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Cytotoxicity and DNA damage caused by 4-demethoxydaunorubicin and its metabolite 4-demethoxy-13-hydroxydaunorubicin in human acute myeloid leukemia cells

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Summary. 4-Demethoxydaunorubicin (4-DMDR) and its major metabolite 4-demethoxy-13-hydroxydaunorubicin (4-DMDRol) were investigated for their cytotoxicity and mode of action against human leukemic cells. The drug and its metabolite appeared to be equally potent as both inhibitors of cell proliferation and inducers of DNA double-strand breaks in the OCI AML-3 cell line and cells derived directly from patients with acute myeloid leukemia (AML). This suggests that 4-DMDRol plays an important role in the antileukemic activity of 4-DMDR.

Introduction

4-Demethoxydaunorubicin (4-DMDR) is an anthracycline [1] with proven clinical antileukemic activity [5, 7, 8]. Clinical pharmacokinetic studies have shown that 4-DMDR is extensively biotransformed to 4-demethoxy-13-hydroxydaunorubicin (4-DMDRol), a metabolite that is eliminated much more slowly than the parent drug [11]. To elucidate the possible role of 4-DMDRol in the antileukemic activity of 4-DMDR, we carried out a series of experiments comparing the cytotoxicity of these drugs and the DNA damage they induce.

Materials and methods

Cells. Peripheral blood was obtained from five adult patients with AML after they had given their informed consent to participate in the study. Mononuclear cells were isolated by Ficoll-Hypaque centrifugation. At this point, the blast-cell content of the samples was >90%. The OCI AML-3 cell line was derived from a patient with acute myelomonocytic leukemia (FAB M4) (Biondi, manuscript in preparation) and was maintained at 37°C in a humidified atmosphere containing 5% CO_2 and cultured in Iscove's medium with 10% fetal calf serum. Four patients had previously been treated with chemotherapeutic agents including ara-C, vincristine, doxorubicin, daunorubicin, mitoxanthrone, VP16 and 4-DMDR; the other subject (patient 5) had not received prior treatment.

Drug treatment. 4-DMDR and 4-DMDRol were synthesized and generously provided by Dr. Suarato of Farmitalia Carlo Erba (Milan, Italy). They were pure as assessed by HPLC. Before their use, the drugs were dissolved in sterile water at a concentration of 1 mg/ml and then diluted in medium at the indicated concentrations. Leukemic blast cells were resuspended in Iscove's medium with glutamine, 25 m*M* HEPES, sodium bicarbonate, 1% sodium piruvate, 20% fetal calf serum and were stimulated with 10% GCT conditioned medium (Gibco) for 24 h before drug treatment. In all experiments, cells were exposed to the drugs for 4 h.

Determination of DNA double-strand breaks. DNA double-strand breaks (DNA-DSB) were determined according to the method described and recently reviewed by Kohn et al. [6]. OCI AML-3 cells were labeled for 48 h using medium supplemented with 0.04 μCi/ml [^{14}C]-thymidine (specific activity, 61 m Ci/mmol), then were washed, resuspended in medium and exposed for 4 h to several concentrations (0.005, 0.01, 0.05, 0.1 and 1 μg/ml) of DMDR or DMDRol. Cell were washed, resuspended in cold PBS and layered on polycarbonate filters (pore size, 0.8 μm; diameter, 47 mm) (Nucleopore Corp., Pleasanton, Calif). Cells ($2-3 \times 10^5$ cells/sample) were lysed with 2 ml lysis solution containing proteinase K and the elution buffer [20 m*M* ethylenediaminetetraacetate (EDTA) solution (adjusted to pH 9.6) containing 0.1% sodium dodecyl sulfate (SDS)] and were pumped for 15 h through the filter at approximately 2 ml/h. Fractions were collected every 3 h and processed as previously described [8].

Colony assays. Colony assays were carried out according to previously described methods [4, 9]. In brief, 10^3 OCI AML-3 cells were plated in 0.5-ml cultures in Iscove's modified Dulbecco's medium (IMDM; Gibco Laboratories, Grand Island, N. Y.) containing 20% heat-inactivated fetal calf serum (FCS; Flow Laboratories, UK) and 0.3% agar (Agar Noble; Difco Laboratories, Detroit, Mich.) over an underlayer containing 0.5% agar in the same medium. After 6–7 days, the agar overlayers were removed from the underlayers, dried on glass slides and fixed in methanol and the colonies were stained with hematoxylin and counted. Each experimental point was determined in quadruplicate.

[^{3}H]-Thymidine incorporation. After treatment with DMDR and DMDRol (0.1 – 1 μg/ml), cells were washed with PBS and incubated in drug-free medium for 72 h. Between 48 and 72 h after the end of drug treatment, cells were exposed to 0.1 μCi/ml [^{3}H]-thymidine (specific activity, 83 Ci/mmol). At 72 h, cells were harvested, washed with PBS

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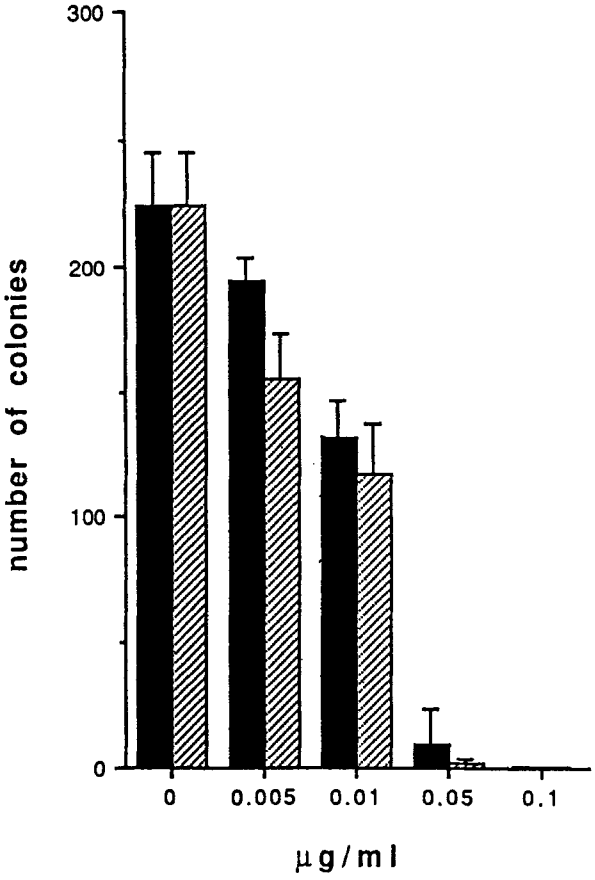

Fig. 1. Cytotoxicity of 4-DMDR (■) and DMDRol (▨) against OCI AML-3 cell as assessed by colony assay. Cells were exposed to the indicated drug concentrations for 4 h. Each value represents the mean of four determinations (±SD)

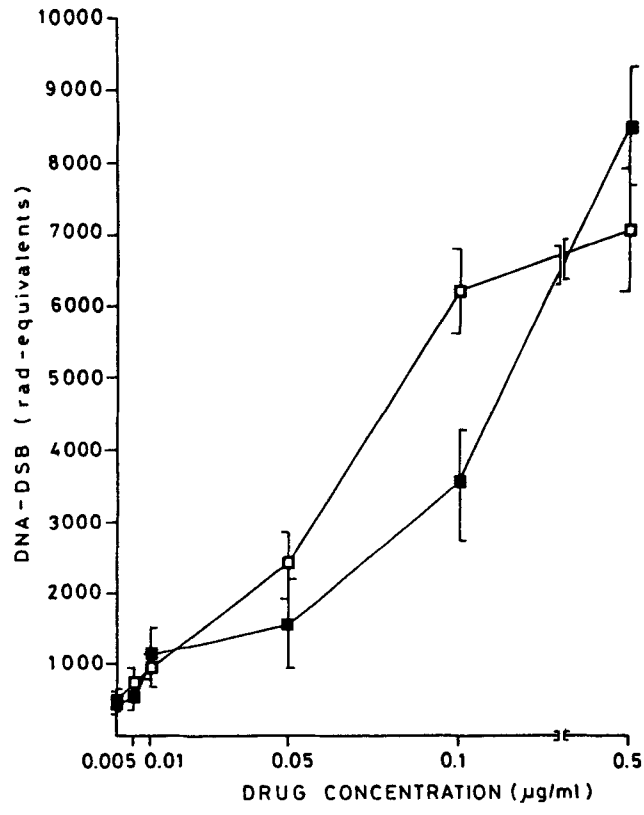

Fig. 2. DNA-DSB induced in OCI AML-3 cells by several concentrations of DMDR (□) and DMDRol (■)

and resuspended in 5% cold trichloroacetic acid (TCA). After at least 6 h at 4° C, the precipitate was collected on a glass microfiber filter (diameter, 2.5 cm; Whatman, GF/C) and washed twice with 2 ml cold 5% TCA and twice again with 2 ml ethanol. The filters were dried and transferred to scintillation vials with 10 ml Pico Fluor 40 Packard solution, and radioactivity was determined using an LS 5800 β-counter (Beckman Instruments, Irvine, Calif.). Each point represented the average of three replications.

Results

Figure 1 compares the cytotoxicity of 4-DMDR and 4-DMDRol against human OCI AML-3 cells. both drugs inhibited colony formation equally in a concentration-dependent manner, with the inhibition being complete at a concentration of 0.1 μg/ml. The inhibition of cell growth by 4-DMDR and 4-DMDRol was comparatively investigated by the assessment of [^{3}H]-thymidine incorporation (Table 1) in the OCI AML-3 cell line and in leukemic cells

Table 1. Inhibition of [^{3}H]-thymidine incorporation by 4-DMDR or 4-DMDRol

Cell type	Control	4-MDR (μg/ml)		4-DMDRol (μg/ml)	
		0.1	1.0	0.1	1.0
OCI AML-3	100±26	0.7±0.6	0.5±0.30	0.9±0.40	0.4±0.05
AML1	100±33	13.5±4.5	2.0±0.01	6.5±0.50	2.0±0.01
AML2	100±7	1.0±0.0	0.5±0.10	1.0±0.01	0.4±0.05
AML3	100±11	9.5±0.6	0.4±0.01	0.4±0.01	0.1±0.01
AML4	100±6	3.3±0.0	3.0±0.05	4.5±0.60	3.4±0.08
AML5	100±33	18.4±7.8	3.3±0.90	22.3±3.50	2.2±0.60

OCI AML-3 or AML cells from five patients were exposed to 4-DMDR or 4-DMDRol for 4 h. Cells were washed and maintained in drug-free medium for 48 h. [^{3}H]-Thymidine was added between 48 and 72 h after the end of drug treatment. Each value represents the mean (±SD) of three determinations and is expressed as the percentage of incorporation as compared with respective controls values

obtaind from five patients with AML. Despite some variability, all cases were sensitive to both 4-DMDR and 4-DMDRol. The OCI-AML-3 cell line showed high sensitivity to both compounds, similar to that found in fresh AML cells from patients 2 and 3 but definitely higher than in the other AML cases (Table 1).

To evaluate whether the mechanisms of cytotoxicity of 4-DMDR and its metabolite 4-DMDRol were similar, we investigated the formation of DNA-DSB in OCI AML-3 cells that had been exposed for 4 h to drug concentrations ranging between 0.005 and 0.5 μg/ml. The two compounds induced similar numbers of DNA-DSB at each dose (Fig. 2).

Discussion

The present study shows that 4-DMDRol is a potent cytotoxic agent showing activity comparable with that of 4-DMDR against both an AML cell line and leukemic cells obtained directly from AML patients. The available pharmacokinetic data clearly indicate that 4-DMDR plasma levels were much lower and of shorter duration than those of its metabolite; moreover, the parent drug can be metabolized to 4-DMDRol by AML cells (data not shown).

Active concentrations of 4-DMDRol are achievable in plasma of patients receiving therapeutic doses of 4-DMDR. For example, concentrations of 0.01 μg/ml that caused a reduction of approx. 50% in OCI AML-3 cell colonies after 4 h exposure were present for several days in plasma of patients receiving a single 4-DMDR dose of either 15 mg i.v. or 30 mg/m^2 p.o. [10, 11].

4-DMDR and 4-DMDRol treatment resulted in similar numbers of DNA-DSB in AML cells, thus confirming previous studies in murine leukemic cells [3] and a human cell line derived from colon cancer [2]. The effect of 4-DMDR can be ascribed to an induction of topoisomerase II DNA cleavable complexes. No information is available on the mechanism of action of 4-DMDRol. Taken together, these data indicate that the cytotoxicity and the mode of action of 4-DMDR and 4-DMDRol are similar but suggest that the metabolite plays a major role in the antileukemic activity of 4-DMDR.

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