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In vitro anthracycline cross-resistance pattern in childhood acute lymphoblastic leukaemia

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Summary Daunorubicin (DNR) is a major front-line drug in the treatment of childhood acute lymphoblastic leukaemia (ALL). Previously, we showed that *in vitro* resistance to DNR at diagnosis is related to a poor long-term clinical outcome in childhood ALL and that relapsed ALL samples are more resistant to DNR than untreated ALL samples. In cell line studies, idarubicin (IDR), aclarubicin (ACR) and mitoxantrone (MIT) showed a (partial) lack of cross-resistance to the conventional anthracyclines DNR and doxorubicin (DOX), but clinical studies in childhood ALL have been inconclusive about the suggested lack of cross-resistance. In the present study we determined the *in vitro* cross-resistance pattern between DNR, DOX, IDR, ACR and MIT in 48 untreated and 39 relapsed samples from children with ALL using the MTT assay. The relapsed ALL group was about twice as resistant to DNR, DOX, IDR, ACR and MIT as the untreated ALL group. Thus, resistance developed to all five drugs. We found a significant cross-resistance between DNR, DOX, IDR, ACR and MIT, although in some individual cases *in vitro* anthracycline cross-resistance was less pronounced. We conclude that IDR, ACR and MIT cannot circumvent *in vitro* resistance to DNR in childhood ALL. Clinical studies may still prove whether IDR, ACR or MIT has a more favourable toxicity profile than DNR.

Keywords: MTT assay; chemosensitivity; drug resistance; daunorubicin; doxorubicin; idarubicin; aclarubicin; mitoxantrone; leukaemia

Two-thirds of children with newly diagnosed acute lymphoblastic leukaemia (ALL) can now be cured with combination chemotherapy, but chemotherapy fails in the remaining third, mainly because the leukaemia relapses (Niemeyer *et al.*, 1991). Despite intensive salvage chemotherapy, children with relapsed ALL still have a poor prognosis, and only one-third of them can be cured (Henze *et al.*, 1991). Anthracyclines, such as daunorubicin (DNR) and doxorubicin (DOX), are commonly used in combination with several other classes of drugs in the treatment of childhood ALL. However, their clinical use is limited by cardiotoxicity and the development of drug resistance (Weiss, 1992). Previously, we showed that samples from children with relapsed ALL are more resistant to DNR than samples from children with untreated ALL (Pieters *et al.*, 1992; Klumper *et al.*, 1993).

Anthracycline analogues lacking cross-resistance may circumvent resistance to DNR or DOX and may improve chemotherapy for relapsed childhood ALL. Many analogues have been developed since DNR and DOX were discovered in the early 1960s, but only a few have reached clinical trials (Muggia and Green, 1991). Idarubicin (IDR), aclarubicin (ACR) and the structurally closely related mitoxantrone (MIT) show a (partial) lack of cross-resistance to DNR and DOX in different cell lines (Hill *et al.*, 1985; Coley *et al.*, 1989; Erttmann *et al.*, 1991). Clinical studies with anthracycline analogues in childhood ALL are inconclusive about the suggested lack of cross-resistance, since no randomised comparative studies have been reported to date. In the present study, we determined the *in vitro* cross-resistance pattern between DNR, DOX, IDR, ACR and MIT within an uniform group of fresh samples obtained from 48 children with untreated ALL and 39 children with relapsed ALL.

Materials and methods

Drugs

We tested the following drugs: DNR (Polyfarma, The Netherlands), DOX and IDR (Montedison, The Netherlands), MIT (Lederle, The Netherlands). ACR was a generous gift from Lundbeck (Copenhagen, Denmark). DNR, DOX and IDR were dissolved in distilled water. ACR was dissolved in ethanol. MIT was obtained in soluble form. All drugs were further diluted with RPMI-1640 (Dutch modification, Gibco, Uxbridge, UK) and stored at −20°C in stock solutions of 50 $\mu g\ ml^{-1}$ (MIT), 100 $\mu g\ ml^{-1}$ (DNR, IDR) and 400 $\mu g\ ml^{-1}$ (DOX, ACR). Microculture plates were prepared with serial 4-fold drug dilutions derived from these stock solutions, and the plates were stored at −20°C to facilitate large-scale testing. We used *in vitro* concentration ranges that covered clinical plasma concentrations (Table I). Anthracyclines can be safely stored at −20°C up to 9 months without decomposition (Scott *et al.*, 1986), and without loss of *in vitro* anti-leukaemic efficacy of all drugs tested (data not shown).

Leukaemic samples

Bone marrow (BM) and or peripheral blood (PB) samples were obtained, with informed consent, from 48 children with newly diagnosed ALL and 39 children with relapsed ALL. All children from the relapsed ALL group had previously been exposed to DNR and or DOX as part of multidrug front-line chemotherapy. None of the children with relapsed ALL had been tested before at initial diagnosis. Samples were processed within 24 h after collection.

Mononuclear cells were isolated by Ficoll density-gradient centrifugation (Ficoll Paque, density 1.077g ml^{-1}; Pharmacia, Sweden) and washed twice in RPMI-1640 containing 0.1% bovine serum albumin. Representative *in vitro* drug resistance data can be generated if more than 70% ALL cells are present after a 4 day cell culture, since *in vitro* drug resistance will be overestimated if more than 30% contaminating non-malignant cells are present (Kaspers *et al.*, 1994). To increase the number of evaluable cell cultures, the leukaemic cell population was enriched in 10 87 samples by removing contaminating non-malignant cells using monoclonal antibodies linked to magnetic beads (Dynabeads M-450, Dynal, Norway). We incubated cell suspensions (50×10^6 cells ml^{-1}) for 30 min at 37°C with one or a combination of the following mouse monoclonal antibodies (ITK, The Netherlands) directed against myeloid cells (CD13 and CD15, dilution 1:50

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Table I *In vitro* and *in vivo* drug concentrations

Drugs	In vitro *Concentration range in μg ml⁻¹*	In vivo *PPC[a] in μg ml⁻¹ (dose mg m⁻² i.v.)*	References
DNR	0.002 - 2.0	0.23 (45)	Speth *et al.* (1989)
DOX	0.008 - 8.0	1.64 (30)	Speth *et al.* (1987)
IDR	0.002 - 2.0	0.05 (10)	Speth *et al.* (1989)
ACR	0.002 - 2.0	0.03 (25)	Yamada *et al.* (1980)
MIT	0.001 - 1.0	0.68 (15)	Van Belle *et al.* (1986)

[a]PPC, peak plasma concentration after one i.v. bolus.

Table II *In vitro* antileukaemic activity and *in vivo* toxicity

Drugs	In vitro LC_{50}[a]	*Relative activity*[b]	*MTD*[c]	In vivo *Relative toxicity*[d]	References
IDR	0.025	1.0	15 - 18	1.0	Ganzina *et al.* (1986)
MIT	0.049	2.0	24 - 33	1.3 - 2.2	Ungerleider *et al.* (1985)
DNR	0.083	3.3	60 - 75	3.3 - 5.0	Carter and Livingston (1982)
ACR	0.118	4.7	85 - 120	4.7 - 8.0	Van Echo *et al.* (1982)
DOX	0.255	10.2	60 - 75	3.3 - 5.0	Carter and Livingston (1982)

[a]Median LC_{50} in μg ml^{-1} of the untreated ALL group. [b]LC_{50} of each drug relative to IDR. [c]Maximum tolerable dose in mg m^{-2} after one i.v. bolus. [d]MTD of each drug relative to IDR.

or 1:100), monocytes (CD14, dilution 1:100) or T lymphocytes in case of non-T-lineage ALL samples (CD2, dilution 1:100). Cell suspensions were washed three times with RPMI and 10% fetal calf serum. Magnetic beads, coated with sheep anti-mouse immunoglobulin G, were added to the cell–antibody suspension (ten beads to one target cell) for 30 min at 37°C. The contaminating normal cells, linked through antibodies to the beads, were separated from the leukaemic cells by magnetic force. The mean percentage of leukaemic cells of the ten samples treated with beads increased from 75% to 89% using this method. The *in vitro* chemosensitivity of leukaemic cells was not influenced by treatment with monoclonal antibodies linked to beads (Kaspers *et al.*, 1994).

In vitro *chemosensitivity*

In vitro chemosensitivity did not differ between BM and PB samples (Kaspers *et al.*, 1991). All samples were freshly cultured with the exception of one cryopreserved sample. The MTT assay was performed as described before (Pieters *et al.*, 1990). Briefly, microculture plates containing 96 wells with 20 μl frozen aliquots of a drug were thawed just before testing and 80 μl of leukaemic cell suspension (2×10^6 ml^{-1}) was added. Leukaemic cells were cultured for 4 days in the absence or presence of six concentrations of each drug in duplicate.

May–Grünwald Giemsa-counterstained cytospins of control cells were made and showed that all samples contained ⩾80% leukaemic cells at onset of the cell culture and ⩾70% leukaemic cells after a 4 day cell culture. After 4 days, we added 10 μl of 5 mg ml^{-1} MTT to each well and the microculture plates were incubated for another 6 h. The tetrazolium salt MTT is reduced to dark-coloured formazan crystals by viable cells only. Formazan crystals were dissolved with 100 μl of acidified isopropanol. The optical density (OD) was measured at 565 nm with an EL-312 microplate reader (Biotek Instruments, Winooski, USA). The OD is linearly related to the number of viable cells (Kaspers *et al.*, 1991). We calculated the leukaemic cell survival (LCS) from the following equation:

$$LCS = \frac{OD_{treated\ cells}}{\text{mean } OD_{control\ cells}} \times 100\%$$

We used the LC_{50}, the drug concentration lethal to 50% of the leukaemic cells, as parameter of *in vitro* chemosensitivity. Comparable *in vitro* drug resistance data can be obtained by repeated testing of samples. LC_{50} values fall within the range of one dilution step (data not shown).

Statistics

The LC_{50} values were non-parametrically distributed. Therefore, differences in the LC_{50} distribution between untreated and relapsed childhood ALL samples were tested using the two-tailed Mann–Whitney *U*-test. Spearman rank correlation coefficients (rho) were calculated to determine the cross-resistance patterns between the drugs tested.

Results

Anti-leukaemic activity

In general, for each drug steep dose–response curves were obtained. *In vitro* chemosensitivity was not influenced by cell culture efficiency: we found no significant correlations between the LC_{50} values of each drug and the control leukaemic cell survival (rho = −0.01 to 0.06, $P > 0.50$) or between the LC_{50} values and the OD per 10^5 viable control cells (rho = −0.01 to 0.19, $P > 0.08$). The *in vitro* chemosensitivity varied 40- to 300-fold among all patients: LC_{50} values (μg ml^{-1}) of DNR ranged from 0.012 to 1.294, of DOX from 0.023 to 1.374, of MIT from 0.003 to >1, of IDR from 0.002 to 0.363 and of ACR from 0.037 to 1.531.

IDR was *in vitro* the most active anti-leukaemic drug. Intra-patient comparisons showed that in general the lowest LC_{50} values were found for IDR, followed by MIT, DNR, ACR and DOX. In Table II the median LC_{50} values of the untreated ALL samples are ranked and the anti-leukaemic activity is given relative to IDR; for example, a 3.3-fold higher DNR concentration is required compared with IDR to obtain an equal *in vitro* anti-leukaemic response. However, Table II shows that an increase in *in vitro* anti-leukaemic activity corresponds to an increase in the clinical toxicity as defined by the maximum tolerable bolus of each drug; for example compared with DNR, IDR is *in vitro* about 3-fold more active, but the equitoxic dose of IDR is about 3-fold lower.

Untreated vs *relapsed childhood ALL*

The control leukaemic cell survival did not differ ($P = 0.66$) between untreated (median 75%, range 35–138%) and

relapsed (median 72%, range 27–259%) childhood ALL samples. The OD per 10^5 control leukaemic cells did not differ ($P = 0.20$) between untreated (median 0.235, range 0.082–0.649) and relapsed (median 0.257, range 0.108–0.675) childhood ALL samples. Despite considerable overlap of the LC_{50} values between both groups, the relapsed ALL group was significantly more resistant ($0.001 < P < 0.033$) to all five drugs tested than the untreated ALL group (Figure 1). We calculated the resistance ratios, i.e. the ratio of the median LC_{50} values from the relapsed and untreated ALL group; the resistance ratios ranged from 1.5 to 2.7 (Table III).

Cross-resistance pattern

We found a significant ($P < 0.001$) correlation between the LC_{50} values of all drugs tested in 87 childhood ALL samples (Table IV). Figure 2 shows the cross-resistance pattern in childhood ALL between the front-line drug DNR *vs* DOX, IDR, ACR and MIT. Separate analyses of the untreated ($n = 48$) and relapsed ($n = 39$) ALL groups gave comparable Spearman rank correlation coefficients. A strong correlation was found between DNR, DOX, IDR and MIT (rho = 0.75–0.84), and to a lesser extent between ACR and the other four drugs (rho = 0.50–0.57).

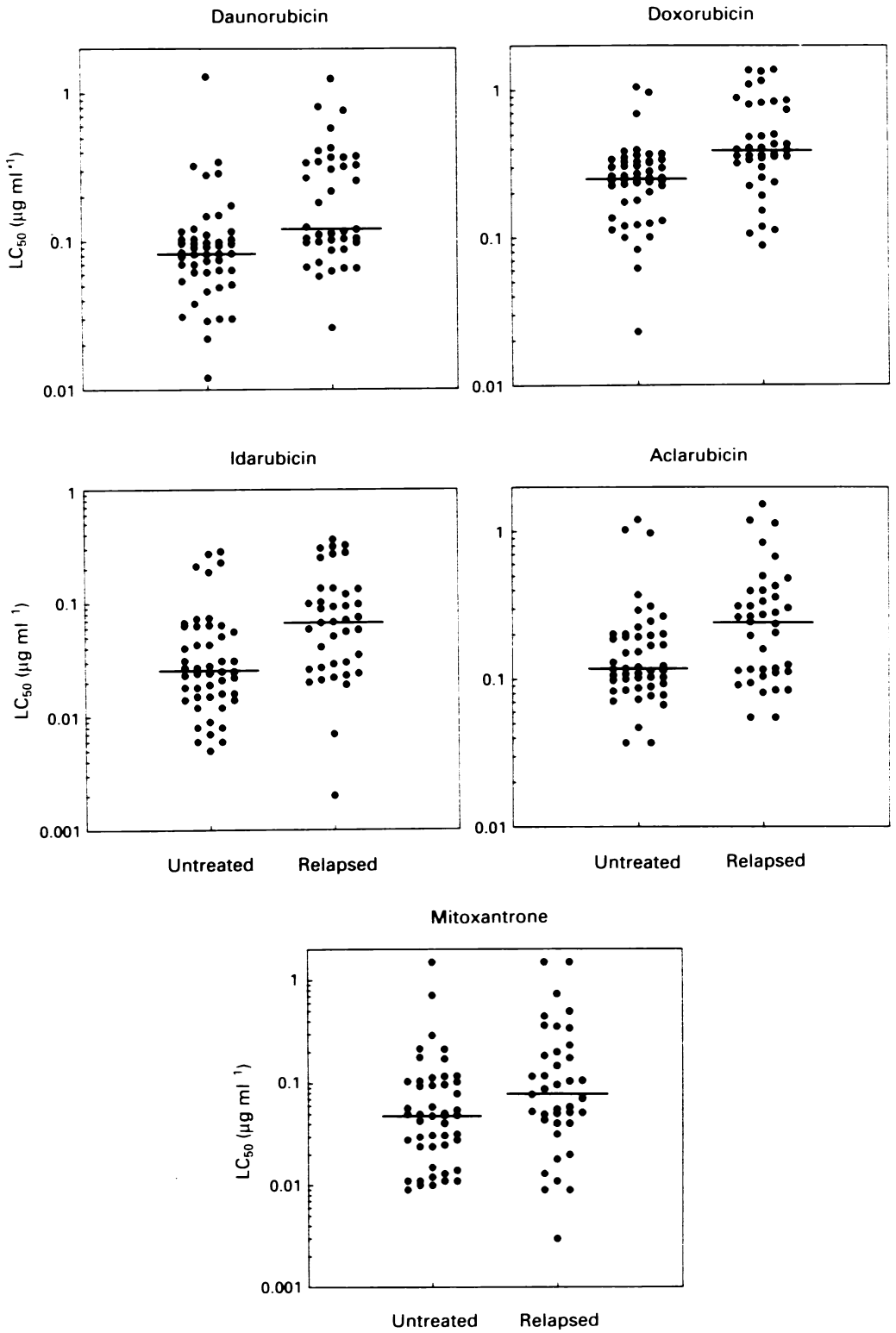

Figure 1 The *in vitro* cytotoxicity of daunorubicin, doxorubicin, idarubicin, aclarubicin and mitoxantrone in 48 untreated compared with 39 relapsed samples from children with acute lymphoblastic leukaemia. Note that different scales for the y-axis are used. Median LC_{50} values are indicated (——).

Discussion

We have shown previously that resistance to DNR at initial diagnosis is correlated with a poorer long-term clinical outcome in childhood ALL (Pieters *et al.*, 1991). Moreover, resistance to DNR and several other drugs such as prednisolone and L-asparaginase, might explain the poor prognosis of relapsed childhood ALL (Klumper *et al.*, 1993). Analogues lacking cross-resistance to DNR can theoretically circumvent DNR resistance, which may improve chemotherapy in relapsed childhood ALL. These analogues, such as IDR, ACR and MIT, have been identified in several cell line studies (Hill *et al.*, 1985; Coley *et al.*, 1989; Erttmann *et al.*, 1991). However, other cell line studies have reported contrasting *in vitro* resistance patterns, for example a full cross-resistance between MIT *vs* DNR and DOX has been found (Scott *et al.*, 1986; Gupta *et al.*, 1988). There is a need for such comparative studies using patient samples.

In the present study, we showed that samples from children with relapsed ALL were 2-fold more resistant not only to DNR and DOX, but also to IDR, ACR and MIT, compared with the untreated childhood ALL group. Moreover, the *in vitro* cytotoxicities of DNR, DOX, IDR, ACR and MIT were closely correlated. Thus, a pronounced cross-resistance developed to all five drugs suggesting that IDR, ACR and MIT cannot circumvent DNR resistance in childhood ALL. However, our results do not exclude the possibility that some individual children with relapsed ALL might benefit from ACR after front-line therapy including DNR, since we found a less pronounced although still significant cross-resistance between ACR and the other drugs tested.

Although relapsed ALL samples were more resistant than untreated ALL samples to all five drugs tested, the LC_{50}

Table III Comparison of the *in vitro* chemosensitivity between 48 untreated and 39 relapsed children with ALL

	Median LC_{50}[a] in μg ml^{-1}			
Drugs	*Untreated*	*Relapsed*	*Resistance ratio*[b]	P-*value*[c]
DNR	0.083	0.121	1.5	<0.001
DOX	0.255	0.390	1.5	<0.001
IDR	0.025	0.068	2.7	<0.001
ACR	0.118	0.244	2.1	0.005
MIT	0.049	0.078	1.6	0.033

[a]LC_{50} drug concentration lethal to 50% of the ALL cells. [b]Resistance ratio, the ratio of the median LC_{50} values from the relapsed and untreated ALL group. [c]Two-tailed Mann–Whitney *U*-test.

Table IV Spearman correlation coefficients[a] of daunorubicin (DNR), doxorubicin (DOX), idarubicin (IDR), mitoxantrone (MIT) and aclarubicin (ACR) in 87 childhood ALL samples

	DNR	*DOX*	*IDR*	*MIT*	*ACR*
DNR	–	0.84	0.84	0.75	0.53
DOX	0.84	–	0.82	0.81	0.57
IDR	0.84	0.82	–	0.81	0.51
MIT	0.75	0.81	0.81	–	0.50
ACR	0.53	0.57	0.51	0.50	–

[a]All correlations were significant at $P<0.001$.

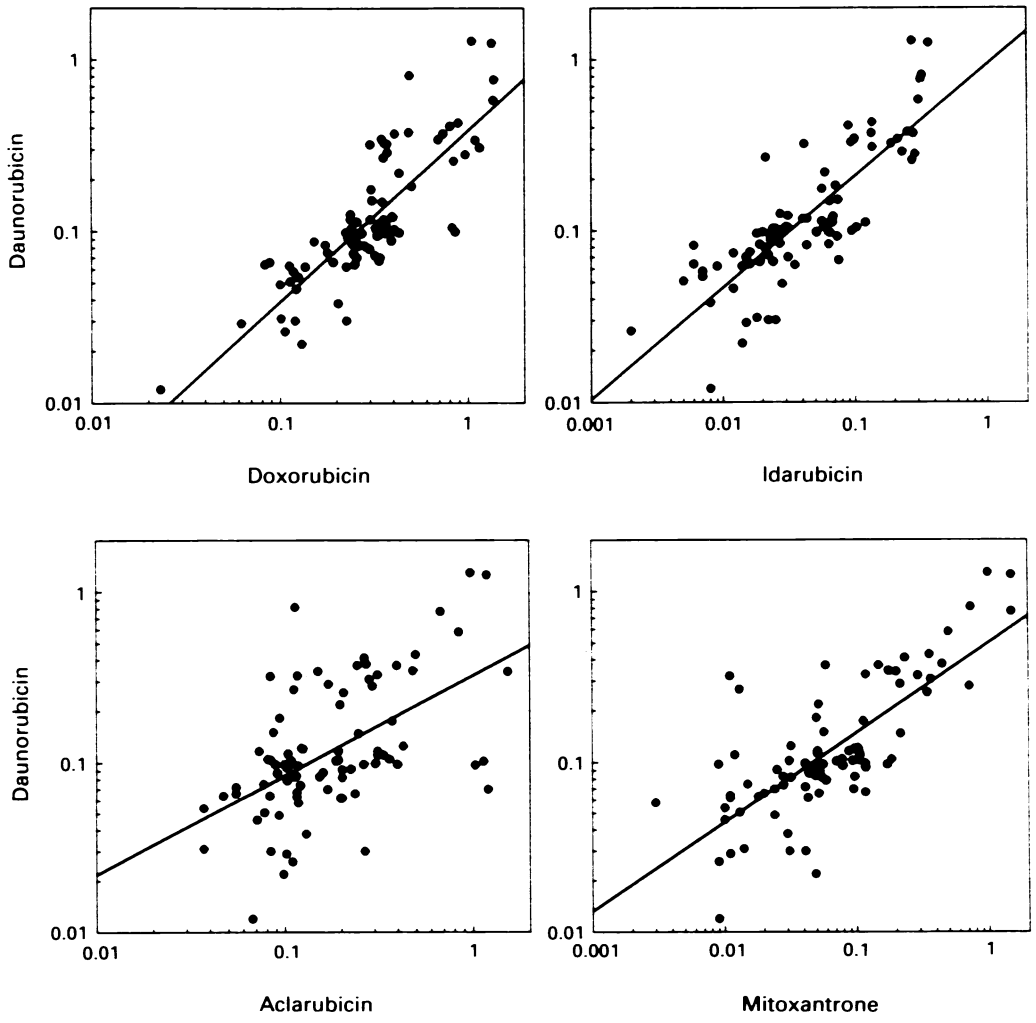

Figure 2 The *in vitro* cross-resistance pattern of daunorubicin *vs* doxorubicin, idarubicin, aclarubicin and mitoxantrone in 87 samples from children with acute lymphoblastic leukaemia. Each point represents a paired LC_{50} value (μg ml^{-1}) obtained from the same patient sample.

values of both groups showed considerable overlap. This suggests that DNR resistance in relapsed ALL may already be present at first diagnosis and that some children with relapsed ALL remained chemosensitive to DNR. Cell lines often express a 10- to more than 100-fold drug-induced resistance, whereas we found relative low resistance ratios for the anthracyclines in childhood relapsed ALL, ranging from 1.5 to 2.7. However, these resistance ratios were based upon the ratio of the median LC_{50} of the relapsed compared with the untreated ALL group; inter-patient chemosensitivities differed over 100-fold. Although Hill *et al.* (1989) have argued that cell lines expressing low levels of drug-induced resistance would be more suitable for the study of clinically relevant resistance mechanisms, our results based on patient samples suggest that cell lines with both low and high levels of drug-induced resistance could be used to investigate drug resistance. These large inter-patient variations probably reflect the clinical heterogeneity of tumour samples, which hampers the extrapolation of results from single cell line studies to clinically relevant results.

No anthracycline resistance mechanisms or strategies to modulate anthracycline resistance with major clinical relevance in childhood ALL have been identified so far. P-glycoprotein expression has been most extensively studied in childhood ALL, but most studies could not detect significant differences in P-glycoprotein expression between untreated and relapsed childhood ALL samples (Ubezio *et al.*, 1989; Tawa *et al.*, 1990; Mizuno *et al.*, 1991; Gekeler *et al.*, 1992; Pieters *et al.*, 1992; Brophy *et al.*, 1994), whereas the latter samples were significantly more resistant to anthracyclines in the present study. Moreover, we showed previously that neither verapamil nor cyclosporin A could modulate *in vitro* DNR resistance in childhood ALL (Pieters *et al.*, 1992). Although multiple factors are likely to cause drug resistance, short-term cell culture drug resistance assays, such as the MTT assay, measure the end point of all possible resistance mechanisms, i.e. leukaemic cell kill, which is shown to be of predictive value in childhood ALL (Pieters *et al.*, 1991).

Our results suggest that IDR, ACR and MIT do not have a higher therapeutic index than DNR since their *in vitro* anti-leukaemic activity was paralleled by their toxicity on normal cells, represented by the MTD. We found that IDR was the most active anti-leukaemic drug *in vitro*, a fact that is well known (Fields and Koeller, 1991). One clinical study reported a higher (statistically not significant) complete remission rate with IDR (75%) than DNR (59%) when both are used in combination chemotherapy in relapsed childhood ALL (Feig *et al.*, 1992). However, this study used increasing IDR doses to determine its MTD in combination chemotherapy, while the dose intensity of DNR was given below the MTD level, as shown by a significant higher toxicity in the group of children treated with IDR. Although our study suggests that the therapeutic index may not differ between IDR and DNR, we did not take into account other advantages of IDR compared with DNR, such as oral administration (Erttmann *et al.*, 1988; Pui *et al.*, 1988) and penetration of the cerebrospinal fluid (Reid *et al.*, 1990). In contrast to childhood ALL, a large randomised study in adult acute myeloid leukaemia (AML) reported a superior response rate for IDR compared with DNR in remission induction chemotherapy (Berman *et al.*, 1991).

Although our results suggest that IDR, ACR and MIT cannot circumvent DNR resistance in childhood ALL, clinical studies with IDR, ACR and MIT showed marked anti-leukaemic responses in children with relapsed ALL previously treated with DNR or DOX (Vietti *et al.*, 1983; Starling *et al.*, 1985; Ungerleider *et al.*, 1985; Fengler *et al.*, 1987; Madon *et al.*, 1987; Tan *et al.*, 1987; Erttmann *et al.*, 1988; Pui *et al.*, 1988; Giona *et al.*, 1990; Graham *et al.*, 1991). However, the response rate for DNR and DOX in such selected patient groups is not known and might well be similar to that of the other three drugs mentioned. Moreover, a second complete remission rate up to 90% can be achieved with combination chemotherapy in relapsed childhood ALL (Henze *et al.*, 1991). Thus, a response to IDR, ACR or MIT in relapsed childhood ALL is not conclusive for a lack of cross-resistance to DNR and or DOX. Phase I II single-agent studies are difficult to compare as small, highly selective patient groups are usually tested, in contrast to our study comparing five drugs within a uniform patient group.

In summary, we showed that the relapsed ALL group was about twice as resistant to DNR, DOX, IDR, ACR and MIT as the untreated ALL group. Significant cross-resistance was observed between DNR, DOX, IDR, ACR and MIT, indicating that IDR, ACR and MIT cannot circumvent *in vitro* resistance to the conventional compounds DNR and DOX in childhood ALL samples. These results suggest that IDR, ACR and MIT are unlikely to enhance the anti-leukaemic response by replacing DNR in combination chemotherapy of relapsed childhood ALL, but these results, based upon cellular drug resistance profiles, do not exclude possible pharmacokinetic advantages of these analogues.

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