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Intracellular uptake and cytotoxic effect in vitro of doxorubicin and epirubicin in human leukemic and normal hematopoietic cells

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Summary. Leukemic cells from patients presenting with acute nonlymphoblastic leukemia and normal hematopoietic bone marrow cells from healthy donors for allogeneic bone marrow transplantation were incubated for 3 h with doxorubicin and epirubicin at different concentrations. The intracellular uptake at the end of the incubation was determined by photofluorometry in leukemic cells from 15 patients and in normal cells from 9 donors for bone marrow transplantation. Cytotoxicity in vitro against granulocyte/macrophage colony-forming units (CFU-GM) was determined in normal cells from 7 donors, and in vitro toxicity against leukemic cells was determined by a clonogenic technique in cells from 6 patients and by vital dye staining (DiSC) following 4 days' culture in cells from 15 patients. Epirubicin was significantly less toxic than doxorubicin to normal hematopoetic cells (72% ± 20% survival of cells for epirubicin vs 45% ± 13% for doxorubicin at a concentration of 0.2 μM; $P \leq 0.005$). As analyzed by the DiSC assay, 0.2 μM epirubicin was slightly more toxic to leukemic cells than was the same concentration of doxorubicin (47% vs 61% survival, $P \leq 0.01$), but the clonogenic assay revealed no difference in toxicity to leukemic cells. At a concentration of 0.2 μM, the mean intracellular uptake of epirubicin in leukemic cells was 0.43 ± 0.26 nmol/mg protein as compared with 0.33 ± 0.14 nmol/mg protein for doxorubicin (not significant). In normal cells, the uptake of epirubicin at a concentration of 0.2 μM was 0.47 ± 0.25 nmol/mg protein as compared with 0.31 ± 0.21 nmol/mg protein for doxorubicin (not significant). The reduced myelotoxicity observed in vitro together with the retained toxicity to leukemic cells indicates that the therapeutic index of epirubicin is better than that of doxorubicin.

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Introduction

Anthracycline antibiotics are widely used to treat leukemia and many solid tumors. Their clinical use is hampered by acute bone marrow toxicity and by chronic cumulative cardiotoxicity [7]. A number of analogues have been synthesized with the aim of reducing the toxicity of these agents and thereby increasing their therapeutic index. One of these analogues, epirubicin, is an epimer of doxorubicin in which the hydroxyl group at position 4 of the aminosugar has been rotated from the L-lyxo configuration of doxorubicin to the L-arabino configuration.

From studies in animals [5] and in patients [2, 5, 6, 18], there are indications that the myelotoxicity and chronic cardiotoxicity induced by epirubicin are less severe than these caused by doxorubicin. The effect of epirubicin on solid tumors appears to be comparable with that of doxorubicin. Some reports have indicated that the former drug also shows activity against doxorubicin-resistant tumors. It has also been reported that epirubicin may exhibit clinical activity against a wider spectrum of solid tumors [2, 6]. Clinical phase I–II studies have revealed activity of epirubicin against acute leukemias [14], but there have been no randomized studies comparing the antileukemic effect of these two drugs. Plasma pharmacokinetic studies have shown a similar pattern but suggest a higher distribution volume and a faster elimination for epirubicin as compared with doxorubicin [3, 4, 13, 19]. Another difference between the two drugs is that the concentrations of glucuronides of epirubicin and of its 13-dihydro metabolite found in plasma and urine are higher than those of the corresponding doxorubicin metabolites [4, 13, 19].

We have previously shown that there are differences in the in vivo intracellular pharmacokinetics of doxorubicin and epirubicin in leukemic cells [17]. Epirubicin reached higher intracellular peak concentrations but was eliminated somewhat faster than doxorubicin. These differences may explain the discrepancies in the clinical activity of the two drugs. The aim of the present study was to evaluate in vitro possible differences of clinical interest between the two drugs with regard to intracellular pharmacokinetics and

toxic effects on both leukemic cells and normal hematopoietic stem cells. We used isolated fresh leukemic blasts from patients presenting with untreated acute nonlymphoblastic leukemia and truly normal hematopoietic stem cells from bone marrow donors. The incubations were performed such that the intracellular drug concentrations mimicked the in vivo concentrations achieved during therapy [16].

Materials and methods

Patients and cell separation. Leukemic cells from patients suffering from newly diagnosed acute nonlymphoblastic leukemia were studied prior to the start of chemotherapy. The cells (80%–90% pure, >90% trypan blue exclusion) were separated from peripheral blood (400 *g* for 20 min) on Lymphoprep (Nyegaard & Co, AS, Oslo, Norway; specific weight, 1.067) and were then washed twice in phosphate-buffered saline (PBS, pH 7.4; 1000 *g* for 10 min). The cytotoxic effect was determined by the differential staining cytotoxicity (DiSC) assay in cells from 15 patients and the clonogenic assay in cells from 6 subjects. Due to low plating efficiency, the clonogenic assay failed in another 5 cases. Intracellular uptake was determined in cells from 15 patients. Normal bone marrow cells were obtained from healthy bone marrow donors for allogeneic bone marrow transplantations. Approximately 2 ml bone marrow was added to a test tube containing 125 units heparin in 3 ml RPMI 1640. Mononuclear cells were separated and washed as described above. The in vitro cytotoxicity was studied in cells from seven donors and the intracellular uptake, in cells from nine donors.

Incubations. After being separated and washed leukemic and normal bone marrow cells were resuspended in RPMI 1640 supplemented with 10% fetal calf serum (FCS) and 1% glutamine. Doxorubicin and epirubicin (Farm-Italia Carlo Erba, Milan, Italy) were diluted in PBS to 10 times the incubation concentrations. Then, 1.8 ml cell suspension was incubated at 37°C with 0.2 ml anthracyclines at final concentrations of 0.2, 0.5 μM and/or 1 μM in a gently shaking bath. Cells were incubated for 3 h, after which steady-state concentrations were achieved. All incubations were performed in duplicate. Dose-response curves for intracellular uptake and in vitro cytotoxicity were established after incubation at concentrations of 0.1–2 μM with cells from four and five of these patients, respectively. In cells from two patients, the intracellular accumulation was followed for 3 h. These cells were exposed to drugs in the manner described above, but in addition to the 3-h exposure, incubations were also terminated at 0, 5, 15, 30, 60, and 120 min.

In vitro anthracycline assay. For in vitro uptake studies in leukemic and normal bone marrow cells, we used a cell concentration of 1×10^6 cells/ml. The incubations were terminated by the addition of 5 ml ice-cold PBS and the cells were thereafter kept on ice. Cells were washed twice in PBS at 4°C and were frozen at –20°C until analysis. After thawing, the cells were sonicated for 20 s at 50 W using a Branson B-12 sonicator (Branson Sonic Power Company, Danbury, Conn.) and the drugs were extracted with trichloroacetic acid (TCA, 27%). The drugs were assayed by photofluorometry as previously described [11] using a model RF-510 Shimadzu spectrofluorometer (excitation and emission wavelengths, 485 and 560 nm, respectively). Anthracycline concentrations in each sample were determined by comparison with identically treated standard solutions and were related to the amount of cell protein determined according to Lowry et al. [10].

In vitro cytotoxic effect, DiSC assay. After incubation of leukemic cells at a cell concentration of 1×10^5 cells/ml with anthracyclines as described above, the cell suspension was centrifuged (400 *g* for 10 min) and the medium was removed. Without being washed the cells were resuspended in fresh medium (2 ml RPMI 1640 supplemented with 10% FCS and 1% glutamine in each tube) and cultured for 4 days at 37°C in a humidified incubator containing 5% CO_2 (ASSAB, T-303). The DiSC assay was performed according to Weisenthal et al. [20], with slight modifications. After being cultured, 0.2 ml of each sample was vitally stained with 0.2 ml 2% fast green together with an internal standard of fixed goose erythrocytes. After 10 min, 0.2 ml of this suspension was cytospin-centrifuged (Shanon Cytospin, 1300 rpm for 7 min) and the living cells were counterstained with hematoxylin-eosin. The cytotoxic effect of the tested drug was determined by the ratio of living cells to goose erythrocytes and was expressed as a percentage of the same ratio in a drug-free control. A minimum of 300 viable cells were counted on each slide.

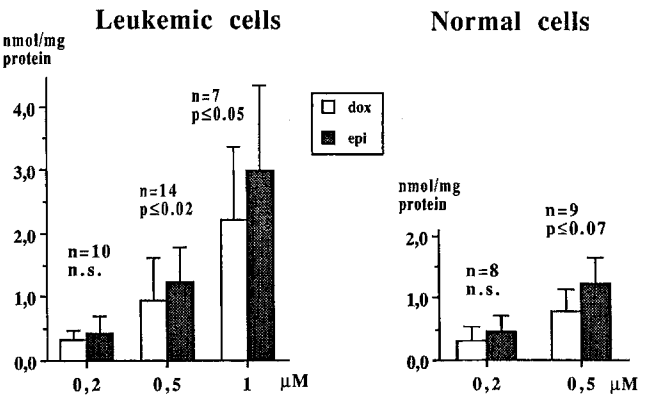

Fig. 1. Mean intracellular uptake (± SD) of epirubicin and doxorubicin at different concentrations in leukemic and normal bone marrow cells in vitro

In vitro cytotoxic effect, clonogenic assay. Leukemic and normal mononuclear bone marrow cells were incubated with drugs as described above. After their incubation, the samples were centrifuged (400 *g*, 10 min), the supernatant was discarded, and in the absence of a wash, the cells were resuspended in 5 ml agar medium and cultured in 35-mm culture dishes (Falcon 3080). The samples were prepared in triplicate, with 1 ml being used in the feeder layer and 1 ml, in the overlayer [9]. One-third of the feeder layer constituted double-concentrated McCoy's medium supplemented with 30% FCS, one-third was 1.5% agar, and the other third comprised human leukocyte-conditioned medium [12], representing a final concentration of 15% FCS and 0.5% agar in McCoy's medium. The cell suspension was added to the overlayer (consisting of 15% FCS and 0.3% agar in McCoy's medium to give a final cell concentration of 1×10^5 cells/ml. The cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2. After 9–12 days, colonies comprising >40 cells were counted and the cytotoxic effect was expressed as a percentage of that of a drug-free control.

Statistical evaluation. For statistical comparison of intracellular uptake and cytotoxic effect, Student's *t*-test for paired data was used.

Results

Intracellular uptake in leukemic cells

The intracellular uptake in vitro of doxorubicin and epirubicin at different incubation concentrations is shown for individual patients in Table 1 and as a mean overall value in Fig. 1. At a concentration of 0.5 μM, the uptake of epirubicin was significantly higher than that of doxorubicin. The mean uptake at this concentration was 1.22 ± 0.6 nmol/mg protein for epirubicin as compared with 0.94 ± 0.7 nmol/mg protein for doxorubicin ($P \leq 0.02$). The uptake of epirubicin at a concentration of 1 μM was also significantly higher than that of doxorubicin (2.98 ± 1.36 vs 2.22 ± 1.14 nmol/mg protein; $P \leq 0.05$).

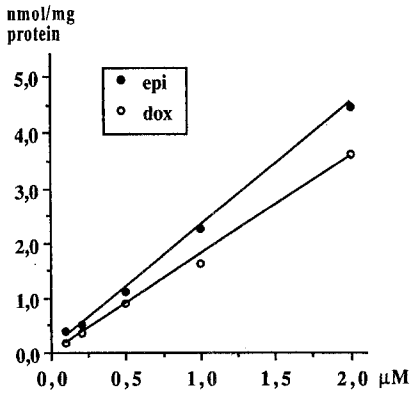

Fig. 2. Dose-response curves for incubation concentration versus intracellular drug uptake of epirubicin and doxorubicin. Each point represents the mean value for cells from 4 patients

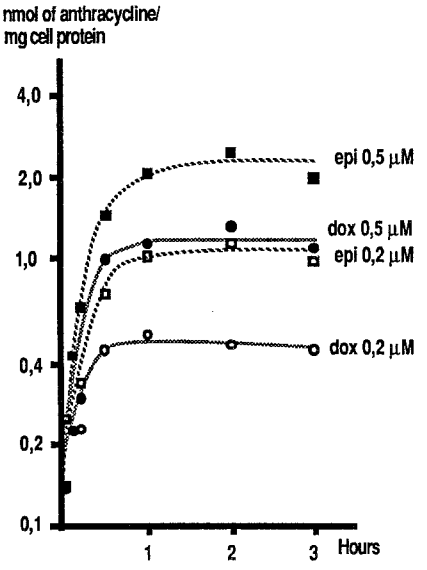

Fig. 3. Intracellular accumulation in vitro of doxorubicin and epirubicin at different concentrations in leukemic cells from one patient

At a concentration of 0.2 μM, the mean uptake of epirubicin was 0.43 nmol/mg protein as compared with 0.33 nmol/mg protein for doxorubicin. This difference was not statistically significant.

There was a linear correlation between the incubation concentration and the intracellular uptake of both drugs (r = 0.99 for doxorubicin and r = 0.99 for epirubicin; Fig. 2). The difference in uptake between the two drugs increased by a factor of 3.7 with increasing incubation concentrations (r = 0.95 for the difference in uptake vs the incubation concentration). This dose-response relationship was also supported by the more uniform and pronounced difference in uptake at the higher incubation concentrations for both drugs (Table 1, Fig. 1). The initial uptake of both drugs was rapid, followed by a slower accumulation (Fig. 3). Steady state was reached after about 2 h. There was a tendency toward faster initial uptake of epirubicin, and the concentrations of this drug were higher than those of doxorubicin at all times studied.

Intracellular uptake in normal bone marrow cells

Although it was not significant, there was also a strong tendency toward higher uptake of epirubicin as compared with doxorubicin in normal hematopoietic cells, and the intracellular levels were similar to those seen in leukemic cells. At a concentration of 0.5 μM, the intracellular uptake of epirubicin was 1.23 ± 0.41 nmol/mg cell protein as compared with 0.79 ± 0.35 nmol/mg cell protein for doxorubi-

Table 1. Intracellular uptake of epirubicin and doxorubicin in vitro in human leukemic and normal hematopoietic cells

Leukemic cells (nmol/mg cell protein)							Normal cells (nmol/mg cell protein)				
Number	0.2 μM		0.5 μM		1 μM		Number	0.2 μM		0.5 μM	
	Dox	Epi	Dox	Epi	Dox	Epi		Dox	Epi	Dox	Epi
L1	0.18	0.49	–	–	–	–	N1	–	–	0.81	1.29
L2	0.33	0.91	0.34	1.37	–	–	N2	0.16	0.32	0.76	1.15
L3	0.15	0.2	0.5	0.69	–	–	N3	0.15	0.92	0.6	1.76
L4	0.45	0.13	0.61	0.68	–	–	N4	0.11	0.33	0.32	1.16
L5	0.41	0.36	0.52	0.81	–	–	N5	0.34	0.61	0.53	1.5
L6	0.3	0.46	0.68	1.17	–	–	N6	0.38	0.16	1.14	0.33
L7	0.61	0.75	1.25	1.52	2.43	2.99	N7	0.13	0.55	0.49	1.33
L8	0.2	0.57	0.45	0.7	0.91	1.37	N8	0.72	0.63	1.36	1.52
L9	0.3	0.21	1.18	1.06	1.65	1.73	N9	0.48	0.23	1.12	1.03
L10	0.4	0.25	0.79	0.78	–	–					
L11	–	–	0.92	1.99	2.32	3.79					
L12	–	–	1.25	1.51	1.47	3.59					
L13	–	–	0.49	0.85	–	–					
L14	–	–	3	2.67	4.5	5.26					
L15	–	–	1.15	1.21	2.26	2.14					
Mean	0.33	0.43	0.94	1.22	2.22	2.98		0.31	0.47	0.79	1.23
$P \leq$	NS		0.02		0.05			NS		0.07	

Dox, Doxorubicin; Epi, epirubicin; L, leukemic cells; N, normal hematopoietic cells; NS, not significant

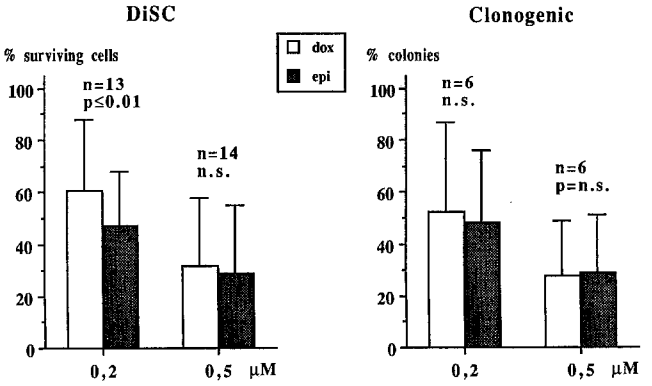

Fig. 4. Mean cytotoxic effect (± SD) of epirubicin and doxorubicin at different concentrations on leukemic cells in vitro as determined using the DiSC or clonogenic assay

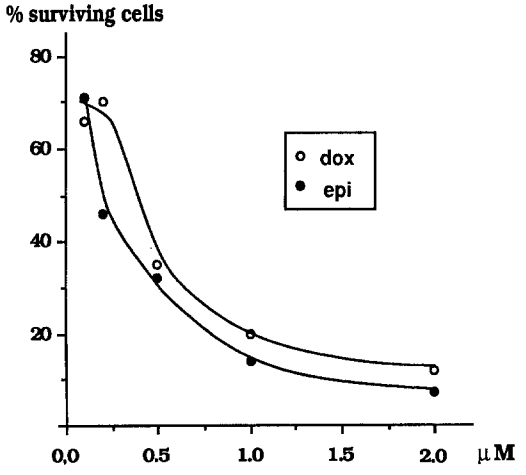

Fig. 5. Dose-response curves for the incubation concentration versus the cytotoxic effect of epirubicin and doxorubicin as determined by the DiSC assay. Each point represents the mean value for cells from 5 patients

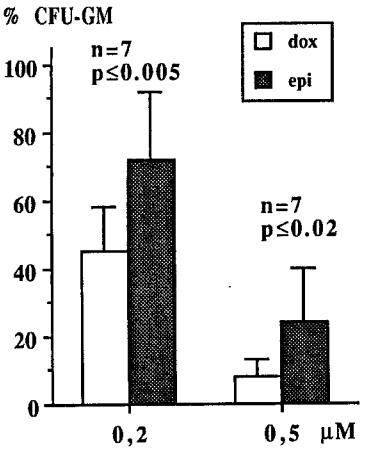

Fig. 6. Mean cytotoxic effect ± SD of epirubicin and doxorubicin determined at different concentrations on normal human hematopoietic stem cells in vitro as determined by clonogenic assay

cin ($P \leq 0.07$). This difference was also more uniform and pronounced at the higher incubation concentrations. This is shown for individual patients in Table 1 and as a mean overall value in Fig. 1. At a concentration of 0.2 µM, the mean uptake of epirubicin was 0.47 nmol/mg protein as compared with 0.31 nmol/mg protein for doxorubicin (not significant).

Cytotoxic effect in vitro on leukemic cells

The cytotoxic effect of epirubicin and doxorubicin on leukemic cells in vitro is shown in Fig. 4 and Tables 2 and 3. At a concentration of 0.2 µM, epirubicin was significantly more toxic than doxorubicin as analyzed by the DiSC assay (61% survival of cells for doxorubicin vs 47% for epirubicin, $P \leq 0.01$). At lower and higher incubation concentrations, this difference was reduced (Fig. 5). The clonogenic assay revealed no significant difference between the toxicity of doxorubicin and that of epirubicin. Both assays showed a clear dose-response relationship for the two drugs at the concentrations studied.

Cytotoxic effect in vitro on normal bone marrow cells

The mean cytotoxic effect of epirubicin and doxorubicin on human mononuclear normal bone marrow cells is

Table 2. Cytotoxic effect of epirubicin and doxorubicin in vitro on leukemic and normal bone marrow cells as determined by clonogenic assay

Leukemic cells (% colonies)					Normal cells (CFU-GM)				
Number	0.2 µM		0.5 µM		Number	0.2 µM		0.5 µM	
	Dox	Epi	Dox	Epi		Dox	Epi	Dox	Epi
L1	83	57	43	35	N6	28	46	2	5
L3	0	22	5	5	N7	34	61	7	16
L5	57	57	53	54	N8	66	81	10	28
L12	85	66	44	38	N9	41	90	11	43
L14	65	79	14	44	N10	45	53	7	13
L20	19	5	10	0	N11	56	100	15	46
					N12	42	72	2	15
Mean	52	48	28	29		45	72	8	24
$P \leq$	NS		NS			0.005		0.02	

Dox, Doxorubicin; Epi, epirubicin; L, leukemic cells; N, normal bone marrow cells; NS, not significant

Table 3. Cytotoxic effect of epirubicin and doxorubicin in vitro on human leukemic cells as determined by the DiSC assay

Number	% Surviving cells			
	0.2 μM		0.5 μM	
	Dox	Epi	Dox	Epi
L2	49	50	25	17
L4	51	29	24	26
L5	26	26	13	10
L6	82	64	–	–
L7	76	23	20	13
L8	26	27	5	8
L9	–	–	4	4
L10	77	54	45	66
L11	34	34	12	8
L12	–	–	37	37
L14	54	44	14	11
L16	39	36	48	29
L17	75	55	39	39
L18	100	78	100	68
L19	100	88	64	76
Mean	61	47	32	29
$P \leq$	0.01		NS	

Dox, Doxorubicin; Epi, epirubicin; NS, not significant

shown in Fig. 6 and Table 2. At both concentrations studied, epirubicin was significantly less toxic than doxorubicin to these cells. The mean value was 72%±20% CFU-GM for epirubicin as compared with 45%±13% CFU-GM for doxorubicin at a concentration of 0.2 μM ($P \leq 0.005$) and 24%±16% CFU-GM for epirubicin vs 8%±5% CFU-GM for doxorubicin at a concentration of 0.5 μM ($P \leq 0.02$). This difference was consistent with epirubicin's being less toxic than doxorubicin to all cells studied.

Discussion

In a previous in vivo study, we found that when patients presenting with acute leukemia were treated with doxorubicin and epirubicin, the peak concentrations of epirubicin in leukemic cells was higher and there was a tendency toward more rapid elimination of this drug as compared with doxorubicin [17]. The present results are in accordance with these previous in vivo findings. At incubation concentrations of 0.5 and 1 μM, the peak concentration of epirubicin was significantly higher than that of doxorubicin. At a concentration of 0.2 μM, the mean uptake of epirubicin was 43% higher than that of doxorubicin, but this difference was not statistically significant.

These results support the hypothesis that the differences in intracellular pharmacokinetics previously found between doxorubicin and epirubicin in vivo originated at the leukemic cell level rather than representing general differences in the metabolism and excretion of the drugs. The pK_a of the amino group of doxorubicin is 8.34 as compared with 8.08 for that of epirubicin, and this parameter can affect the membrane binding of drugs [8]. In addition, the lipid solubility of epirubicin is slightly higher than that of doxorubicin [15], and lipophilicity affects drug transport across cell membranes. Moreover, the affinity of epirubicin for DNA is somewhat lower than that of doxorubicin [8]. Taken together, these differences in physicochemical properties result in the higher initial uptake and lower intracellular retention of epirubicin.

Previous findings indicate that a high intracellular peak concentration is important for the antileukemic effects of anthracyclines [1]. However, the effect of the higher peak concentration can be offset by more rapid elimination, which might explain why our results did not convincingly demonstrate that epirubicin was more toxic than doxorubicin to tumor cells. Incubation of leukemic cells with doxorubicin at a concentration of 0.2 μM for 3 h is the most clinically relevant treatment since it gives intracellular drug concentrations that closely mimic those achieved in vivo during therapy [16]. Under these conditions, the DiSC assay revealed an increased cytotoxicity of epirubicin to leukemic cells. This was not confirmed at higher incubation concentrations or by the clonogenic assay. The probable reason for this discrepancy between the assays is that the difference in cytotoxicity between the two drugs was comparatively small and often lay within the margin of error for the assays (a difference of ≤10% in the survival of cells or colonies).

As evaluated by the survival of CFU-GM, in contrast to our findings in leukemic cells, the toxicity of epirubicin to normal hematopoietic stem cells was significantly lower than that of doxorubicin. This difference was pronounced and was consistently observed in cells from different bone marrow donors. Thus, it appears clear that at least in vitro, the intracellular pharmacokinetic profile of epirubicin, involving higher peak concentration and lower retention, results in lower toxicity to normal hematopoietic stem cells but at least similar toxicity to leukemic cells. The clearly reduced toxicity of epirubicin to normal hematopoietic cells at incubation concentrations that are equitoxic to leukemic cells indicate a more favorable therapeutic index for epirubicin as compared with doxorubicin. Our findings in the present study and in the previous in vivo study suggest that epirubicin could be of value in the treatment of acute leukemia and deserves to be clinically evaluated in this disease.

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