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Uptake and Intracellular Distribution of Doxorubicin Metabolites in B-Lymphocytes of Chronic Lymphocytic Leukemia[1]

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ABSTRACT

The toxicity of doxorubicin metabolites was evaluated on lymphocytes of B-cell chronic lymphocytic leukemia. Only doxorubicinol was found to be cytotoxic for these lymphocytes, whereas exposure to aglycones at concentrations as high as 5 μM for 1 h had no effect on the proliferative capacity of these cells. After exposure of cells to isomolar concentrations of doxorubicin or its metabolites, uptake/retention of doxorubicinol was 23% of doxorubicin, and uptake/retention of aglycones was 5 to 13% of doxorubicin. Seventy to 90% of doxorubicin and 60 to 90% of doxorubicinol taken up/retained by the cells were detected in the cell nuclear fraction, whereas only 20 to 40% of the aglycones were localized in the cell nucleus. Cytotoxicity of metabolites was generally related to the proportion of drug taken up/retained by the cells and localized to the nuclei. The low uptake and nuclear localization may be at least partially responsible for the lack of cytotoxicity of aglycones on B-lymphocytes from chronic lymphocytic leukemia.

INTRODUCTION

Previous studies have demonstrated that, among the various metabolites of doxorubicin, only doxorubicinol is cytotoxic to human bone marrow erythroid and myeloid progenitors *in vitro* (1, 2). Since all doxorubicin metabolites share the quinone ring in their molecule, and the quinone ring has been thought to represent an active, oxygen-cycling part of the molecular structure of these compounds (3, 4), the reasons for the lack of cytotoxicity of doxorubicin aglycones and the significantly lower toxicity of doxorubicinol compared to the parent compound remain unclear. We investigated the cellular handling of these compounds with the hypothesis that differences in uptake, accumulation, retention, or intracellular distribution of these metabolites might be responsible for the differences in cytotoxicity *in vitro* among them.

Although our initial studies on the toxicity of doxorubicin metabolites were performed on bone marrow mononuclear cells (2), in the present study we have used a more uniform population of target cells, namely B-lymphocytes from CLL.[4] The lymphocytes were chosen because they are easily available, easily purified, and represent a homogeneous population of cells capable of proliferating *in vitro* under appropriate experimental conditions. We have found that, similar to bone marrow erythroid and myeloid progenitors among the various metabolites of doxorubicin, only doxorubicinol had *in vitro* cytotoxicity against the B-CLL lymphocytes. Furthermore, the uptake and/or retention of the various metabolites by these lymphocytes and the percentage of the metabolites detectable in the nuclear fraction of these cells were found to correlate with the presence or lack of toxicity.

MATERIALS AND METHODS

Materials. Doxorubicin hydrochloride was purchased as commercial material from Adria Laboratories or was obtained courtesy of the National Cancer Institute. Purity was documented by a single peak on a previously described HPLC assay (5) at maximum sensitivity (1.0 pmol of doxorubicin injected in methanol stock solution). Other chemical reagents were obtained from Fisher laboratories and were standard laboratory grade.

For the cytotoxicity assay, IMEM was obtained from Gibco, Grand Island, NY; bovine serum albumin and FCS were from Reheis, Phoenix, AZ; and phytohemagglutinin was from Calbiochem, Detroit, MI. [^{3}H]dThd was obtained from New England Nuclear, Boston, MA.

Synthesis and Purification of Doxorubicin Metabolites. The following metabolites were synthesized according to the published procedures of Takanashi and Bachur (6): doxorubicinol; doxorubicin aglycone; 7-deoxydoxorubicin aglycone; doxorubicinol aglycone; and 7-deoxydoxorubicinol aglycone. The identity of the standards was confirmed by mass spectroscopy. The purity of all aglycone standards was determined as >95% by HPLC analysis (5). Doxorubicinol was 100% pure by HPLC analysis. All compounds were prepared for the experiments with B-CLL lymphocytes in normal saline.

For use in the cell culture assay, parent compound and aglycone metabolites were added to normal saline. There were no difficulties in obtaining high concentration (100 μM) solutions of doxorubicin and doxorubicinol. The aglycones are less soluble in water. To maximize concentrations, supersaturated solutions of aglycones were incubated in normal saline at 27°C for 4 to 6 h and protected from light. Following repetitive vigorous mixing, the solution was centrifuged at 2500 rpm for 10 min at 4°C. The supernatant was removed and stored overnight at −20°C. The next morning, the solution was thawed and recentrifuged as above. The concentration was determined based upon doxorubicin fluorescence equivalents as described previously (5). The solution was frozen one more night and recentrifuged the next morning. No sediment was found after the second night's storage. The solutions were then used in the cell culture system. Maximum concentrations obtained for the aglycones in saline solution using this technique are: doxorubicin aglycone, 20 μM; doxorubicinol aglycone, 25 μM; 7-deoxydoxorubicin aglycone, 6 μM; and 7-deoxydoxorubicinol aglycone, 16 μM. After the freezing and rethawing procedure discussed above, the aglycone concentrations in the 5, 10, or 20% DMSO solutions were no higher than those obtained in normal saline without DMSO.

Cytotoxicity Assay. After informed consent, peripheral blood from five patients with CLL was collected in heparin (5 units/ml). All patients were in a chronic asymptomatic phase of CLL and had never received cytotoxic chemotherapy. The mononuclear cells were separated by velocity sedimentation over Ficoll-Hypaque (7). Cells at the interface were collected and washed twice, and the adherent cells were removed by adherence to plastic (8) and T-lymphocytes by 2-aminoethylisothiouronium bromide-treated sheep red cell rosetting (9). The recovered cell population consisted of greater than 85% B-lymphocytes as determined by the presence of surface immunoglobulin (10). B-lymphocytes (5×10^6) were incubated with varying concentrations of doxorubicin or its metabolites in 1 ml of IMEM for 1 h at 37°C in a water bath with a 5% CO_2 atmosphere. The cells were then washed twice with 5 ml of IMEM containing 1% bovine albumin, and 5×10^6

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[4] The abbreviations used are: CLL, chronic lymphocytic leukemia; IC_{50}, concentration of drug causing 50% inhibition of cell thymidine uptake or causing 50% of cells to die; [^{3}H]dThd, tritiated thymidine; IMEM, Iscove's modification of Dulbecco's minimal essential medium; HPLC, high-pressure liquid chromatography; FCS, fetal calf serum; DMSO, dimethyl sulfoxide.

cells were cultured in 1 ml of IMEM with 15% FCS in the presence of 2×10^6 autologous, mitomycin-treated T-lymphocytes (11) and 10 μg/ml of phytohemagglutinin (12). After 3 to 4 days of incubation at 37°C in a 5% CO_2 atmosphere, the cultures were pulsed with [^{3}H]dThd. Following 8 h of incubation, the cells were harvested and washed twice with isotonic saline, twice with 5% trichloroacetic acid, and then with absolute methanol. The pellet was dissolved in hyamine and added to vials containing scintillation fluid. In this system, incorporation of [^{3}H]dThd into B-lymphocytes is linear with relation to the number of viable cells added to cultures. Toxicity of doxorubicin or its metabolites was expressed as the percentage of counts from cultured B-CLL lymphocytes after incubation with medium alone.

Cellular Uptake and Distribution. For studying the cellular uptake/retention of doxorubicin or its metabolites by these lymphocytes, 2.5×10^6 cells were incubated in the presence of 5 μM doxorubicin or its metabolites in IMEM for 1 h at 37°C in a 5% CO_2 atmosphere. The cells were then washed 4 times with 5 ml of ice-cold phosphate-buffered saline containing 10% FCS. Doxorubicin or its metabolites were assayed as described below.

To study the intracellular distribution of doxorubicin or its metabolites, cells exposed to the drug were lysed with 0.5% Nonidet P-40, and the nuclear as well as the nonnuclear fractions were prepared according to the method of Long *et al.* (13). Doxorubicin and its metabolites were extracted from the pelleted nuclear and nonnuclear fractions and assayed as described below. All experiments were performed in quadruplicate.

In those experiments aiming at determining the time course of uptake/retention of doxorubicin metabolites by B-CLL lymphocytes, 2.5×10^6 cells were incubated as above at 37°C for 3, 5, 15, 30, 60, and 90 min. Immediately upon termination of incubation, the cells were separated from the drug solution by centrifugation at $15,000 \times g$ for 1 min at 4°C over a 0.5-ml mixture of 90% *n*-butylphthalate and 10% mineral oil. The drug solution and the oil mixture were discarded, and doxorubicin metabolites were extracted from the cell pellet and measured as described below.

Quantitation of Doxorubicin and Metabolites. Total drug fluorescence in the extracts, a measure of doxorubicin and its fluorescent metabolites, was determined after extraction with methanol:0.6 N HCl (3:1) as previously published (14). The extraction efficiency is 90% and reproducible in duplicate specimens. The concentrations were quantitated against a standard curve which was linear over concentrations ranging from 10 nmol/ml of extract to 1000 nmol/ml of extract. Concentrations were determined based upon doxorubicin fluorescence equivalents as described previously (5).

RESULTS

Exposure of B-CLL lymphocytes to doxorubicin or doxorubicinol following by a 3- to 4-day culture in the presence of T-lymphocytes and phytohemagglutinin resulted in a significant decline of the amount of [^{3}H]dThd incorporated into these cells. Exposure of cells to aglycones at concentrations as high as 5 μM for 1 h had no effect on [^{3}H]dThd incorporation. These findings indicated that, among the metabolites of doxorubicin, only doxorubicinol exerts a toxic effect upon these lymphocytes. The IC_{50} of doxorubicinol was approximately 6.2% of that exerted by the parent compound (Fig. 1).

When B-CLL lymphocytes were exposed to isomolar concentrations (5 μM) of doxorubicin or its metabolites, the amount of drug that was taken up/retained by these cells was higher for doxorubicin (675 ± 19 nmol/2.5×10^6 cells), less for doxorubicinol (158 ± 17 nmol/2.5×10^6 cells), and even lower for aglycones (doxorubicin aglycone, 36 ± 12; 7-deoxydoxorubicin aglycone, 98 ± 38; 7-deoxydoxorubicinol aglycone, 93 ± 25 nmol/2.5×10^6 cells). Doxorubicinol uptake/retention was 23% of parent compound. Aglycone uptake/retention was even lower, 5% to 14% of doxorubicin (Fig. 2). The percentage of the drugs taken up/retained by these cells did not change when the incubation medium contained 1% human albumin or 10% human serum.

The differences of uptake/retention between the parent compound and its metabolites do not appear to be attributed to differences in efflux. In time course experiments, both doxorubicin and its metabolites were found to be taken up/retained by B-CLL lymphocytes in a similar fashion. Uptake/retention of doxorubicin or its metabolites by these cells increased progressively over 15 min and reached a maximum within 30 to 60 min (Fig. 3).

The wide differences in the amount of the drug that was detected in the cells between doxorubicin, doxorubicinol, and aglycones became greater when the cells were lysed and the concentration of the drug was determined in the pelleted nuclei (Fig. 4). The proportions of the metabolite detected in the nuclear fraction as the percentage of total drug taken up/retained by the cells were 70 to 90% for doxorubicin and 60 to

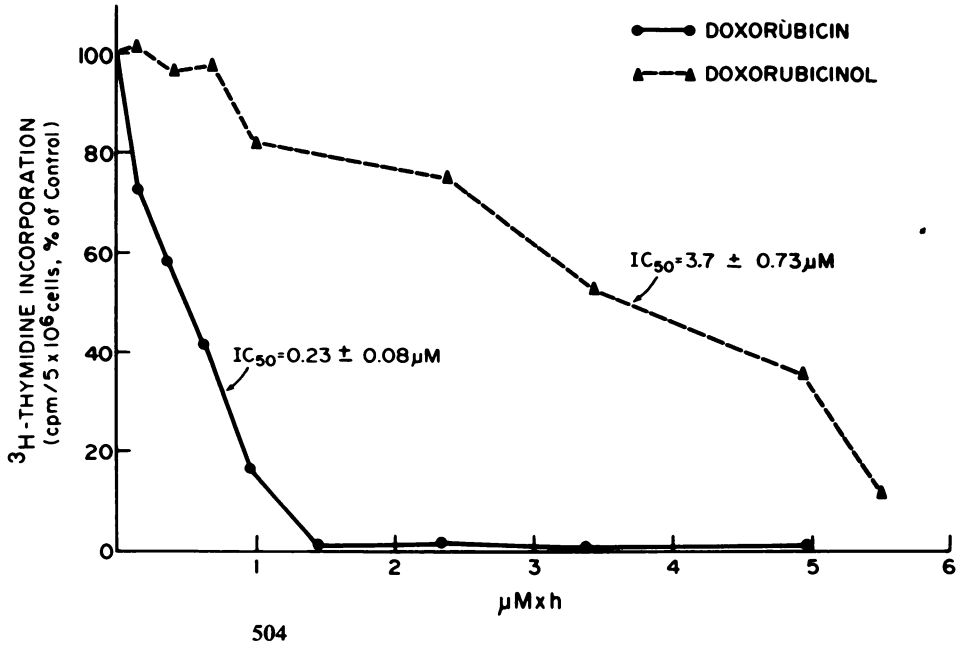

Fig. 1. Effect of doxorubicin and doxorubicinol on [^{3}H]dThd incorporation into B-CLL lymphocytes. Results are expressed as the percentage of counts of B-CLL lymphocytes cultured under the same conditions without prior exposure to drugs. In all experiments the cells were incubated with the drug or its metabolites for 1 h. Each *point* represents the mean from three experiments.

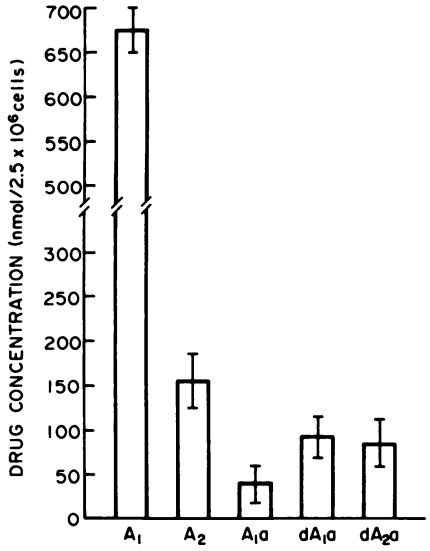

Fig. 2. Concentration of doxorubicin and its metabolites in B-CLL lymphocytes. Each *column* represents the mean from three experiments; *bars*, SEM. A_1, doxorubicin; A_2, doxorubicinol; A_1a, doxorubicin aglycone; dA_1a, 7-deoxydoxorubicin aglycone; dA_2a, 7-deoxydoxorubicinol aglycone.

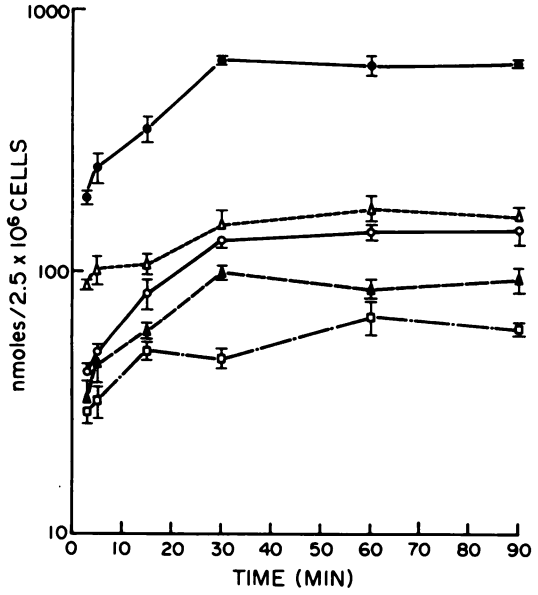

Fig. 3. Time course of uptake/retention of doxorubicin and its metabolites by B-CLL lymphocytes. B-CLL lymphocytes (2.5×10^5) were incubated at 37°C for various periods of time and then centrifuged for 1 min at 15,000 × *g* over a 90/10 mixture of *n*-butyl-phthalate/mineral oil. Doxorubicin metabolites were extracted from the cell pellet and measured as described in "Materials and Methods." *Points*, mean from quadruplicates representing nmol/2.5×10^6 cells; *bars*, SEM. ●, doxorubicin; △, doxorubicinol; ○, doxorubicin aglycone; ▲, 7-deoxydoxorubicin aglycone; □, 7-deoxydoxorubicinol aglycone.

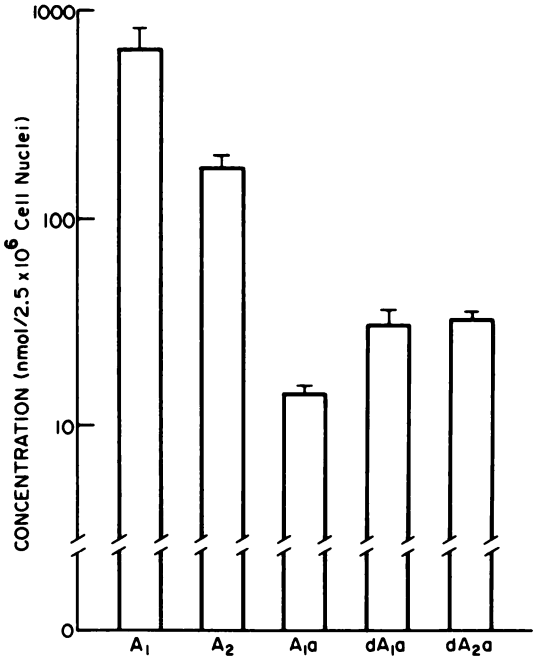

Fig. 4. Concentration of doxorubicin and its metabolites in the nuclei of B-CLL lymphocytes. *Columns*, mean from triplicates of a representative experiment; *bars*, SEM. A_1, A_2, etc. denote same metabolites as in Fig. 2.

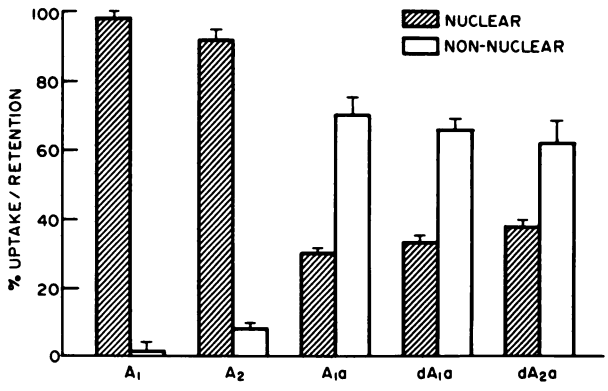

Fig. 5. Amount of doxorubicin and its metabolites detected in the nuclear and nonnuclear cell fractions expressed as percentage of total cellular retention. *Columns*, mean of triplicates from a representative experiment; *bars*, SEM. A_1, A_2, etc. as in Fig. 2.

90% for doxorubicinol. Only 20 to 40% of the aglycones were localized in the cell nucleus (Fig. 5).

DISCUSSION

The metabolism of anthracyclines is complex, resulting in the appearance in plasma and in cells of reduced glycoside metabolites and aglycones. From studies using human urinary extracts (6) and liver and kidney extracts (15–17), doxorubicinol and its aglycones have been shown to be major metabolites. Takanashi and Bachur (6), Asbell *et al.* (15, 16), and Bullock *et al.* (17) have studied the metabolic pathways for both doxorubicin and its analogue daunorubicinol. Asbell *et al.* (16) have compared the toxicities on L1210 cells of the alcohol metabolite of daunorubicin, daunorubicinol, and an unidentified metabolite (presumably the aglycone) to the parent compound. The IC_{50} for daunorubicinol was found to be 6-fold less than that of the parent compound, while that of the presumed aglycone was 80-fold less cytotoxic. Yesair *et al.* (18) expanded these findings by evaluating the effects of the 7-deoxyaglycones of doxorubicin, doxorubicinol, daunorubicin, and daunorubicinol upon L1210 growth, colony formation, and drug uptake. IC_{50}s of aglycone metabolites were 100-fold higher, and those of the alcohol metabolite were 10-fold higher than those of the parent compound. Furthermore, Yesair *et al.* found lower uptake of daunorubicinol in L1210 cells as well as evidence of intracellular metabolism of daunorubicin to daunorubicinol. Ozols *et al.* (19) found that doxorubicin aglycone at high concentrations (1.0 μg/ml) was equally toxic for ovarian carcinoma colony formation to doxorubicinol, but less cytotoxic than the parent compound. At more physiological concentrations, the aglycone

was not as toxic as either the parent compound or the reduced glycoside.

The work presented here extends our recently published cytotoxicity studies using human bone marrow erythroid and myeloid progenitor cell assays. The IC_{50} ratio of doxorubicin to doxorubicinol inhibition of [^{3}H]thymidine incorporation in CLL lymphocytes was 16.1 compared to 11.8 in myeloid progenitor cell and bone marrow erythroid colony assays (2). The results are also similar to previously published data by other investigators (16, 19) including those by Yesair *et al.* (18), who used P388 and L1210 leukemia cell lines. While the work of Yesair *et al.* focused upon established, sensitive, screening cell lines, the current study emphasizes the use of *ex vivo* human leukemic cells. In all studies, cytotoxicity of doxorubicinol is less than that of the parent drug and is associated with decreased cellular uptake. The current studies have extended these findings to the aglycones and have made an assessment of the intracellular localization of these metabolites. The tested aglycone metabolites, doxorubicin aglycone, 7-deoxydoxorubicin aglycone, and 7-deoxydoxorubicinol aglycone, which are all detected in human plasma (5), did not cause any decrease in thymidine incorporation up to an incubation concentration of 5 μM over 1 h.

The glycoside forms of doxorubicin, the parent compound, and doxorubicinol are handled differently from the aglycones by B-CLL lymphocytes. The parent compound is most avidly taken up/retained by the cells. Doxorubicinol uptake is 23% of the doxorubicin uptake. Both glycosides localize to the nucleus. Ninety-nine % of the total doxorubicin and 92% of the total doxorubicinol were found in the cell nuclear fraction. At the same incubation concentrations, intracellular aglycone concentrations were only 7% to 15% of the intracellular concentration of doxorubicin. Of the total aglycone that was present intracellularly, only 20% to 40% was localized in the nucleus. These findings suggest that a much lower proportion of the aglycones either enter or are associated with the membrane of the cells compared to the glycosides. Furthermore, the majority of the aglycones that enter the cell do not associate themselves with the nucleus. Although anthracyclines may cause cytotoxicity without nuclear localization (20, 21), nuclear localization of the cytotoxic glycosides *vis-a-vis* the noncytotoxic aglycones suggests that nuclear localization may be a marker for doxorubicin-induced cytotoxic activity. It is possible that the low concentrations of aglycones which gain cellular entry may be insufficient to cause cytotoxicity. The maximal aglycone incubation concentrations used in these cytotoxicity experiments, 5 μM, are 100 times the maximum concentrations that are clinically found in human plasma (5). Therefore, it is unlikely that *in vivo* higher concentrations will be obtained intracellularly than what was obtained in the present study *in vitro*. Aglycones, if found intracellularly in clinical specimens, are probably the result of intracellular metabolism or anaerobic free radical formation (22, 23) rather than the result of transmembrane passage of metabolites from plasma.

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