat2, Cells treated with 100 μM Prednisolone and the MCL1 silencing sequence; Treat3, Cells treated with 700 μM Prednisolone and the MCL1 silencing sequence; siRNA+Predni, The mean expression levels of Treat1, Treat2, and Treat3 experiments; Predni, Cells treated with 100 μM Prednisolone and no MCL1 silencing sequence)

(Fig. 8a). Similarly, significant differences were observed between the mean expression levels of all treatments and cells treated with Prednisolone without transfection (Fig. 8b).

Interestingly, cells treated with different concentrations of Prednisolone and silenced for the *MCL1* gene manifested a significant reverse correlation ($r = -0.83$) with respect to the observed apoptosis at 72 h.

4   Discussion

Corticosteroids are used as therapeutics for almost over half a century. They include some of the most studied substances, especially in leukemia treatment. It is generally accepted that corticosteroids inhibit growth and induce apoptosis of immune system cells. Several studies have attempted to identify gene targets responsible for GC action as well as resistance to GC-induced apoptosis. Interesting examples include the identification of *mpk-1*, which appeared to sensitize cells to glucocorticoids while interference with *bim* expression resulted in inhibition of apoptosis [32, 33]. Elucidation of the mechanisms of GC action may lead to identification of gene targets responsible for glucocorticoid resistance. This may allow discovery of drugs, such as inhibitors of overexpressed genes, which in combination with glucocorticoids may increase the effectiveness of anti-leukemia therapies. Key tools in this

process are high-throughput technologies such as microarray-based gene expression analysis.

In this study, we have observed resistance behavior consistent with these studies as well as more recent reports for CCRF-CEM cells [34, 35]. Although it is possible that these cells are clonally inhomogeneous, that is the case too with the in vivo situation in patients. Moreover, the large number of the CCRF-CEM subclone studies in the literature makes it difficult to do comparisons of different subclone behaviors and provide an absolute frame of reference in terms of a resistant cell model. Thus, we believe that the cell line used for this study is useful in simulating glucocorticoid action and resistance in leukemic cells.

In the present report we have observed two significant phenomena: the resistance of the CCRF-CEM cell line under Prednisolone treatment and the mitogenic effect Prednisolone has on these cells. Two genes were identified as interesting in this cell line and in particular *MCL1* and *GRIM19*. To the best of our knowledge, there are no previous reports concerning the role of *GRIM19* in leukemia except our previous report where we highlighted the role of *GRIM19* in acute leukemia [1, 12]. On the other hand, numerous reports have investigated the role of *MCL1* in acute lymphoblastic leukemia. In particular, all recent and previous studies agree on the fact that *MCL1* is an anti-apoptotic gene, which when inhibited it promotes cell death and inhibits cell

proliferation [36, 37]. *MCL1* is a gene whose physiological function concerns the natural homeostatic response of survival against oxidative stress. At the same time, *MCL1* is believed to be degraded by the proteasome, a mechanism that is accelerated by the action of GCs [38]. Our findings agreed with previous studies, which have indicated that silencing of *MCL1* reduced the gene's expression levels as well as sensitized leukemia cells to Prednisolone [39]. However, in our study *MCL1* silencing did not result into cell sensitization to Prednisolone, indicating the presence of additional mechanisms of resistance to GC-induced apoptosis. This finding was in agreement with recent reports, which stated that not all MCL1-inhibiting molecules are able to sensitize leukemic cells to GCs [40]. At the same time, it has been shown that *MCL1* downregulation is accompanied by *BCL2* family of gene downregulation suggesting a common signaling mechanism for both genes and GC-induced apoptosis [41]. In the present study, our observed *MCL1* expression was in agreement with the expected *MCL1* function since we have found that *MCL1* levels decreased with increasing concentration and increasing apoptosis. Interestingly, cells treated with 10 nM Prednisolone manifested the highest levels of expression as compared to the other treatments. At the same time, *MCL1* silencing at 10 nM had the least effect on the gene's levels indicating a competitive phenomenon between Prednisolone and *MCL1* expression.

4.1   Conclusions

To conclude, the current study showed that *GRIM19* and *MCL1* were two genes whose *early* expression was closely related to GC's *late* effect. Also, the fact that the *very early* expression of both genes indicated that cells probably were not inherently resistant, but they adapted to the challenging environment reacting in such a way that probably triggered a resistance mechanism. Finally, our results are a probable hint that *MCL1* and *GRIM19* are possible therapeutic targets for GC-resistant leukemia as suggested also by previous reports.

References

1. Lambrou GI et al (2009) Prednisolone exerts late mitogenic and biphasic effects on resistant acute lymphoblastic leukemia cells: relation to early gene expression. Leuk Res 33(12):1684–1695. https://doi.org/10.1016/j.leukres.2009.04.018
2. Lauten M et al (2001) Clinical outcome of patients with childhood acute lymphoblastic leukaemia and an initial leukaemic blood blast count of less than 1000 per microliter. Klin Padiatr 213(4):169–174
3. Yeh JM et al (2020) Life expectancy of adult survivors of childhood cancer over 3 decades. JAMA Oncol 6(3):350–357. https://doi.org/10.1001/jamaoncol.2019.5582
4. Aisyi M et al (2019) The effect of combination of steroid and L-asparaginase on hyperglycemia in children with acute lymphoblastic leukemia (ALL). APJCP 20(9):2619–2624. https://doi.org/10.31557/apjcp.2019.20.9.2619
5. Kowalczyk JR et al (2019) Long-term treatment results of polish pediatric and adolescent patients enrolled in the ALL IC-BFM 2002 trial. Am J Hematol 94(11):E307–e310. https://doi.org/10.1002/ajh.25619
6. Li C et al (2019) Long-term outcomes of modified BFM-95 regimen in adults with newly diagnosed standard-risk acute lymphoblastic leukemia: a retrospective single-center study. Int J Hematol 110(4):458–465. https://doi.org/10.1007/s12185-019-02703-0
7. Tantiworawit A et al (2019) Outcomes of adult acute lymphoblastic leukemia in the era of pediatric-inspired regimens: a single-center experience. Int J Hematol 110(3):295–305. https://doi.org/10.1007/s12185-019-02678-y
8. Distelhorst CW et al (1987) Neutrophil elastase produces 52-kD and 30-kD glucocorticoid receptor fragments in the cytosol of human leukemia cells. Blood 70(3):860–868
9. Tissing WJ et al (2007) Genomewide identification of prednisolone-responsive genes in acute lymphoblastic leukemia cells. Blood 109(9):3929–3935
10. Tissing WJ et al (2005) Genetic variations in the glucocorticoid receptor gene are not related to glucocorticoid resistance in childhood acute lymphoblastic leukemia. Clin Cancer Res 11(16):6050–6056
11. Mousavian Z et al (2019) Differential network analysis and protein-protein interaction study reveals active protein modules in glucocorticoid resistance for infant acute lymphoblastic leukemia. Mol Med 25(1):36. https://doi.org/10.1186/s10020-019-0106-1
12. Lambrou GI et al (2020) Gene expression and resistance to glucocorticoid-induced apoptosis in acute lymphoblastic leukemia: a brief review and update. Curr Drug Res Rev 12(2):131–149. https://doi.org/10.2174/2589977512666200220122650
13. Ghasemi A et al (2018) Evaluation of BAX and BCL-2 gene expression and apoptosis induction in acute lymphoblastic leukemia cell line CCRFCEM after high- dose prednisolone treatment.

APJCP 19(8):2319–2323. https://doi.org/10.22034/apjcp.2018.19.8.2319

14. Marcopoulou CE et al (2003) Proliferative effect of growth factors TGF-beta1, PDGF-BB and rhBMP-2 on human gingival fibroblasts and periodontal ligament cells. J Int Acad Periodontol 5(3):63–70

15. Rocamora-Reverte L et al (2017) T-cell autonomous death induced by regeneration of inert glucocorticoid metabolites. Cell Death Dis 8(7):e2948. https://doi.org/10.1038/cddis.2017.344

16. McKay LI, Cidlowski JA (2000) CBP (CREB binding protein) integrates NF-kappaB (nuclear factor-kappaB) and glucocorticoid receptor physical interactions and antagonism. Mol Endocrinol 14(8):1222–1234

17. Pruett SB et al (2003) Characterization of glucocorticoid receptor translocation, cytoplasmic IkappaB, nuclear NFkappaB, and activation of NFkappaB in T lymphocytes exposed to stress-inducible concentrations of corticosterone in vivo. Int Immunopharmacol 3(1):1–16

18. Almawi WY, Melemedjian OK (2002) Molecular mechanisms of glucocorticoid antiproliferative effects: antagonism of transcription factor activity by glucocorticoid receptor. J Leukoc Biol 71(1):9–15

19. Magnani M et al (2000) The ubiquitin-dependent proteolytic system and other potential targets for the modulation of nuclear factor-kB (NF-kB). Curr Drug Targets 1(4):387–399

20. Lambrou GI et al (2012) Glucocorticoid and proteasome inhibitor impact on the leukemic lymphoblast: multiple, diverse signals converging on a few key downstream regulators. Mol Cell Endocrinol 351(2):142–151. https://doi.org/10.1016/j.mce.2012.01.003

21. Adamaki M et al (2017) Aberrant AML1 gene expression in the diagnosis of childhood leukemias not characterized by AML1-involved cytogenetic abnormalities. Tumour Biol 39(3):1010428317694308. https://doi.org/10.1177/1010428317694308

22. Adamaki M et al (2013) Implication of IRF4 aberrant gene expression in the acute leukemias of childhood. PLoS One 8(8):e72326. https://doi.org/10.1371/journal.pone.0072326

23. Adamaki M et al (2015) HOXA9 and MEIS1 gene overexpression in the diagnosis of childhood acute leukemias: significant correlation with relapse and overall survival. Leuk Res 39(8):874–882. https://doi.org/10.1016/j.leukres.2015.04.012

24. Uzman BG et al (1966) Morphologic variations in human leukemic lymphoblasts (CCRF-CEM cells) after long-term culture and exposure to chemotherapeutic agents. A study with the electron microscope. Cancer 19(11):1725–1742. https://doi.org/10.1002/1097-0142(196611)19:11<1725::aid-cncr2820191142>3.0.co;2-t

25. Tindall EA et al (2007) Novel Plexor SNP genotyping technology: comparisons with TaqMan and homog-

enous MassEXTEND MALDI-TOF mass spectrometry. Hum Mutat 28(9):922–927

26. Anderer G et al (2000) Polymorphisms within glutathione S-transferase genes and initial response to glucocorticoids in childhood acute lymphoblastic leukaemia. Pharmacogenetics 10(8):715–726

27. Zhang B et al (2005) WebGestalt: an integrated system for exploring gene sets in various biological contexts. Nucleic Acids Res 33(Web Server issue):W741–W748. https://doi.org/10.1093/nar/gki475

28. Wang J et al (2013) WEB-based GEne SeT AnaLysis Toolkit (WebGestalt): update 2013. Nucleic Acids Res 41(W1):W77–W83. https://doi.org/10.1093/nar/gkt439

29. Wang J et al (2017) WebGestalt 2017: a more comprehensive, powerful, flexible and interactive gene set enrichment analysis toolkit. Nucleic Acids Res 45(W1):W130–W137. https://doi.org/10.1093/nar/gkx356

30. Watson JV et al (1987) A pragmatic approach to the analysis of DNA histograms with a definable G1 peak. Cytometry 8(1):1–8

31. Ormerod MG et al (1987) Improved program for the analysis of DNA histograms. Cytometry 8(6):637–641

32. Abrams MT et al (2005) Evaluation of glucocorticoid sensitivity in 697 pre-B acute lymphoblastic leukemia cells after overexpression or silencing of MAP kinase phosphatase-1. J Cancer Res Clin Oncol 131(6):347–354

33. Abrams MT et al (2004) Inhibition of glucocorticoid-induced apoptosis by targeting the major splice variants of BIM mRNA with small interfering RNA and short hairpin RNA. J Biol Chem 279(53):55809–55817

34. Laane E et al (2007) Dexamethasone-induced apoptosis in acute lymphoblastic leukemia involves differential regulation of Bcl-2 family members. Haematologica 92(11):1460–1469

35. Dyczynski M et al (2018) Metabolic reprogramming of acute lymphoblastic leukemia cells in response to glucocorticoid treatment. Cell Death Dis 9(9):846. https://doi.org/10.1038/s41419-018-0625-7

36. Li Z et al (2019) The MCL1-specific inhibitor S63845 acts synergistically with venetoclax/ABT-199 to induce apoptosis in T-cell acute lymphoblastic leukemia cells. Leukemia 33(1):262–266. https://doi.org/10.1038/s41375-018-0201-2

37. Seipel K et al (2019) Rationale for a combination therapy consisting of MCL1- and MEK-inhibitors in acute myeloid leukemia. Cancers 11(11):1179. https://doi.org/10.3390/cancers11111779

38. Inoue C et al (2020) Involvement of MCL1, c-myc, and cyclin D2 protein degradation in ponatinib-induced cytotoxicity against T315I(+) Ph+leukemia cells. Biochem Biophys Res Commun 525(4):1074–1080. https://doi.org/10.1016/j.bbrc.2020.02.165

39. Aries IM et al (2013) The synergism of MCL1 and glycolysis on pediatric acute lymphoblastic leukemia cell survival and Prednisolone resistance.

Haematologica 98(12):1905–1911. https://doi.org/10.3324/haematol.2013.093823

40. Spijkers-Hagelstein JA et al (2014) Glucocorticoid sensitisation in Mixed Lineage Leukaemia-rearranged acute lymphoblastic leukaemia by the pan-BCL-2 family inhibitors gossypol and AT-101. Eur J Cancer 50(9):1665–1674. https://doi.org/10.1016/j.ejca.2014.03.011

41. Zhou F et al (2000) The delayed induction of c-jun in apoptotic human leukemic lymphoblasts is primarily transcriptional. J Steroid Biochem Mol Biol 75(2-3):91–99