Important role of Caspase-8 for chemosensitivity of ALL cells

Harald Ehrhardt¹,², Franziska Wachter¹, Martina Maurer¹, Karsten Stahnke³, Irmela Jeremias¹,⁴

¹ Helmholtz Center Munich – German Research Center for Environmental Health, Marchioninistrasse 25, 81377 Munich, Germany
² Division of Neonatology, University Children's Hospital, Perinatal Center, Ludwig-Maximilians-University Munich, Campus Großhadern, Marchioninistr. 15, Munich D-81377, Germany
³ University Children's Hospital, Eythstr. 24, D-89075 Ulm, Germany
⁴ Department of Oncology / Hematology, Dr. von Haunersches Kinderspital, Lindwurmstr 4, 80337 München, Germany

running title: Role of Caspase-8 for chemosensitivity in ALL

key words: Caspase-8, p53, cytotoxic drugs, cell death, leukemia, MTX

financial support: This work was supported by Else Kroener Fresenius Stiftung, FöFoLe #19-2005 (both to HE and IJ) and Dr. Helmut Legerlotz Stiftung (to IJ).

Total word count: 4744
Total figure count: 5

* to whom correspondence should be send:
Irmela Jeremias
Helmholtz Center Munich – German Research Center for Environmental Health
Department of Gene Vectors
Marchioninistrasse 25
D-81377 München
Irmela.Jeremias@helmholtz-muenchen.de
Abstract

**Purpose:** Sensitivity of tumor cells towards chemotherapy mainly determines the prognosis of patients suffering from acute lymphoblastic leukemia (ALL); nevertheless, underlying mechanisms regulating chemo-sensitivity remain poorly understood. Here, we aimed at characterizing the role of Caspase-8 for chemo-sensitivity of B- and T-ALL cells.

**Experimental Design:** Primary tumor cells from children with ALL were evaluated for expression levels of the Caspase-8 protein, were amplified in NSG-mice, transfected with siRNA and evaluated for their chemo-sensitivity in vitro.

**Results:** Effective cell death in B- and T-ALL cells depended on the presence of Caspase-8 for the majority of cytotoxic drugs routinely used in anti-leukemia treatment. Caspase-8 was activated independently from extrinsic apoptosis signaling. Accordingly in primary ALL cells, the expression level of Caspase-8 protein correlated with cell death sensitivity towards defined cytotoxic drugs in vitro. In the subgroup of primary ALL cells with low expression of Caspase-8, methotrexate upregulated the expression of Caspase-8 mediated by the transcription factor p53 suggesting epigenetic silencing of Caspase-8. RNA interference in patient-derived B- and T-ALL cells revealed that effective cell death induction by most routine drug combinations involving methotrexate depended on the presence of Caspase-8.

**Conclusion:** Our results indicate that Caspase-8 is crucial for the high anti-leukemic efficiency of numerous routine cytotoxic drugs. Re-expression of epigenetically downregulated Caspase-8 represents a promising approach to increase efficiency of anti-leukemic therapy.

**Translational relevance:**
Our data represent the basis to select targeted drug combinations for future treatment trials of ALL: We describe that the pro-apoptotic intracellular signaling protein Caspase-8 is required for anti-leukemia efficiency of a high number of cytotoxic drugs including asparaginase, cyclophosphamide, dexamethasone and doxorubicin.

Epigenetic silencing of pro-apoptotic proteins represents a central regulatory mechanism how tumor cells escape chemo-sensitivity, and we found epigenetic silencing of Caspase-8 expression in a number of primary ALL samples. Expression
of Caspase-8 should be evaluated as a potential biomarker for risk stratification in ALL. Methotrexate induces re-expression of epigenetically downregulated Caspase-8. Therefore, targeted drug combinations can consist of methotrexate together with drugs depending on the expression of Caspase-8. Retrospectively, our data might explain on a molecular level, why clinical empirical studies already revealed a high anti-leukemic efficiency for some of these drug combinations over decades.
Introduction

Downregulation of pro-apoptotic signaling proteins represents an important mechanism, how tumor cells evade anti-cancer treatment. As an example, Caspase-8 is frequently downregulated in tumor cells of different origins, mostly due to epigenetic gene silencing by promoter hypermethylation\textsuperscript{1-3}. De-methylating agents like azacytidine induce re-expression of Caspase-8 and sensitize for Caspase-8 dependent apoptosis\textsuperscript{1,2}. Loss or dysfunction of Caspase-8 result in increased cellular transformation, enhanced tumor progression, poor response to chemotherapy and impaired prognosis of patients of different cancer entities\textsuperscript{4-8}.

Several attempts were undertaken to upregulate the expression of Caspase-8 in tumor cells. The Caspase-8 promoter contains binding sites for NF\(\kappa\)B, SP-1, IRF-1 and p53, and efficient upregulation of Caspase-8 was obtained upon stimulation of the STAT-1/IRF-1 pathway\textsuperscript{2}. For epigenetically downregulated Caspase-8, azacytidine and IFN-\(\gamma\) were effective\textsuperscript{9,10}. In addition to azacytidine, we have recently shown that epigenetically downregulated Caspase-8 can be upregulated by routinely used cytotoxic drugs like methotrexate (MTX) via the transcription factor p53\textsuperscript{11}.

Caspase-8 is mainly known for its important role within the extrinsic apoptosis pathway, where it signals from proximal death receptor activation to activation of caspases or mitochondria\textsuperscript{12,13}. In contrast, cytotoxic drugs mainly signal via the intrinsic apoptosis signaling pathway where activated mitochondria release apoptogenic factors like Cytochrome c for cleavage of downstream effector caspases\textsuperscript{14,15}. The role of Caspase-8 for intrinsic apoptosis signaling has been controversially discussed\textsuperscript{1,16-19}: Recent data provided evidence for an downstream amplifier function of Caspase-8 during drug-induced cell death in some, but not all tumor types\textsuperscript{20,21}. For acute lymphoblastic leukemia (ALL), the role of Caspase-8 remains poorly defined. Interestingly, a recent paper showed that in patients with childhood ALL, high mRNA levels of Caspase-8 were predictive for a good initial response to polychemotherapy\textsuperscript{8}.

We aimed to characterize the functional relevance of Caspase-8 for cytotoxic drug-induced apoptosis in patient-derived childhood ALL cells. Using molecular studies, we found a surprisingly important function of Caspase-8 for chemo-sensitivity of ALL cells.
Materials and Methods

Materials
TRAIL from Pepro (Peprotech, Hamburg, Germany) without his-tag was used exclusively for all experiments. Anti-apo1, Fas receptor blocking antibody, DR4 and DR5 Fc fragments and zIETD were obtained by Alexis Corp. (Grünberg, Germany), methotrexate from Calbiochem (San Diego, CA), 4-hydroperoxy-cyclophosphamide from Baxter oncology GmbH (Frankfurt, Germany). All further reagents from Sigma (Deisenhofen, Germany). For Western Blot, the following antibodies were used: anti-GAPDH from Thermo Fisher (Waltham, MA), anti-p53 and anti-Lamin A/C from Santa Cruz (Santa Cruz, CA) and anti-Caspase-8 from Alexis Corp.

Cell lines, primary samples and amplification in NOD/SCID mice, cell death assays
All parental cell lines were obtained from DSMZ (Braunschweig, Germany), CEM-TR and J-TR cells had been described previously. After thawing, parental cells were amplified and numerous aliquots were frozen within 1 month after thawing of original cells. Each frozen aliquot was than re-thawn and used for a maximum of three months after thawing. Derivative cells stably transfected with shRNA against Caspase-8 or p53 have been published before and cells were maintained as described there.

For all cell line experiments, cells were seeded as described before and incubated with cytotoxic drugs for 48 hours. For combined stimulation, cells were incubated with MTX for 48 hours followed by the second stimulant for additional 48 hours. Cell death was measured by forward side scatter analysis using FACscan (Becton Dickinson, Heidelberg, Germany). For biochemical inhibition of Caspase-8 or death receptor blockade, cell lines were pretreated for 6 hours before stimulation. Primary leukemia blasts were investigated from n=29 children treated for acute leukemia at the Ludwig Maximilians University's children's hospital and the children's hospital of the TU Munich during 2005 and 2008. All experiments were approved by the local ethics boards according to the declaration of Helsinki. Samples were obtained, isolated and seeded as described. Cells were stimulated simultaneously with methotrexate (30 µM) and TRAIL (1µg/ml) or cytotoxic drugs at peak plasma concentration as described previously. TRAIL resistance was defined as specific...
cell death by TRAIL < 10 %. For molecular studies, primary cells were xenografted in NOD/SCID mice and amplified before molecular modulation.

**Western Blot analysis**

Western Blot analysis of total cellular protein was performed for all cell line experiments as described\(^\text{16}\). For investigation of primary samples, Icemans lysis buffer (Tris HCl 10 mM pH 7.5, NaCl 50 mM, Triton X-100 0.5 %, desoxycholic acid 0.5 %, sodium duodecyl sulfate 0.5 %) was used. For quantification of Caspase-8 expression in primary samples, AIDA Image Analyzer was applied.

**Transfection experiments**

In addition to formerly described stably transfected cell lines, parental CEM and JURKAT cells were stably transfected with previously specified vectors expressing shRNA against Caspase-8 or a mock sequence using the Amaxa Cell Line Nucleofactor kit V (Lonza, Basel, Switzerland) according to the manufacturers’ instructions\(^\text{11}\). As alternative shRNA target for Caspase-8 the following sequence was studied: 5´-TCTATTAATTCCGAAGAGC-3´. Primary samples were transfected using the identical transfection technique as for the cell lines and the following siRNAs at a concentration of 20µM: silencer validated siRNA-p53 (5´-GGGUUAGUUUACAAUCAGC-3´, Ambion, Austin, TX), siRNA-Caspase-8 (5´-GCUCUCCGAUUAAUAGATT-3´) and as control sequence siRNA-Lamin (5´-ACUGCAGCAUGUAUAACTT-3´, both from Eurofins MWG Operon, Ebersberg, Germany). Primary cells were resuspended in RPMI medium supplemented with 20% FCS, 1% penicillin/streptomycin, 1% gentamycin, 6µl/ml mixture of insulin, transferrin and selenium (Invitrogen, Carlsbad, CA), 1mM sodium pyruvate and 50µM 1-thioglycerol. 12 hours after transfection, cells were simultaneously stimulated with MTX and cytotoxic drugs for 48 hours. Efficacy of inhibition of Caspase-8 upregulation by siRNA interference after MTX was demonstrated by Western Blot analysis at the end of total incubation time for each experimental setting.

**Statistical analysis**

**Normalized isobolograms were generated using CompuSyn software.** For cell line experiments, data are presented as mean values of at least three independent
experiments ± SEM if not stated differently. To test for statistically significant differences, one way RM ANOVA was applied. Primary samples were evaluated using one way RM ANOVA on ranks or Spearman’s rank order correlation test. Statistical significance was accepted with p-values <0.05.
Results

Caspase-8 plays a pivotal role for apoptosis induction via the extrinsic apoptosis signaling cascade, while its role for intrinsic apoptosis signaling induced by cytotoxic drugs appears tumor type specific\textsuperscript{20,21}. Here, we characterized the role of Caspase-8 for cell death induction by cytotoxic drugs in B- and T-ALL cells.

Cytotoxic drug-induced cell death depends on Caspase-8

The T-cell ALL cell lines CEM and JURKAT showed abundant expression of Caspase-8 and certain sensitivity towards cell death induction by numerous cytotoxic drugs of current anti-leukemia treatment protocols, except dexamethasone. Upon induction of cell death, all cytotoxic drugs induced activation of p53 (Supplementary Figure 1A) and cleavage of Caspase-8 (Supplementary Figure 1B).

To study the causative role of Caspase-8 for mediating cell death induction by cytotoxic drugs, we compared parental T-ALL CEM and JURKAT cells with several derivative cell lines. In the derivative cell lines, expression of Caspase-8 was downregulated either by stable expression of a small hairpin RNA directed against Caspase-8 or by epigenetic silencing of the Caspase-8 promoter\textsuperscript{11,16,23}. Caspase-8 low-expressing ALL cell lines are known to be resistant against cell death induction by TRAIL and CD95L\textsuperscript{11} and partially resistant towards daunorubicin and camptothecin-induced cell death\textsuperscript{21}. In addition to these known agents, downregulation of Caspase-8 disabled effective cell death induction by the majority of routinely used cytotoxic drugs including asparaginase, cyclophosphamide, doxorubicin, 6-thioguanine and vincristine (Figure 1A, Supplementary Figure 1C and D). In this experiment, CEM-TR cells were more resistant towards drug-induced apoptosis compared to CEM cells stably expressing the shRNA against caspase-8 eventually due to the incomplete knockdown in the latter as observed in Western Blot. In addition to T-ALL cell lines, the B-ALL lines BJAB, SEM and REH were tested and all showed certain dependency on Caspase-8 for drug-induced apoptosis, although to a minor extent than JURKAT and CEM cells (Figure 1B, Supplementary Figure 1E and data not shown). Unfortunately, corticoids could not be studied in this setting, due to complete apoptosis resistance (data not shown). Thus, the majority of cytotoxic drugs used in the treatment of ALL depended on the presence of Caspase-8 for effective cell death induction.
In line with these results, biochemical inhibition of Caspase-8 activation by the Caspase-8 directed inhibitor zIETD or a second independent shRNA against Caspase-8 inhibited cell death induction by the same drugs (Supplementary Figure 1F and G and data not shown). Upon downregulation of Caspase-8, high concentrations of drugs like doxorubicin were required for efficient cell death induction, although these concentrations are measured only in some patients and for short periods of time, while clinically more relevant concentrations were less effective in cells with downregulated Caspase-8 (Figure 1C and data not shown). Thus, Caspase-8 represents an important intracellular signaling mediator for a high number of cytotoxic drugs used in routine anti-leukemia treatment.

**Caspase-8 is downregulated in a subset of primary ALL cells**

As we found an important role of Caspase-8 for drug induced cell death in ALL cell lines, we next investigated the Caspase-8 expression levels in primary ALL cells. In many solid tumor entities like neuroectodermal tumors, downregulation of Caspase-8 is a frequent event in patients’ tumors associated with drug resistance and poor prognosis. In line, high expression levels of Caspase-8 mRNA were prognostically significant for favorable response to initial chemotherapy in childhood ALL. To further clarify the role of Caspase-8 for efficient apoptosis induction, we determined the expression level in 29 primary childhood ALL samples derived from bone marrow aspiration before onset of therapy using Western Blot analysis standardized to GAPDH (Supplementary Figure 2). 24 / 29 (83%) samples showed expression levels of Caspase-8 lower than that in JURKAT cells with apparently undetectable expression of Caspase-8 in 6 / 29 (21%) of samples. Clinical parameters were available for 4 out of the 6 patients with ALL cells showing apparently absent expression of Caspase-8. Interestingly, all 4 patients had enhanced clinical risk factors, 3/4 showing high risk criteria at primary disease and one patient with ALL relapse. These 6 samples showed complete resistance to extrinsic cell death signaling, e.g. by the death inducing ligand TRAIL (data not shown).

In addition to the determination of Caspase-8 protein levels, primary ALL cells were screened for in vitro cell death sensitivity against a subset of cytotoxic drugs. Despite of low sample numbers, expression levels of Caspase-8 significantly correlated to in vitro cell death induction by doxorubicin and dexamethasone (Figure 2A and B) and
to some extent to 6-thioguanin (p = 0.087). In contrast, cell death induction by cytarabine and MTX which showed Caspase-8 independent cell death in cell line experiments (Figure 1) were completely independent from Caspase-8 levels (p = 0.96 for cytarabine and p = 0.58 for MTX).

The data show that downregulation of Caspase-8 protein is not restricted to solid tumor cells, but also occurs in a relevant fraction of ALL samples. Downregulation of Caspase-8 leads to apoptosis resistance of primary ALL cells towards defined routinely used cytotoxic drugs in vitro.

**Cytotoxic drugs induce cell death independently from death receptor signaling**

During cell death signaling, Caspase-8 can be activated by two different mechanisms: Upon extrinsic apoptosis signaling, Caspase-8 becomes activated in the proximal cell death signaling pathway at the death receptors. Within the intrinsic apoptosis signaling pathway, Caspase-8 is activated downstream of mitochondria by executioner Caspases to fulfill an amplifier function. During drug-induced cell death, the activation of the death receptor system has been controversially discussed.

To discriminate, whether Caspase-8 becomes activated by the extrinsic or the intrinsic apoptosis signaling pathway upon stimulation by cytotoxic drugs in ALL cells, death receptors were blocked by inhibitory antibodies. These antibodies were able to completely block cell death induction by death inducing ligands (Figure 3). Nevertheless, death receptor blockade did not alter cell death induction by cytotoxic drugs (Figure 3 and Supplementary Figure 3A and B) arguing towards a death-receptor independent activation of Caspase-8 in our model of CEM and JURKAT T-cell leukemia cells.

**MTX upregulates epigenetically downregulated Caspase-8**

So far, we have shown that Caspase-8 plays an important role for cell death induction by a number of cytotoxic drugs in ALL cells (Figure 1 and 2). Surprisingly, a relevant subset of primary ALL cells expressed only low amounts of Caspase-8 protein followed by reduced in vitro sensitivity for cytotoxic drugs (Figure 2). As patients carrying these tumor cells might be of high risk for poor response to initial polychemotherapy, they might benefit from strategies restoring the expression of Caspase-8 protein. Therefore, we next asked, whether apoptosis resistance against
cytotoxic drugs that depend on functional Caspase-8 could be overcome by upregulation of epigenetically downregulated Caspase-8. As a first model, we used our derivative CEM and JURKAT cell lines with low expression of Caspase-8 which were generated by weekly incubation of JURKAT and CEM cells with increasing sublethal doses of TRAIL over 30 weeks. These cells upregulated expression of Caspase-8 by stimulation with the demethylating agent azacytidine¹¹,¹⁶. We had shown previously that MTX upregulated the expression of Caspase-8 protein by a mechanism dependent on the transcription factor p53 and thereby sensitized these resistant ALL cells towards TRAIL-induced apoptosis¹¹ (Figure 4C and D, Supplementary Figure 4C and D and data not shown). Here, we investigated whether further cytotoxic drugs were able to upregulate the expression of Caspase-8. None of the drugs tested upregulated expression of Caspase-8 except MTX (Supplementary Figure 4A).

Upregulation of Caspase-8 enables cell death induction by cytotoxic drugs
When the treatment of MTX was combined with doxorubicin, asparaginase or cyclophosphamide, highly synergistic apoptosis induction was detected using normalized isobolograms (Figure 4A and B, Supplementary Figure 4B and data not shown). Interestingly, MTX-mediated upregulation of Caspase-8 enabled significant cell death induction by the Caspase-8 dependent drugs tested (Figure 4C and D and Supplementary Figure 4C and D). To prove the role of Caspase-8 for this cell death, MTX-induced upregulation of Caspase-8 was inhibited by shRNA directed against either Caspase-8 or p53. If upregulation of Caspase-8 was inhibited on a molecular level, MTX was no longer able to sensitize ALL cells towards apoptosis induction by the Caspase-8 dependent drugs (Figure 4C and D and Supplementary Figure 4C and D). These data show that upregulation of epigenetically silenced Caspase-8, e.g., by MTX overcomes apoptosis resistance of ALL cells against cytotoxic drugs with Caspase-8 dependent signaling such as asparaginase, cyclophosphamide and doxorubicin.
In patient-derived ALL cells, upregulation of Caspase-8 sensitzes for drug-induced apoptosis

ALL cell lines frequently show mutations in p53, although primary ALL cells rarely do so. To avoid working on cell line artefacts and to approximate the clinical situation, we investigated upregulation of Caspase-8 in our primary ALL samples with low expression of Caspase-8. We used MTX for upregulation of Caspase-8 and the 6 samples from Figure 2 with lowest expression of Caspase-8. MTX induced marked upregulation of Caspase-8 in all 6 primary ALL samples (Figure 5A).

We next aimed to prove a causative role of p53-mediated regulation of Caspase-8 to enable apoptosis induction by Caspase-8-dependent drugs by MTX in primary ALL cells. Unfortunately, the small sample volumes of these cells disabled performing molecular modulations in primary samples. To remain as close as possible to the characteristics of primary ALL cells, we amplified them in mice using the xenograft NOD/SCID mouse model. For molecular experiments, we used our recently established technique allowing electroporation-based transient transfection of siRNA into patient-derived ALL cells freshly isolated from mice. In contrast to CEM and JURKAT cells, these cells were not completely resistant against corticoid-induced apoptosis enabling additional studies on dexamethasone-induced apoptosis.

To study the impact of p53-mediated upregulation of Caspase-8 on a molecular level, mouse-amplified ALL cells from two different patients with low constitutive expression of Caspase-8 (#6, T-ALL, initial diagnosis, female, 7 years and #4, pre-B-ALL, initial diagnosis, female, 5 years) from Figure 5A were transfected with siRNA against Caspase-8 or p53; this transfection prevented upregulation of Caspase-8 upon stimulation with MTX (Figure 5B and C). On a functional level, transient transfection with siRNA against Caspase-8 or p53 diminished or completely prevented that MTX increased apoptosis by drugs dependent on Caspase-8 such as TRAIL, asparaginase, cyclophosphamide, dexamethasone or doxorubicin (Figure 5B and C).

Interestingly, dexamethasone-induced cell death also depended on expression levels of Caspase-8 adding dexamethasone to the list of Caspase-8 dependent routine drugs of ALL.

Taken together, Caspase-8 represents an important mediator for apoptosis induction by most, but not all drugs of current treatment of ALL. MTX upregulates expression of Caspase-8 via p53 and thereby sensitizes for cell death induction by Caspase-8 dependent drugs (Figure 5D). Regulation of...
Caspase-8 *might represent a possible explanation*, why Caspase-8 dependent drugs are particularly effective when combined with MTX as realized in clinical treatment protocols of ALL since decades.
Discussion

The molecular studies unravel a surprisingly important function of Caspase-8 during apoptosis induction by cytotoxic drugs in these cells. The relevance of Caspase-8 for effective cell death induction was clarified for two different situations: 1. In B- and T-ALL cell lines and primary B- and T-ALL cells with basic expression of Caspase-8, efficiency of cell death induction by the majority of routine cytotoxic drugs depended on Caspase-8. 2. In the subset of ALL cells with downregulated Caspase-8 due to epigenetic regulatory mechanisms, MTX was able to upregulate Caspase-8. Here, the efficiency of defined drug combinations involving MTX was coupled to the re-expression of Caspase-8.

The role of Caspase-8 for chemosensitivity was shown in (i) primary tumor cells from children with ALL; (ii) primary ALL cells after amplification in NOD/SCID mice and (iii) ALL cell lines. Using modern molecular technologies, we confirmed a causative role of Caspase-8 on patient-derived ALL cells and provided the molecular proof for the clinically observed association between polychemotherapy efficiency and Caspase-8 expression.

While a role of Caspase-8 was known only for daunorubicin and camptothecin-induced cell death so far, our data broadly widen the number of clinically used cytotoxic drugs which depend on Caspase-8 for induction of apoptosis. The downstream amplifier function of Caspase-8 in the intrinsic apoptosis signaling pathway is thus highly important for chemotherapy in ALL cells, in contrast to, e.g., Ewing sarcoma cells. Our data represent the first molecular studies on patient-derived ALL cells on the role of Caspase-8 for apoptosis induction by routine cytotoxic drugs.

From the 9 drugs studied, 6 drugs depended on Caspase-8 for induction of apoptosis. For yet unknown mechanisms, cytarabine, etoposide and MTX activated Caspase-8, but induced apoptosis independently from Caspase-8 (Figure 5D). Routinely, cytotoxic drugs are classified regarding their main mechanism of action. While, e.g., the topoisomerase inhibitor doxorubicin induced Caspase-8-dependent cell death, the topoisomerase inhibitor etoposide did not. No correlation was detected between the mechanism of action of the drugs used and their dependence on Caspase-8 for induction of...
apoptosis. Additional studies are required to clarify how dependency of Caspase-8 is related to the classifying mechanism of action of cytotoxic drugs. Since decades, MTX is successfully used in combination with nearly all drugs used in polychemotherapy protocols of ALL\textsuperscript{29-31}. Our data show that a possible explanation for the favorable effects of some of these drug combinations might rely on regulation of Caspase-8. In addition, MTX sensitized for apoptosis induction by drugs signaling Caspase-8 independent cell death. MTX sensitized ALL-cells for cytarabine and etopside by a Caspase-8 and p53 independent mechanism (data not shown). To our surprise, we were not able to identify a specific group of p53 regulators able to restore Caspase-8 within our experimental setting, but MTX was the only drug besides the formerly described 5-FU\textsuperscript{11,32}.

More recently, it has become increasingly clear that anti-tumor therapy has to target epigenetic tumor alterations\textsuperscript{33,34}. Caspase-8 is one of the frequently downregulated proteins due to epigenetic silencing\textsuperscript{1,3,10}. We can show here that MTX that is part of anti-leukemia protocols for decades, is able to upregulate epigenetically downregulated Caspase-8 and thereby sensitizes tumor cells towards drug-induced apoptosis. Thus and without awareness of the underlying mechanisms, current polychemotherapy protocols obviously target epigenetic tumor alterations. The old well known MTX is identified here as acting like a modern, epigenetic drug.

On a more general level, our work encourages the future search for targeted drug combinations which are based on the molecular understanding of the underlying signaling mechanism. Cytotoxic drugs that regulate Caspase-8 should be combined with drugs that depend on the expression of Caspase-8. These drug combinations inherit the potential of higher anti-tumor efficiency compared to empirically determined drug combinations. This approach is in line with current concepts of targeted therapies and the re-evaluation of drug combinations\textsuperscript{35-37}. The current search of targeted therapeutics should be accompanied by a search for targeted drug combinations.
Acknowledgement

The skilled technical work of L. Mura is kindly appreciated. We thank R. Besch for providing the second Caspase-8 target sequence, the animal facility for caring for the mice and K. Schneider for providing patient-derived xenograft leukemia cells.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.
Grant Support

This work was supported by Else Kroener Fresenius Stiftung, FöFoLe #19-2005 (both to HE and IJ) and Dr. Helmut Legerlotz Stiftung (to IJ).
References


10. Ruiz-Ruiz C, Ruiz de Almodovar C, Rodriguez A, Ortiz-Ferron G, Redondo JM, Lopez-Rivas A. The up-regulation of human caspase-8 by interferon-


Titles and legends to figures

Figure 1  Enhanced cell death induction by cytotoxic drugs in the presence of Caspase-8 in leukemia cell lines
A) Parental CEM cells were stably transfected with shRNA against Casp-8 (shCasp-8) or a mock sequence (mock) and Western blot analysis was performed of total cellular protein including derivate CEM-TR cells with permanent downregulation of Caspase-8 due to epigenetic regulatory mechanisms. Cells were stimulated for 48 hours with death inducing ligand TRAIL (300 ng/ml) together with Apo-1 (1 µg/ml) and protein A (5 ng/ml) (DIL), asparaginase (Asp, 1 U/ml), 4-hydroperoxycyclophosphamide (Cyclo, 0.3 µM), doxorubicin (Doxo, 100 ng/ml) or etoposide (VP-16, 0.3 µM) for 48 hours. *comparing parental or mock transfected cells with cells transfected with shCasp-8 or TR cells.

B) BJAB cells stably transfected with shRNA against Casp-8 (shCasp-8) or a mock sequence (mock) were stimulated with DIL, Asp, Cyclo, Doxo and vincristine (VCR) and analyzed as in Figure 1A and Supplementary Figure 1D. Doxo and VCR were applied at 30ng/ml.

C) CEM cells from Figure 1A were stimulated with Doxo for 48 hours as indicated. Cell death was measured using forward side scatter analysis. Data are presented as mean ± SEM of at least three independent experiments. *p<0.05, ANOVA, comparing parental and mock transfected cells with cells transfected with shCasp-8 or TR cells.

Figure 2  Downregulation of Caspase-8 in primary childhood acute leukemia cells
A,B) n=29 primary childhood acute leukemia samples were evaluated for Caspase-8 expression on protein level by Western blot analysis. Caspase-8 expression was standardized to GAPDH and is depicted as relative expression as shown in detail in Supplementary Figure 2. Relative Caspase-8 expression was correlated to cell death induction by Doxo (A; 0.5µM; p=0.0085) or dexamethasone (Dexa; B; 10-5M; p=0.0465). Statistical analysis was performed using Spearman’s rank order correlation test. r=correlation coefficient.
Figure 3 Activation of Caspase-8 during drug-induced apoptosis independent of death receptors

Parental CEM cells were pretreated with Fas blocking antibody (1 µg/ml) and DR4 and DR5 Fc fragments (1 µg/ml each) (R-block) for 6 hours followed by stimulation with cytotoxic drugs as in Figure 1A.

Apoptosis measurement, presentation and statistical analysis were performed as in Figure 1.

Figure 4 MTX-mediated sensitization for drug-induced cell death by p53 dependent regulation of Caspase-8 in leukemia cells

A,B) CEM-TR cells were stimulated with MTX (10, 30, 100, 300nM) for 48 hours followed by Doxo (10, 30, 100 and 300 ng/ml; A) or cyclo (0,1, 0,3, 0,6 and 1µM; B) for another 48 hours. The effect of the combined stimulation is presented using normalized isobolograms.

C,D) CEM-TR cells stably transfected with shRNA against Caspase-8 (shCasp-8; C) or with shRNA against p53 (shp53; D), a mock sequence (mock) or left untreated (co) were pretreated with MTX (6 nM) for 48 hours followed by incubation with death inducing ligand TRAIL (300 ng/ml) together with Apo-1 (1 µg/ml) and protein A (5 ng/ml) (DIL), Asp (1 U/ml), Cyclo (0,3 µM) or Doxo (100 ng/ml) for additional 48 hours. In parallel, Western Blot analysis was performed of unstimulated and MTX treated cells. For ease of reading, apoptosis induction by single agents is presented only for parental cells, but did not differ from cell death induced in derivative cells.

Cell death measurement, statistical analysis, presentation of data and Western Blot were performed as in Figure 1.

Figure 5 Augmented cell death induction by cytotoxic drugs in the presence of Caspase-8 in patient-derived acute leukemia cells

A) The 6 primary samples with downregulated Caspase-8 from Figure 2 were left untreated or stimulated with MTX (30µM) for 48 hours and analyzed as in Figure 2.

B) Primary cells from sample #6 (T-ALL, initial diagnosis, female, 7 years) from Figure 5A with downregulated Caspase-8 were amplified in NOD/SCID mice.
and transfected with a non-specific sequence (mock) or siRNA against Caspase-8 (Casp-8) or p53. After 12 hours, cells were simultaneously stimulated with MTX (30µM) and TRAIL (1µg/ml), Asp (0.3 U/ml), Cyclo (0.3 µM), Dexta (10⁻⁹ M) or Doxo (0.05 µM) for 48 hours. In parallel, Western Blot analysis was performed of cells incubated with MTX for 48 hours. Depicted are mean values from n=6 independent experiments. Statistical analysis was performed comparing cell death induction in samples transfected with a mock sequence or siRNA against Casp-8 or p53 for each stimulatory setting, p<0.05, one way RM ANOVA on ranks. NS=not significant

C) Primary cells from sample #4 (pre-B-ALL initial diagnosis, female, 5 years) from Figure 5A with downregulated Caspase-8 were obtained, transfected, stimulated and analyzed as in Figure 5B. Depicted are mean values of n=9 independent experiments.

D) Cytotoxic drugs investigated were classified according to their ability to activate p53, to regulate the expression of Caspase-8 and their dependency of efficient cell death induction on the presence of Caspase-8.

Cell death measurement and Western Blot were performed as in Figure 1 and presentation of data as in Figure 4 if not stated differently.
Figure 1

**C**

- ○ parental
- ○ mock
- ● shCasp-8
- ● CEM-TR

**Specific apoptosis (%)**

**Doxo (ng/ml)**

- 0 10 20 30 40 50 60 80 100 200 300
Figure 2

A

Specific apoptosis (%) by Doxo

Fold expression of Caspase-8 protein

B

Specific apoptosis (%) by Dexa

Fold expression of Caspase-8 protein

Fold expression of Caspase-8 protein

Fold expression of Caspase-8 protein

Fold expression of Caspase-8 protein

p = 0.0085

p = 0.0465
Figure 4
**Figure 4**

**C**

MTX & Caspase-8 & GAPDH

<table>
<thead>
<tr>
<th></th>
<th>parental</th>
<th>mock</th>
<th>shCasp-8</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTX</td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Caspase-8</td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>GAPDH</td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

- **co**
- **mock**
- **shCasp-8**

**specific apoptosis (%)**

MTX & DIL & Asp & Cyclo & Doxo

<table>
<thead>
<tr>
<th>MTX</th>
<th>DIL</th>
<th>Asp</th>
<th>Cyclo</th>
<th>Doxo</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**D**

MTX & Caspase-8 & GAPDH

<table>
<thead>
<tr>
<th></th>
<th>parental</th>
<th>mock</th>
<th>shp53</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTX</td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Caspase-8</td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>GAPDH</td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

- **co**
- **mock**
- **shp53**

**specific apoptosis (%)**

MTX & DIL & Asp & Cyclo & Doxo

<table>
<thead>
<tr>
<th>MTX</th>
<th>DIL</th>
<th>Asp</th>
<th>Cyclo</th>
<th>Doxo</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**A**

![Graph showing fold expression of Caspase-8 protein](image)

<table>
<thead>
<tr>
<th>Patient #</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>mock</td>
<td>co</td>
<td>co</td>
<td>co</td>
<td>MTX</td>
<td>MTX</td>
<td>MTX</td>
</tr>
<tr>
<td>siCasp-8</td>
<td>MTX</td>
<td>MTX</td>
<td>MTX</td>
<td>MTX</td>
<td>MTX</td>
<td>MTX</td>
</tr>
<tr>
<td>sip53</td>
<td>co</td>
<td>co</td>
<td>MTX</td>
<td>MTX</td>
<td>MTX</td>
<td>MTX</td>
</tr>
</tbody>
</table>

**B**

![Graph showing specific apoptosis](image)

- MTX: +
- DIL: +
- Asp: +
- Cyclo: +
- Dexa: +
- Doxo: +

**Figure 5**
Figure 5

Caspase-8 and p53, intrinsic apoptosis signaling

Dil, Asp, Cyclo, Dexamethasone, Doxorubicin

MTX, Ara-C, VP-16

p53, transcriptional regulation

Cell death
Important role of Caspase-8 for chemo-sensitivity of ALL cells
Harald Ehrhardt, Franziska Wachter, Martina Maurer, et al.

Clin Cancer Res  Published OnlineFirst October 18, 2011.

Updated version  Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-11-0513

Supplementary Material  Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2011/10/18/1078-0432.CCR-11-0513.DC1

Author Manuscript  Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.