CHAPTER 5

IN VITRO STUDIES IN PEDIATRIC ACUTE LYMPHOBLASTIC LEUKEMIA
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NEED FOR CORRECT PROGNOSTICATION

Twenty-five years ago, the prognosis of children suffering from ALL was very poor, and most of them survived only 2 or 3 months after diagnosis (Margolin and Poplack, 1997). Over the past few years, cure rate has dramatically improved for ALL, mainly as a result of the identification of better prognostic indicators and newer therapeutic modalities. Long term event free survival (EFS), only 5% in 1965, has now increased to 70% (Pui, 1994 and 1995). However, current protocols fail in about 30% of children with newly diagnosed ALL; bone marrow relapses representing the most common treatment failures (Pui, 1994). Clinical markers have been used to define high, low and intermediate risk groups. However, these have limitations because some patients with no adverse clinical or chromosomal markers do not respond to therapy. Therefore, identification of newer clinical and biological features that accurately predict patient response to therapy has been a continuing goal of leukemia specialists. The combination of such biologic parameters will allow better identification of standard and high-risk groups, the latter being characterized by less remission and lowest survival rates.

Cellular drug resistance is related to a high risk of treatment failure in childhood leukemia. It is well known from early single agent studies during the development of ALL treatment, that a proportion of children with initial ALL were not responsive to a single drug (Kaspers et al., 1994). Primary resistance to
chemotherapeutic agents may be an important reason for relapses in ALL. Altered expression of P-glycoprotein, the multidrug resistance-associated protein has been implicated as a cause of drug resistance in vitro (Ivy et al., 1996; Gottesman et al., 1993).

**Figure 42**: Schematic diagram representing drug resistance mechanisms in leukemia cells

(Pgp and MRP can effectively efflux cytotoxic drugs from the cell. GST enzymes can detoxify drugs in the cytoplasm and LRP vaults may be involved in the redistribution of drugs away from...
The fact that tumors may develop resistance to multiple drugs even before treatment has major implications for cancer therapy (Pieters et al., 1991). Earlier studies have shown clinical importance of de novo in vitro drug resistance. When children with ALL at diagnosis were divided into a resistant and a sensitive group by the median LC50 value of specific drugs, the risk of relapse was significantly higher in the resistant group (Hongo et al., 1997). Clinical resistance can also be due to the reduced efficacy of the apoptotic machinery.

**Drug resistance in leukemia - P-Glycoprotein (P-gp)**

Clinical drug resistance may be attributed to the concerted implementation of events that ultimately results in reduced uptake, increased metabolism of drugs, increased repair capacity by cells or reduced propensity of cells to undergo apoptosis. The most extensively studied form of MDR in human cells is due to P-Glycoprotein. It is a member of the ATP binding cassette (ABC) super family of transport proteins (Higgins, 1992). P-glycoprotein functions as a membrane-associated protein pump working against concentration gradient, whose increased expression results in resistance to anthracyclins, epipodophylotoxins, Vinca alkaloids and some alkylating agents (Eudicott and Ling, 1989; Gottesman and Pastan, 1993). This type of resistance occurs in both de novo and acquired resistance to therapy for leukemia. During past few years, the phenomenon of multidrug resistance (MDR) has been described and some of its molecular aspects have been clarified (McKenna, 1997). P-gp mediated drug resistance...
results in reduced drug accumulation in tumor cells as a result of increased efflux.

It has now been established that most (if not all) of the cytotoxic agents used in treatment protocols for leukemia kill cells by inducing apoptosis (Ling et al., 1993; Tosi et al., 1994; Begleiter et al., 1994). Expression of the cell death controlling genes has been shown to affect chemosensitivity. Thus preliminary studies have suggested that the nature of genes that define the capability of cells to undergo apoptosis after drug treatment may be an important arbiter of therapeutic response in leukemia.

We have in the present study looked for associations between in vitro drug sensitivity as detected by MTT assay and resistance pattern detected by P-gp in a group of pediatric patients with ALL. The results of this study will delineate those patients in whom therapeutic strategies should be altered so as to achieve maximum clinical outcome with reduced deaths due to toxic effects of drugs.

**NEED FOR INDIVIDUALIZATION OF TREATMENT: ROLE OF DRUG SENSITIVITY EVALUATION**

Chemotherapy is the mainstream treatment protocol for children with ALL. At most centers in India, pediatric ALL patients are treated according to the M C P - 841 regimen which includes induction, consolidation and maintenance phases consisting of Daunorubicin (30 mg/m² IV; days 8, 15, 29), Vincristine (1.4 mg/m² IV; days 1, 8, 15, 22, 29), Asparginase (6000 u/m² IM; days 2, 4, 6, 8, 10, 12, 14, 16, 18, 20), Prednisone (40 mg/m² p.o.; 28 days) and Methotrexate (6-12 mg IT;
days 1, 8, 15, 22). Most of the children achieve complete remission after induction treatment while a small percentage of group tend for induction failure and further relapse. Another good percentage of patients further enters into complications due to the drug toxicity which some times proves fatal.

Glucocorticoids are highly cytotoxic to lymphocytes and thus have been a key component of treatment regimen for ALL; Prednisone being the most commonly used of these compounds in ALL therapy. However, toxicity often causes complex problems including peptic ulcer, obesity, diabetes, osteoporosis and phsyosics. Anthracyclins such as Daunomycin and Doxorubicin are currently used in combination with several other classes of drugs in treatment for ALL. However, clinical use is limited by cardio toxicity and development of drug resistance. Effects caused by other agents have been summarised in Table14.

Thus with the current method of treatment, some patients do develop resistance to chemotherapy or relapse at a later stage. Standard therapy dose may not be therefore sufficient enough in some group of patients to completely eradicate tumor cells, while in others the drug concentration may be in excess of optimum higher enough to exert adverse effects. This difference arises due to the changes in the tumor cellular characteristics manifested in these patients. It is therefore vital to develop effective therapy for children with ALL in whom no remission occurs or later suffer relapse with current protocols. It is also important to protect the patient from toxicity due to over dosage. It is also an accepted fact that overdosage of drugs leads to the further complications during the treatment period. Therefore, monitoring of individual sensitivity of these drugs becomes
mandatory in these patients undergoing treatment. Advance prognostic information can also stimulate clinical trials to reduce toxic effects or can indicate the need of more intense treatment.

Table 14-Chemotherapeutic agents used and its documented toxic effects

<table>
<thead>
<tr>
<th>Drug used</th>
<th>Toxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corticosteroids</td>
<td>Peptic ulcer, Obesity, Diabetes, Osteoporosis, Psychosis</td>
</tr>
<tr>
<td>Anthracyclins</td>
<td>Cardiac Toxicity, Myelosuppression, Phlebitis, Mucositis</td>
</tr>
<tr>
<td>Vincristine</td>
<td>Neuropathy, Hair loss, Alopecia, Paresthesias</td>
</tr>
<tr>
<td>L- Asparginase</td>
<td>Hypersensitivity, Low albumin and Coagulation factors, Pancreatitis, CNS toxicity</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>Mouth ulcers, Gut toxicity, Hepatotoxicity,</td>
</tr>
<tr>
<td>6- Mercapto Purine</td>
<td>Jaundice, GI Ulceration, Anorexia</td>
</tr>
<tr>
<td>Ara-C</td>
<td>Gut toxicity, Hemolytic Anemia, Stomatitis</td>
</tr>
<tr>
<td>Hydroxy Urea</td>
<td>Gut toxicity, Atrophy</td>
</tr>
<tr>
<td>Adriamycin</td>
<td>Loss of hair, dose related Cardiac toxicity</td>
</tr>
<tr>
<td>Cyclophoshmamide,</td>
<td>Cardiomyopathy, Loss of hair, Marrow Aplasia,</td>
</tr>
<tr>
<td>Chlorambul,</td>
<td>Pulmonary Fibrosis, Hyper Pigmentation</td>
</tr>
<tr>
<td>Bulsulfan</td>
<td></td>
</tr>
</tbody>
</table>

Importance of in vitro assays for drug sensitivity

In vitro cell culture drug sensitivity assays have a number of potentially valuable applications in leukemia as well as in other malignancies. Recently, a number of studies have reported the clinical relevance of in vitro drug sensitivity assays in childhood leukemia (Hongo et al., 1997; Kaspers et al., 1997). Although they are already being used for drug screening, there is a reluctance to use these assays
for selection of patients for risk stratified therapy and individualised tailored therapy. Earlier studies show that children with ALL with in vitro drug resistant leukemic cells have a poorer prognosis compared to patients with relatively sensitive cells at initial diagnosis (Hongo et al., 1997). The poor proliferative capacity of ALL cells in vitro limits the use of long-term clonogenic assays. Recently there has been an increase in reports of a short term culture drug resistance assay using MTT (3 - [4,5- dimethyl thiazol - 2 - yl] - 2, 5 - diphenyl tetrazolium) dye (Hongo et al., 1997; Carmichael et al., 1987). These assays are based on measuring total cell kill of both proliferating and non-proliferating cells and in fact, measures the end effect of actual resistance mechanisms. This assay first described by Black and Speer in 1954 and revised by Mossmann in 1983 (Mosmann, 1983) is now being adapted for testing ALL cells. This assay can be applied clinically to select effective drugs as it can be performed in a 96 well plate, and results can be analyzed using a scanning multiwell spectrophotometer. Thus the 4-day semi automated MTT assay is an efficient tool for large scale drug resistance testing. A precise identification of those children with poor prognostic features will greatly increase the probability of applying more effective therapeutic approaches. Individual tumors, even those of the same histologic type, show sensitivity to a specific cytostatic agent. This variation in sensitivity can help attempt to develop an individualized chemotherapy programme and thus sensitivity testing serves as an orientational aid in planning chemotherapy.
WORKING HYPOTHESIS

- Cellular drug resistance may play an important role in induction failure and relapse.
- Clinical outcome is often highly altered by the process of drug resistance.
- In vivo drug resistance/sensitivity may be effectively predicted by performing a dye based in vitro drug resistance assay.
- In vitro drug resistance may be related to apoptotic process.
- The above data of drug sensitivity may have clinical application, to either reduce toxic doses or to initiate more intensive treatment.

SPECIFIC AIMS

- Evaluation of in vitro drug sensitivity of individual drugs by MTT assay in pediatric ALL.
- Since drug resistance can be due to multi drug resistant gene product or due to reduced apoptosis, determination of P-gp and apoptosis in these patients.
- Correlation of in vitro drug sensitivity and apoptosis induction to in vivo response of the individual patient.
- Correlation of the drug resistance assay to final clinical outcome and prognosis.
METHODOLOGY

Tumor cell samples

Five ml of blood was collected by venipuncture into sterile heparinized tubes, before the commencement of chemotherapy. In most cases, the mononuclear cells compartment was flooded with lymphoblasts. Mononuclear lymphoid cells were isolated by density gradient centrifugation using the method we have standardized before. Cells were washed twice in PBS and resuspended in RPMI 1640(GibcoBRL) containing 10% fetal calf serum (FCS). The mean percentage of lymphoblasts (range, 80 to 95%) form these patients were ~90%. Leukemic lymphoblasts thus obtained were looked for viability by Trypan Blue dye exclusion assay and were then considered for the study.

Immunocytochemical analysis

The expression of P-gp was analyzed in all samples by immunocytochemistry using a monoclonal antibody, which detects anti- P-gp, (Oncogene Science Inc, Uniondale, NY, USA). The details of the protocol are explained in Appendix 1. Percentage of positive cells was assessed by counting at least 500 cells. A negative control was run, using PBS instead of the primary antibody.

In vitro sensitivity assay

The in vitro drug sensitivity of drugs was tested by MTT assay with drug dilutions in five serial fold dilutions [the used drug concentration range covered the clinical plasma concentrations (Klumper et al., 1995). Briefly, 96 well micro culture plates contained 100µL cell suspensions with 6 concentrations of each drug. The details of drug preparations are explained in Appendix 1. Six wells contained leukemic
cells in drug free medium to determine the control cell survival and the percentage of leukemic cells after culture. Another six wells contained medium only, which served as blank. The following drugs and range of concentration were tested: Doxorubicin (0.008 to 8µg/mL), Prednisone (0.08 to 250µg/mL), Vincristine (0.05 to 50µg/mL), L-Asparaginase (0.003 to 10 IU/mL), 6-Mercaptopurine (15.6 to 500 µg/mL), Cytarabine (0.002 to 2.5µg/mL) and Dexamethsone (0.0002 to 8µg/mL). Methotrexate was not tested because this drug is not cytotoxic to ALL cells in short-term assays such as the MTT assay (Pieters et al., 1990). All the drugs were obtained from Sigma (MO, USA). Drugs were newly prepared in RPMI-1640 and stored at -70°C. After 4 days of incubation of cultures in 5% carbon dioxide at 37°C in a humidified incubator, 10µL of MTT was added to each well and subsequently incubated for another 6 hours. The yellowish tetrazolium salt MT is reduced to dark coloured formazan by viable cells only. Formazan crystals were dissolved in acidified isopropanol. The OD was measured at 570nm with a Multiskan MS ELISA Reader (Labsystems, Helsinki, Finland). The leukemic cell survival (LCS) was calculated as follows:

\[ \text{LCS} = \left( \frac{\text{OD}_{\text{drug exposed cell}}}{\text{mean OD}_{\text{control wells}}} \right) \times 100\% \]

The drug concentration lethal to 50% of the leukemic cells (the LC_{50}) was used as a measure of resistance. In vitro drug resistance were reproducible. For each single drug, patients were classified into two groups, either as sensitive (S) (lower than median LCS) or resistant (R) (median or higher than the median concentration). Median values of the tested drugs are represented in Table 15. Patients were also classified into three categories super sensitive (SS),
intermediate sensitivity (IS) and relative resistance (RR) by sensitivity to the combination of four drugs (DPAV; Doxorubicin, Prednisone, Asparginase, Vincristine). For each of these four drugs, patients were classified as either S or R according to the definitions given above, and for the DPAV combinations, SS was defined as S to all four drugs, IS as S to two or three drugs, and RR as S to no drugs or to one drug.

Clinical outcome

In vitro drug sensitivity was correlated with both short and long term clinical outcome. CR was defined as less than 5% leukemic blasts in representative BM containing megakaryocytes and granulocytic precursors with some degree of maturation, and no manifestation of leukemia elsewhere. Status after five-year follow up was used to find out overall survival (OS).

STATISTICAL ANALYSIS

The Spearman rank correlation was used to analyse the relationship of LC50 values of drugs with clinical parameters. The Kaplan Meier method for estimation of overall survival, log rank test was conducted for assessing the difference between two curves, using the SPSS statistical package.

RESULTS

We could do only forty-five samples during our study period. Five samples were excluded because of insufficient number of cells (3 samples) for testing and low absorbances in control well (2 samples). However, the technical success rate
was over 88%. LD<sub>50</sub> values were determined from individual dose response curves. LD<sub>50</sub> values varied markedly between the patient samples for all drugs. The distribution of LD<sub>50</sub> values for the drugs tested is shown in figure 43.

**Correlation with apoptosis and clinical parameters**

It is now understood that anticancer drugs act through induction of apoptotic program. Therefore, it was possible that there could be an association between the resistant pattern exhibited by patients with regard to DPAV sensitivity and propensity for apoptosis. In order to investigate whether any such correlation exist between drug sensitivity and apoptosis, Spearman correlation analysis was done. No significant association was found for bax and bcl-2 with the DPAV sensitivity status. However, patients with a lower p53 protein expression showed predominantly a super sensitive pattern (r value = 0.3421; p = 0.032). Likewise patients with a lower apoptotic index exhibited a RR pattern for DPAV sensitivity (r value = -0.3325; p = 0.036). These results further substantiate that defective apoptosis could be the cause for the patients being resistant to front line drugs. There was no correlation between DPAV sensitivity and any of the clinical parameters analyzed.

Patients were classified into two groups for each single drug, as one having drug concentration less than median and those having greater than the median values. Analysis for the mean values p53, bcl-2 and bax protein yielded following results. Patients sensitive for L-Asparginase had a high bax percentage than the resistant group (13.06±3.23 vs. 5.83±1.28; p value = 0.051). Likewise, patients sensitive for Prednisone showed a lower bcl-2 mean value than the resistant one
FIGURE 43: LD 50 VALUES FOR MOST COMMONLY USED DRUGS IN INDUCTION THERAPY IN CHILDHOOD ALL

*L.D50 values are in µg/mL except for L-Asparaginase (IU/mL)*
(6.67±1.90% vs. 13.22± 2.5%; p value = 0.045). Patients sensitive for Vincristine also showed a lower bcl-2 mean value than the resistant one (6.56 ±1.68% vs.13.3±2.6%; p value = 0.036). Patients sensitive for Dex, 6 MP, Ara-C and Dox did not show any significant difference in mean values for the above proteins. However, the implications of the above result remains to be explained.

P-gp was detected as membrane/cytoplasmic immunoreactivity among leukemic cells and was seen in 40% (48/122) of the samples (Figure 44). A cut off value of 8% (median value) was fixed, below which cases were considered to be negative for P-gp. Presence of spontaneous drug resistance in pretreatment leukemia was thus evident from the study. Whether it had implications on the other cellular functions and survival were analyzed by correlation and survival analysis. Associations for P-gp and clinical and experimental variables were done by Spearman correlation analysis. Its expression was not significantly associated with any of the clinical parameters except for the complete remission status; patients not achieving CR had increase in P-gp immunoreactivity. However, it reached only a borderline statistical significance (r value = -0.2361; p value = 0.08). Likewise, a positive correlation was also documented with PCNA immunoreactivity (r value = 0.2317; p value = 0.074). A significant association was also documented between sensitivity detected as DPAV sensitivity and by P-gp immunoreactivity (p value = 0.04).

**Drug sensitivity testing and prognosis**

Investigations regarding the relationship between drug sensitivity and prognosis yielded the following results. De novo drug resistance was definitely involved in
Figure 44. De novo & acquired Pgp expression in pediatric ALL

Fig 44A. Cytoplasmic immunoreactivity for Pgp (Magnification 1080 x)

Fig 44B. Intense immunoreactivity for Pgp in a patient undergoing treatment. The patient relapsed and died during therapy (Magnification 1200 x)
childhood ALL. More specifically, patients sensitive to Prednisone, Asparginase, Vincristine and 6- Mercapto purine had higher overall survival compared to patients whose blast cells were resistant to these drugs (P < 0.01). For the other drugs tested overall survival did not vary from that of the resistant patients.

Table 15 - Median concentration of LC₅₀ and range of each drug tested

<table>
<thead>
<tr>
<th>Abbreviation (Drug Name)</th>
<th>Median Concentration (µg/mL) of LC₅₀</th>
<th>Range (µg/mL) Minimum- Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin (DOX)</td>
<td>0.231</td>
<td>0.005-10.322</td>
</tr>
<tr>
<td>Vincristine (VCR)</td>
<td>1.336</td>
<td>0.008-53.657</td>
</tr>
<tr>
<td>L- Asparginase (L-ASP)</td>
<td>0.139#</td>
<td>0.02-8.470#</td>
</tr>
<tr>
<td>Prednisone (Pred)</td>
<td>5.178</td>
<td>0.015-339.96</td>
</tr>
<tr>
<td>6- mercapto purine (6 MP)</td>
<td>67.125</td>
<td>9.35-523.64</td>
</tr>
<tr>
<td>Cytarabine (Ara-C)</td>
<td>0.066</td>
<td>0.02-1.824</td>
</tr>
<tr>
<td>Dexamethasone (DEX)</td>
<td>0.072</td>
<td>0.001-0.979</td>
</tr>
</tbody>
</table>

*Values are units per millilitre

Univariate analysis

Sensitivity to glucocorticoids has long been considered having significance in predicting survival. When patients were classified into two groups according to their median LD₅₀ values, the following results were obtained.
The patients showing a LD$_{50}$ value greater than the median values had lower survival rates than those showing a lower LD$_{50}$ values.

Five year overall survival of SS group (n= 17) was 60%, that of IS (n=8) was 39% and that of RR (n=15) was ~8.5% (log rank test, p value = 0.0205; Breslow, p value = 0.0560, Table 17). For DPAV sensitivity, there was a significant worsening of prognosis from the extremely sensitive patients through an intermediate sensitive group to the most resistant group. Moreover, the RR patient group relapsed earlier than the other groups.

**Table 16- Drug resistance profile (for DPAV) and the occurrence of leukemia related terminal events**

<table>
<thead>
<tr>
<th></th>
<th>SS</th>
<th>IS</th>
<th>RR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Patients</td>
<td>17</td>
<td>8</td>
<td>15</td>
</tr>
<tr>
<td>Event (%)</td>
<td>6 (35%)</td>
<td>4 (50%)</td>
<td>13 (87%)</td>
</tr>
</tbody>
</table>
Figure 46- Kaplan Meier survival graph for patients classified into SS, IS and RR groups by sensitivity to four drug combination

![Kaplan Meier survival graph]

Table 17 - The relationship between DPAV sensitivity and survival in childhood ALL patients

<table>
<thead>
<tr>
<th>DPAV sensitivity</th>
<th>Cumulative survival</th>
<th>Duration Mean±SE</th>
<th>Confidence interval</th>
<th>Percentage censored</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SS</td>
<td>60%</td>
<td>40±7</td>
<td>(26-54)</td>
<td>62.5%</td>
<td></td>
</tr>
<tr>
<td>IS</td>
<td>39%</td>
<td>32±8</td>
<td>(16-47)</td>
<td>50%</td>
<td>0.0205</td>
</tr>
<tr>
<td>RR</td>
<td>8%</td>
<td>16±6</td>
<td>(5-27)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(SS - super sensitivity, IS- Intermediate sensitivity, RR- relative resistance)
DISCUSSION

The most important finding of the present study appears to be that we have in a cohort of newly diagnosed ALL patients been able to confirm the clinical relevance of in vitro leukemia cells survival to the short and long term clinical outcome. A number of recent studies have by indirect means suggested that the survival capacity of neoplastic cells may be of significance to clinical drug resistance (Krajewski et al., 1995; Wattel et al, 1994; Campos et al., 1993). However, number of studies that directly correlate in vitro survival capacity of the neoplastic cells to clinical outcome is small. In this context, it is reasonable to point out that although it has during recent years been generally assumed that a link exists between the capacity of neoplastic cells to survive per se and clinical drug resistance, only few studies have documented such a link. Turning to in vitro studies, the literature becomes more abundant. Several studies have over the years focussed on the link between the in vitro spontaneous tendency of the neoplastic cells to undergo cell death and the tendency to undergo drug or irradiation induced cell death (Miyashita and Reed, 1993; Campos et al., 1993). From these studies it has been almost unequivocally suggested that spontaneous ability of the neoplastic cells to survive under in vitro circumstances is associated with low tendency to undergo drug induced cell death. However, one possible exception from this apparent general rule may well be in the case of drugs requiring cell proliferation for excretion of the cytotoxic drug effect.

Resistance to prednisone monotherapy has been considered as an important factor in the failure of chemotherapy in childhood ALL (Pieters et al., 1991). In
our study it was shown that additionally, resistance of Vincristine, Asparginase and 6 MP too was related to prognosis. When children with ALL at initial diagnosis were divided into a resistant and a sensitive group by median LD$_{50}$ value of specific drugs, the risk of relapse was significantly higher in the resistant group.

From our results, the probability of CCR was significantly lower in patients with resistant cells than those with sensitive cells for single drugs such as Prednisone (p < 0.006) Vincristine (p < 0.01), Asparginase (p < 0.002) and 6 MP (p < 0.005). De novo resistance to specific classes of drugs can be overcome by combination chemotherapy. Thus we classified patients into three categories (SS, IS and RR) by sensitivity to DPAV combination (this is the widely used drugs for the treatment of induction therapy). Five year overall survival of SS group (n= 17) was 60%, that of IS (n=8) was 39% and that of RR (n=15) was ~8.5% (log rank test, p value = 0.0205). Children with DPAV sensitivity had good clinical outcomes, whereas children with DPAV resistant leukemia underwent induction failure and or faster relapse when treated with the same drug combination.

The present result clearly agrees with that of Kaspers et al., (1997), where they had classified patients according to the three groups as sensitive (33% lowest LD$_{50}$ values), intermediate sensitive (33% intermediate LD$_{50}$ values), or resistant (33% highest LD$_{50}$ values). When Kaplan Meier curves were plotted, resistance to Prednisolone, L - Asparginase and Vincristine were found to be prognostic significance.
Thus patients can be stratified into different groups according to their sensitivity to the four front line drugs (DPAV). Further, patients who show resistance could be additionally supplied with these drugs or other drugs to which their cells are sensitive. But since this is a preliminary observation with limited sample population, further studies only will tell the actual implications of this study.

The standard way to validate an in vitro drug sensitivity assay is to demonstrate such a study in a prospective study. A comparison between responders and non-responders in terms of achieving a complete remission is hard to make because the group of non-responders is too small in children with newly diagnosed ALL. Predictive value of this assay has been validated recently in a number of studies (Norgaard et al., 1999; Kaspers et al., 1997; Hongo et al., 1997). Clinical relevant data, assessed with the MTT assay, have emerged to be of prognostic significance in newly diagnosed childhood ALL (Pieters et al., 1991; Kaspers et al., 1994). Previous studies suggest an overlapping of LD$_{50}$ values for samples with initial and relapsed ALL shows the possibility of a group of patients being already drug resistant at initial diagnosis (Klumper et al., 1995).

The present study clearly shows that in vitro drug sensitivity testing provides significant prognostic information in childhood ALL at the time chemotherapy commences and that early detection of drug resistance may provide a successful strategy for individualizing treatment. Relapsed leukemia requires the development of a method for the rapid and accurate prediction of clinical response to specific chemotherapeutic agents. Thus this study could be further
continued by evaluating the drug resistance pattern at initial diagnosis and at relapse.

An assay in which large number of drugs can be tested is likely to be more beneficial to the patient, especially when the disease has become resistant to first line chemotherapy. In such a situation there is often a feeling of urgency that time not wasted on a trial with a compound that will not be effective, and it is helpful to select an effective drug and to eliminate those drugs that will have little or no antileukemic effect. In future, patients may be treated selectively only with those drugs to which their leukemic cells are sensitive, avoiding unnecessary exposure to ineffective and potentially toxic agents.

**Clinical importance of P-gp overexpression**

Clinical importance of P-gp overexpression as a mechanism of drug resistance in malignant cells has been well characterised (Campos et al 1992; Goasguen et al, 1993; Yuen & Sikic, 1994). Contradictory results have been reported in childhood leukemia; in some studies P-gp expression was higher at relapse compared with initial leukemia or was related to long term survival or relapse whereas in other studies no such associations were found (Dhooge et al., 1999; Beck et al., 1995; Ivy et al., 1996; Pieters et al., 1992).
Kaplan Meier survival analysis showed no significant differences in the overall survival of these patients who were P-pg positive and negative (51% vs. 63%; p value = 0.25).

P-gp expression did not clearly differ between the various risk groups identified as age and WBC and any other clinical parameters. Partial association between P-gp expression and in vitro cytotoxicity for both MDR related drugs and non-MDR related drugs were also recorded (p value = 0.06). There was no correlation between P-gp immunoreactivity and the other apoptosis regulatory proteins indicating that these are two independent resistance mechanisms working in tumor cells. In summary, our data suggest that while P-gp expression is related to drug resistance in childhood ALL but is not able to predict survival when compared to drug resistance assayed by in vitro methods.