Resistance to different classes of drugs is associated with impaired apoptosis in childhood acute lymphoblastic leukaemia.

Running title: Decreased apoptosis in drug-resistant ALL patients.

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Abstract

Resistance of leukaemic cells to chemotherapeutic agents is associated with an unfavourable outcome in paediatric ALL. To investigate underlying mechanisms of cellular drug resistance, the activation of various apoptotic parameters in leukemic cells from 50 children with ALL was studied after in vitro exposure with 4 important drugs in ALL therapy (prednisolone, vincristine, L-asparaginase and daunorubicin). Exposure to each drug resulted in early induction of phosphatidylserine (PS) externalisation and mitochondrial transmembrane (ΔΨm) depolarisation followed by caspase-3 activation and PARP inactivation in the majority of patients. For all four drugs, a significant inverse correlation was found between cellular drug resistance and (1) the percentage of cells with PS externalisation (<0.001<P<0.008) and (2) the percentage of cells with ΔΨm depolarisation (0.002<P<0.02). However, the percentage of cells with caspase-3 activation and the percentage of cells with PARP inactivation showed a significant inverse correlation with cellular resistance for prednisolone (P=0.001; P=0.001) and L-asparaginase (P=0.01; P=0.001) only. This suggests that caspase-3 activation and PARP inactivation are not essential for vincristine and daunorubicin-induced apoptosis. In conclusion, resistance to 4 unrelated drugs is associated with defect(s) upstream or at the level of PS externalisation and ΔΨm depolarisation. This leads to decreased activation of apoptotic parameters in resistant cases of paediatric ALL.

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**Introduction**

Although combination chemotherapy has improved the prognosis of childhood ALL over the last few decades, relapse still occurs in 20 to 30% of the cases. Cellular drug resistance measured at initial diagnosis is associated with an increased relapse risk and unfavourable clinical outcome in childhood ALL. In addition, the presence of adverse clinical prognostic factors such as older age (>10 years) and pro-B and T-lineage immunophenotype have been shown to be associated with cellular resistance to drugs in children with ALL. These findings indicate that cellular drug resistance (measured *in vitro*) can be used as tool to identify patients at higher risk of treatment failure.

Chemotherapeutic agents have been described to induce apoptosis in malignant cells. There are two major routes by which apoptosis can be induced: the extrinsic or death receptor-associated route and the intrinsic or mitochondrial route. Although there is disagreement concerning the role of the extrinsic route in chemotherapy-induced apoptosis, there is a general agreement regarding the importance of the intrinsic route. The intrinsic route can be subdivided into three general phases; (1) insult generation, (2) signal transduction and (3) execution. During the insult generation-phase, chemotherapeutic agents interact with and cause damage to their specific cellular targets. The signal transduction phase is the least-defined phase and is thought to involve integration of pro- and anti-apoptotic signals. The relative abundances of pro- and anti-apoptotic signals, that can be influenced by anticancer drugs, ultimately determines if the execution phase is initiated. The execution phase is initiated by release of cytochrome c and other polypeptides from the mitochondrial intermembrane space. This release is accompanied by a dissipation of mitochondrial inner transmembrane potential ($\Delta Y_m$). Once released in the cytoplasm, cytochrome c interacts with Apaf-1 (apoptotic protease-activating factor-1), ATP/dATP and procaspase-9 to form a complex known as the apoptosome. In the apoptosome, caspase-9 is activated which in turn activates effector caspases, like procaspase-3 and –7. The effector caspases cleave a number of structural and regulatory cellular proteins (e.g., poly(ADP-ribose) polymerase (PARP), lamins) and are responsible for the typical morphological and biochemical features of an apoptotic cell. A simplified overview of the events taking place during chemotherapy-induced apoptosis is given in Figure 1.

The fact that a point of convergence in the cellular response to cytotoxic drugs appears to be apoptosis and that leukaemic cells display cross-resistance to drugs with different mechanisms of action has led to the hypothesis that cellular drug resistance may be related to defects in the apoptotic route. Aberrations at various levels of the apoptotic route have been linked to a drug resistant phenotype in cell lines: absence of cytochrome c
release, defective Apaf-1 activity and caspase deficiency. However, the occurrence of apoptotic defects has not been studied in children with ALL. Therefore, the aim of this study was to determine whether cellular drug resistance is associated with defects in drug-induced apoptosis in paediatric ALL. To this aim, leukaemic cells of 50 children with newly diagnosed ALL were exposed in vitro to four structurally unrelated drugs used in induction therapy of ALL, and activation of various apoptotic parameters was evaluated (Figure 1).
Materials and methods

Patient samples

Bone marrow (BM) and/or peripheral blood (PB) were obtained from children with newly diagnosed ALL that entered the Sophia Children’s Hospital or one of the hospitals participating in the German COALL study. Within 24 hours after sampling, mononuclear cells were isolated by density gradient centrifugation with a Ficoll-Isopaque gradient (Lymphoprep 1.077 mg/ml; Nycomed Pharma, Oslo, Norway). Cells were resuspended in culture medium consisting of RPMI 1640 Dutch modification without L-glutamine (Gibco BRL, Breda, The Netherlands) supplemented with 20% foetal calf serum (FCS; Integro, Zaandam, The Netherlands), 2 mM L-glutamine, 200 µg/ml gentamycin (Gibco BRL) 100 IU/ml penicillin, 100 µg/ml streptomycin, 0.125 µg/ml fungizone (Gibco BRL), and 5 µg/ml insulin, 5 µg/ml transferrin and 5 ng/ml sodium selenite (ITS media supplement; Sigma Aldrich, Zwijndrecht, The Netherlands). If necessary, the lymphoid cells were further purified to at least 90% leukaemic blasts by removing non-malignant cells with immunomagnetic beads (DynaBeads, Dynal Inc., Norway).

In vitro drug resistance assay

In vitro drug resistance for daunorubicin (DNR; Cerubidine, Rhône-Poulenc Rorer, Amstelveen, The Netherlands), vincristine (VCR; TEVA Pharma, Mijdrecht, The Netherlands), L-asparaginase (ASP; Paronal, Christiaens, Breda, The Netherlands) and prednisolone (PRED; Bufa Pharmaceutical Products, Uitgeest, The Netherlands) was determined using the 4-day MTT assay as described previously by Pieters et al.\textsuperscript{25} Briefly, round-bottomed 96-well microculture plates were filled with 20 µl of different dilutions of a drug and stored at –20°C. Six concentrations of each drug were tested in duplicate. The range of final concentration of these drugs were: DNR: 0.002-2.0 µg/ml; VCR: 0.05-50 µg/ml; ASP: 0.003-10 IU/ml and PRED: 0.008-250 µg/ml. Aliquots of 80 µl cell suspension (2 x 10^6 cells/ml) were added to each well. Four wells contained 100 µl culture medium without drugs or cells for blanking the plate reader and 8 wells contained 100 µl culture medium with cells and without drug for measuring control cell viability. After incubating plates for 4 days at 37°C in a humidified incubator in 5% CO₂, 10 µl of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT, 5mg/ml; Sigma) was added and the plates were incubated for an additional 6 hours. During these 6 hours, the
living cells present in each well will reduce the yellow MTT tetrazolium salt to purple-blue formazan crystals. The formazan crystals were dissolved with 100 µl of 0.04 N HCl-isopropanyl alcohol (acidified isopropanol). The optical density (OD) of the wells, which is linearly related to cell number, was measured spectrophotometrically at 562 nm. Leukaemic cell survival (LCS) was calculated by the equation: LCS = (OD_{day4 treated well}/mean OD_{day4 control wells}) x 100%. The drug concentration lethal to 50% of the ALL cells, the LC50 value was used as a measure for cellular drug resistance. MTT-Assay results were only used if the drug-free control wells contained ≥70% leukaemic cells after 4 days of culture.

**In vitro drug exposure for measuring apoptotic features**

Fresh leukaemic cells (2.0 x 10^6 cells/ml) with a purity of at least 90% leukaemic blasts were cultured in the presence of drugs at 37˚C in a humidified incubator in 5% CO2. The range of final drug concentrations were: PRED: 0.061-250 µg/ml; VCR: 0.195-50 µg/ml; ASP: 0.016-10 IU/ml and DNR: 0.008-2.0 µg/ml.

**Measurement of aberrant phosphatidylserine externalisation on the outer cell membrane.**

During the early stages of apoptosis, phosphatidylserine (PS) is translocated from the inner side of the plasma membrane to the outer leaflet of the cell membrane. Annexin V is a Ca^{2+}-dependent phospholipid-binding protein with high affinity for PS and can therefore be used to detect apoptotic cells. Leukaemic cells were resuspended in 200 µl Annexin-V-Alexa™ 488 Reagent, (Nexins Research BV, Kattendijke, The Netherlands) and incubated for 15 minutes at 4˚C. A total of 5,000 events were analysed by flow cytometry (FACSCalibur, Becton Dickinson, Erembodegem, Belgium). Drug-induced apoptosis was calculated according to the following formula: percentage of apoptotic cells = 100% x (D-C)/(100-C), where D represents the percentage of Annexin-V positive cells in the presence of a drug and C the percentage of Annexin V positive cells in the absence of a drug (spontaneous apoptosis). The intra-assay coefficient of variation for measurements of PS externalisation was 3.4%.
Detection of apoptosis-associated alterations in $\Delta \Psi_m$

Disruption of $\Delta \Psi_m$ was determined using 3,3’-dihexyloxacarbocyanine iodide (DiOC$_6$(3); Molecular Probes Inc., Eugene, OR), a lipophilic cationic dye which accumulates in the mitochondrial matrix driven by $\Delta \Psi_m$. Loss of $\Delta \Psi_m$ was visualised as a reduction in the signal in the FL1 channel. Leukaemic cells were incubated in 200 µl PBS containing 40 nM DiOC$_6$(3) solution and incubated in a humidified incubator for 30 minutes at 37°C in 5% CO$_2$. A total of 5,000 events were analysed by flow cytometry. Percentage of cells with decreased mitochondrial transmembrane depolarisation ($\Delta \Psi_m$) was calculated with the following formula: $100\% \times (D-C)/(100-C)$, where $D$ represents the percentage of cells with reduced DiOC$_6$(3) accumulation in drug-treated samples and $C$ represents % cells with reduced DiOC$_6$(3) accumulation in untreated samples. The intra-assay coefficient of variation for measurements of disruption of $\Delta \Psi_m$ was 4.5%.

Measurement of caspase-3 and PARP cleavage

Leukaemic cells were fixed using 2% (v/v) 37% formaldehyde solution in 100% acetone. Fixed cells were washed twice with PBS/0.1% BSA and incubated with an antibody directed against cleaved caspase-3 (Cell Signalling Technology, Beverly, MA, USA) or cleaved PARP (Cell Signalling Technology) at room temperature for 30 minutes. Both antibodies recognise an epitope exposed only when both proteins are cleaved during apoptosis. Subsequently, cells were washed and incubated with fluorescein isothiocyanate (FITC) conjugated rabbit anti-rabbit F(ab’)$_2$ (DAKO, Glostrup, Denmark) for caspase-3 and FITC-conjugated pork anti-mouse F(ab’)$_2$ (DAKO) for PARP at room temperature for 30 minutes. A total of 5,000 events were measured by flow cytometry. Caspase-induced PARP cleavage leads to PARP inactivation, hence we measure caspase-3 activation and PARP inactivation. The percentage of cells with caspase-3 activation or PARP inactivation was determined with the following formula: $100\% \times (D-C)/(100-C)$, where $D$ represents the percentage of cells that stain positive for the antibody in drug-treated samples, and $C$ in untreated samples. Intra-assay variation of caspase-3 and PARP cleavage measurements was 11.2 and 11.8% respectively.
Statistics

Correlations between different apoptotic parameters as well as between the LC\textsubscript{50} values and apoptotic parameters were calculated using the Spearman’s rank ($r_s$) correlation test. Statistical tests were performed at a two-tailed significance level of 0.05.
Results

Time-dependent induction of apoptotic parameters was studied in 5 children with ALL in order to determine the most suitable time point for testing a larger group of children with ALL. \textit{In vitro} exposure to each of the 4 drugs tested caused a time-dependent activation of apoptotic parameters in ALL cells as assessed by an increase of cells with PS externalisation, $\Delta \Psi_m$ depolarisation, caspase-3 activation and PARP inactivation (Figure 2). In only one patient sufficient cells were available to perform an extensive concentration-series. The data indicated a concentration-dependent increase in the activity of all apoptotic parameters (data not shown).

Although exposure to all 4 drugs resulted in activation of similar apoptotic parameters, a difference in apoptosis kinetics was observed. Whereas daunorubicin and vincristine trigger a relatively fast activation of apoptotic parameters, L-asparaginase and prednisolone consistently induced apoptosis more slowly (Figure 2). After 18 hrs daunorubicin or vincristine exposure the mean percentage of cells with PS externalisation in the 5 ALL samples is 52±23% and 36±21% respectively. In contrast, the mean percentage of cells with PS externalisation after 18 hrs L-asparaginase and prednisolone exposure was 20±15% and 21±31% compared to 30±18% and 19±19% respectively after 42 hrs. The two types of kinetics could be confirmed in subsequent experiments; the mean percentage of cells with PS externalisation in the 50 patients measured in this study after 18 hrs daunorubicin or vincristine treatment are 60±24% and 42±25% respectively compared to 31±19% and 30±30% after 42 hrs L-asparaginase and prednisolone exposure respectively. To be able to study the relationship between apoptosis and cellular drug resistance in a large group of patients, activation of apoptotic parameters was measured after 18 hrs of incubation with daunorubicin and vincristine and after 42 hrs of incubation with L-asparaginase and prednisolone in further experiments.

Figure 3 shows that the percentage of cells with PS externalisation is proportional to the percentage of cells with reduction in mitochondrial transmembrane potential ($r_s=0.75$, $P<0.001$), caspase-3 activation ($r_s=0.72$, $P<0.001$) and the percentage of cells with PARP inactivation ($r_s=0.67$, $P<0.001$). Significant correlations were also found when analysing data from each of the 4 drugs separately (Table 1). The slopes of the regression lines in Figure 3A-C are $a=0.75$, $a=0.52$ and $a=0.44$ respectively. The closer the slope of the regression line approaches $a=1.0$, the closer the event probably follows after PS externalisation. This indicates that upon drug exposure PS externalisation and $\Delta \Psi_m$ depolarisation are early events, whereas caspase-3 activation and PARP inactivation are relatively occurring later.

Large inter-individual variability in the extent of drug-induced activation of apoptotic parameters was observed between patients. For instance, prednisolone-induced PS externalisation after 42 hours ranged between
–26% and 86% (median: 27%). Figure 4 and Table 2 show for each individual drug highly significant inverse correlations between the LC₅₀ and (1) the percentage of cells with PS externalisation and (2) the percentage of cells with ΔΨᵋ depolarisation. However, caspase-3 activation and PARP inactivation showed a less consistent inverse correlation pattern with cellular drug resistance. A significant inverse correlation between cellular drug resistance and the percentage of cells with caspase-3 activation was observed for prednisolone (rₛ=–0.60, P=0.001) and L-asparaginase (rₛ=–0.46, P=0.01) but not for vincristine and daunorubicin. Likewise, PARP inactivation was inversely correlated to cellular drug resistance for prednisolone (rₛ=–0.58, P=0.001) and L-asparaginase (rₛ=–0.58, P=0.001) only (Table 2).
Discussion

Cellular drug resistance may reflect disruptions in the apoptotic route.\textsuperscript{17-24} Low caspase-3 activity has been previously linked to a poor prognosis in adult chronic myelogenous leukaemia (CML)\textsuperscript{25} and high levels of caspase-3 with improved survival in adult acute myeloid leukaemia (AML).\textsuperscript{26} In addition, loss of spontaneous caspase-3 activation \textit{in vivo} is associated with relapse in adults with ALL.\textsuperscript{30} However, the presence and clinical significance of these disruptions in the apoptotic route have not been studied well in paediatric ALL. In the present study, we have analysed drug-induced activation of apoptotic parameters in leukaemic cells taken at initial diagnosis of ALL. PS externalisation, $\Delta \Psi_m$ disruption, caspase-3 activation and PARP inactivation were measured after \textit{in vitro} exposure to four cytotoxic drugs that form the backbone of ALL therapy: prednisolone, vincristine, L-asparaginase and daunorubicin.

Time series experiments showed a fast activation of apoptotic parameters for daunorubicin and vincristine and a slower activation for L-asparaginase and prednisolone (Figure 2). One may speculate that this reflects differences in primary cellular targets of the different drugs. Hypothetically, a cell is likely to respond quickly to the direct damaging effect of daunorubicin and vincristine treatment, i.e. DNA damage and microtubule damage respectively. In contrast, it may take a cell relatively longer to respond to the indirect effects of L-asparaginase and prednisolone treatment, i.e. induction of gene expression or depletion of the intracellular stock of the amino acid asparagine.

Our data suggest that PS externalisation and disruption of $\Delta \Psi_m$ are both early features of apoptosis induced by 4 structurally unrelated drugs in childhood ALL (Figure 2,3). The spread of data points around the line $x=y$ in Figure 3 indicates that in half of the patients disruption of $\Delta \Psi_m$ appears to precede PS externalisation (dots above the line $x=y$). However, in the other half of patients, disruption of $\Delta \Psi_m$ follows or coincides with PS externalisation. No consensus is reached in literature concerning the sequence of these two apoptotic events. Conflicting reports have been published showing that disruption of $\Delta \Psi_m$ either preceded or coincided with or followed PS externalisation.\textsuperscript{31-34} An explanation for this phenomenon is proposed by Denecker \textit{et al}, who suggests that both $\Delta \Psi_m$ disruption and PS externalisation are not necessarily two dependent but rather parallel events initiated after an apoptotic stimulus.\textsuperscript{33} Consequently the sequence of these two apoptotic events may be cell type-, stimulus- and apparently also patient-specific.

The present data show that resistance of leukemic cells to each of 4 unrelated drugs is associated with decreased PS externalisation and $\Delta \Psi_m$ depolarisation compared to sensitive cells. Caspase-3 activation or PARP
inactivation were linked to cellular resistance to prednisolone and L-asparaginase, but not with cellular resistance towards vincristine and daunorubicin (Table 2). A possible explanation for this observation is that caspase-3 and PARP cleavage may be an epiphenomenon, which is not essential for vincristine and daunorubicin-induced apoptosis. Multiple caspases, which are redundant in function, are expressed in acute leukaemic cells. Possibly, in case of vincristine and daunorubicin-induced apoptosis another caspase than caspase-3 may function as the main effector caspase in primary ALL cells.

We found that cellular drug resistance is associated with decreased PS externalisation and ΔΨm depolarisation compared to sensitive cells. Decreased activation of these apoptotic parameters is likely to result from a defect upstream or at the level of both PS externalisation and disruption of ΔΨm (Figure 5). Aberrations in the expression of various molecules associated with cellular drug resistance in mainly adult leukaemia and cell lines have been described in literature. Treatment with chemotherapeutic drugs increases intracellular ceramide levels. Significantly reduced ceramide levels have been linked to drug resistance in adult patients with ALL, CML and AML. Deficient upregulation of CD95-ligand and downregulation of CD95-receptor expression has been shown to confer drug resistance in leukaemic cell lines. Aberrant expression of both anti- and pro-apoptotic Bcl-2 family members is known to prevent mitochondrial permeability transition pore opening and release of apoptogenic proteins from mitochondria. Data regarding the role of the expression levels of Bcl-2 family members and clinical outcome in ALL are contradictory. Overexpression of the p53 regulator MDM2 has been associated with early relapse, adriamycin resistance, and failure to respond to re-induction therapy in childhood leukaemia. In addition, constitutive activation of anti-apoptotic proteins such as both Akt/PKB and c-Raf as well as inactivation of the pro-apoptotic protein PTEN have been linked to drug resistance in various types of cancers. Other proteins which overexpression is associated with resistance to apoptosis in acute leukaemia are members of the heat shock protein family like Hsp27 and Hsp70. To find out (1) which molecules play an actual role in cellular drug resistance in children with ALL and (2) whether resistance to different drugs is associated with drug-specific defects we currently perform gene expression studies using high-density oligonucleotide microarrays.

In conclusion, the present study shows that decreased PS externalisation and ΔΨm depolarisation are found in children with ALL who are in vitro resistant to structurally unrelated drugs. These data suggest that cellular resistance to these drugs is caused by defects upstream or at the level of mitochondrial function. Caspase-3 activation and PARP inactivation are suggested to play a role in prednisolone- and L-asparaginase-induced apoptosis, but are not essential to vincristine- and daunorubicin-induced apoptosis. The nature of the
defects upstream or at the level of PS externalisation and $\Delta \Psi_m$ depolarisation in resistant cells of children with ALL are not elucidated and will be subject of further research.

Acknowledgements:

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References


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Table 1: Correlation between PS externalisation and the downstream apoptotic parameters upon drug exposure in paediatric ALL.

<table>
<thead>
<tr>
<th>Drug</th>
<th>( \Delta \Psi_m ) depolarisation</th>
<th>caspase-3 activation</th>
<th>PARP inactivation</th>
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<tr>
<td>prednisolone</td>
<td>Correlation coefficient P-value N</td>
<td>( .81 ) (&lt; .001 ) 31</td>
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<td>daunorubicin</td>
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<td>( .63 ) (&lt; .001 ) 29</td>
<td>( .50 ) (.005 ) 31</td>
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Table 2: Inverse correlation between cellular drug resistance and the activation of parameters along the effector route of apoptosis in paediatric ALL.

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<tr>
<th>apoptotic parameter</th>
<th>LC$_{50}$ prednisolone</th>
<th>LC$_{50}$ vincristine</th>
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Figure Legends:

Figure 1: Simplified overview of the events taking place during drug-induced activation of apoptotic parameters.
Drug A and B represent two structurally unrelated drugs. Numbers 1, 2 and 3 refer to the three phases of the drug-induced apoptotic route as described in the Introduction section; 1: insult generation, 2: signal transduction and 3: execution. Parameters with an asterisk (*) are measured in this study using flow cytometry, the right column indicates the detection method used.

Figure 2: Time-dependent drug-induced apoptosis in ALL.
Freshly isolated ALL cells were cultured in the presence of 2.0 µg/ml daunorubicin (DNR; solid line with squares), 50 µg/ml vincristine (VCR; dotted line with circles), 10 IU/ml L-asparaginase (ASP; dashed line with triangles) or 250 µg/ml prednisolone (PRED; dotted and solid line with diamonds) for the indicated time points. Drug-induced PS externalisation (A), mitochondrial transmembrane disruption (B), caspase-3 activation (C) and PARP inactivation (D) were determined by flow cytometry and calculated by the formula described in Materials and Methods. Results are expressed as mean ± SD of 5 patients with ALL.

Figure 3: Correlation between drug-induced apoptotic parameters in paediatric ALL.
Correlation between the percentage of cells with PS externalisation and ΔΨm depolarisation (A), activated caspase-3 (B) or inactivated PARP (C) in leukaemic cells in vitro incubated with prednisolone, vincristine, L-asparaginase or daunorubicin in 50 children with ALL. The dashed line represent the line x = y and the solid line represents the linear regression line.

Figure 4: Drug-induced apoptosis inversely correlates with cellular drug resistance in paediatric ALL.
Freshly isolated ALL cells were incubated in the presence of vincristine or daunorubicin for 18 hrs or prednisolone or L-asparaginase for 42 hrs at 37°C in a humidified incubator in 5% CO2. See Figure 2 for drug concentrations used. Each dot corresponds to a patient with ALL.

Figure 5: Impaired apoptosis in resistant compared to sensitive ALL cells.
A defect localised upstream of the mitochondria may lead to decreased activation of downstream apoptotic parameters in resistant ALL patients. Potential sites of defects are indicated with a cross. Decreased activation of apoptotic parameters is illustrated by the decreased size of the arrows in resistant compared to sensitive patients.
Table 1: Correlation between PS externalisation and the downstream apoptotic parameters upon drug exposure in paediatric ALL.

Freshly isolated ALL cells were cultured for 18 hours in the presence of 50 µg/ml vincristine or 2.0 µg/ml daunorubicin or 42 hrs in the presence of 250 µg/ml prednisolone or 10 IU/ml L-asparaginase. Drug-induced activation of apoptotic parameters was determined by flow cytometry. Correlation between apoptotic parameters was calculated using the Spearman’s rank correlation test.

Table 2: Inverse correlation between cellular drug resistance and the activation of parameters along the effector route of apoptosis in paediatric ALL.

See Table 1 for legends
**Figure 1 Holleman et al.**

**Measured by:**

- Annexin V staining
- DiOC₆(3) accumulation
- Cleaved caspase-3 staining
- Cleaved PARP staining

1. Drug A → Drug B → Phosphatidylserine externalisation *
2. ΔΨₘ ↓ *
3. Cytochrome c release → Active caspase-9 → Active effector caspases * → Cleavage of intracellular targets *
Time-dependent drug-induced activation in ALL.

A.

B.

Figure 2 A-B Holleman et al
Time-dependent drug-induced activation in ALL.

C.

D.

Figure 2 C-D Holleman et al
Correlation between drug-activated apoptotic parameters in paediatric ALL.

A.

Figure 3 A-C Holleman et al.
Drug-induced apoptosis inversely correlates with cellular drug resistance in paediatric ALL.

A. Prednisolone

B. Vincristine

C. L-asparaginase

Figure 4 A-C Holleman et al.
D. Daunorubicin

Figure 4 D Holleman et al.
Impaired apoptosis in resistant compared to sensitive ALL cells.

Figure 5 Holleman et al.
Resistance to different classes of drugs is associated with impaired apoptosis in childhood acute lymphoblastic leukaemia

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