In vitro effect of multidrug resistance modifiers on idarubicinol efflux in blasts of acute myeloid leukemia

Abstract Recent results show that the intracellular uptake pattern of idarubicin (IDA) in multidrug-resistant (MDR) cells is nearly identical to that seen in the drug-sensitive parent cell line, whereas the MDR cells have minimal daunorubicin (DNR) uptake compared with the drug-sensitive parent cells. It is known that the major metabolite of IDA, idarubicinol (IDA-OL), has nearly the same cytotoxicity as IDA, while the cytotoxicity of daunorubicinol (DNR-OL) is about 1/30th of that of DNR. We examined the effect of the MDR modifiers verapamil and dexniguldipine on the efflux of IDA, DNR and their hydroxylated metabolites IDA-OL and DNR-OL in blast populations of acute myeloid leukemia (AML), in the MDR-negative cell line CEM-CCRF and in their MDR-positive counterpart (CEM-VBL). All patients with relapsed or persistent AML had been pretreated with IDA and cytosine arabinoside. The efflux of the anthracyclines was estimated by flow cytometry. A total of 36 patients with AML were investigated; 18 out of 36 AML blast populations showed an efflux of DNR, DNR-OL and IDA-OL. The efflux of DNR, DNR-OL and particularly IDA-OL could be reversed by 10 μM verapamil or 1 μM dexniguldipine. For IDA we found an effusion of 40 ± 11% in all blast populations which could not be significantly inhibited by the modulators. Similar results for IDA were found in the MDR-positive cell line (CEM-VBL 100) and in their MDR-negative counterpart (CEM-CCRF). The incubation of CEM-CCRF cells with DNR, DNR-OL, IDA-OL and especially IDA led to MDR induction as determined by reverse transcription/polymerase chain reaction analysis with MDR-specific primer and by cellular efflux studies. We conclude that the outcome of chemotherapy with idarubicin is influenced by MDR, although IDA is not essentially MDR-dependent itself, but because IDA-OL is actively involved in multidrug resistance. Further investigations should consider the question of whether the combination of IDA and MDR modifiers can enhance the serum level of the active metabolite IDA-OL and can reverse the MDR pattern in cells treated with IDA.

Key words Acute myeloid leukemia · Multidrug resistance · MDR modifiers · Idarubicinol

Abbreviations MDR multidrug resistant · DNR daunorubicin · DNR-OL daunorubicinol · IDA idarubicin · IDA-OL idarubicinol · VER verapamil · DNIG dexniguldipine · AML acute myeloid leukemia · AraC cytosine arabinoside · RT-PCR reverse transcription/polymerase chain reaction

Introduction Chemotherapeutic treatment of acute myeloid leukemia (AML) usually includes the pyrimidine analogue cytosine arabinoside (AraC) and an anthracycline derivative. In comparison with more widely used anthracyclines, such as daunorubicin (DNR) and doxorubicin, idarubicin (IDA) has greater growth-inhibiting activity against human tumor cells in vitro, shows superior activity in DNA damage assays, and may be less cardiotoxic (Ames and Speafo 1992; Kuffel et al. 1992; Cersosimo 1992). IDA is a highly lipophilic anthracycline with substantial activity in adult and pediatric leukemias (Carella et al. 1990). Recent results show that the intracellular uptake pattern of IDA in multidrug-resistant (MDR) cells is nearly identical to that seen in the drug-sensitive parent cell lines, whereas the MDR cells have minimal DNR uptake compared with the drug-sensitive parent cells (Berman and McBride 1992). It is known that the major metabolite of DNR, daunorubicinol (DNR-OL) is
about 1/30th as cytotoxic as DNR, while the cytotoxicity of idarubicinol (IDA-OL) is nearly the same as that of IDA (Kuffel et al. 1992; Schott and Robert 1989). Judging from the pronounced formation and long terminal half-life of idarubicinol, it may significantly contribute to the cytotoxicity of the treatment of leukemias with IDA (Ames and Sprafka 1992; Carella et al. 1990; Berman and McBride 1992; Speth et al. 1986).

In an earlier investigation we could demonstrate that patients with de novo AML, treated with IDA and cytosine arabinoside as induction chemotherapy, show an increase in the MDR phenotype at the time of relapse compared with the initial MDR pattern (Schröder et al. 1996a). In the present study, we investigate the effect of verapamil (VER) and dextrigulidine (DNIG) on the efflux of DNR, IDA and their hydroxylated metabolites DNR-OL and IDA-OL in blast populations of AML and also in the human MDR cell line CEM-VBL and their MDR-negative counterpart.

To omit other mechanisms of resistance against anthracyclines, not based on P-glycoprotein, caused by the overexpression of membrane transporters such as MRP (multidrug-resistance-associated protein), the p95 protein or the p110 protein, which are not amenable to reversal by MDR modulators, we included in this investigation only blast populations expressing the p170 glycoprotein (P-glycoprotein) on the cell surface, determined by flow-cytometric analysis and blast populations with a positive DNR efflux pattern (Cole et al. 1992; Chen et al. 1990; Scheper et al. 1993; Doyle et al. 1993).

**Materials and methods**

**Patients**

Bone marrow or peripheral blood blast samples were obtained from 21 patients with newly diagnosed, previously untreated AML, and from 15 patients at the time of first or subsequent relapse. The diagnosis, classification and remission status of AML were assessed according to standard criteria (Cheson et al. 1990). Patients with antecedent hematological disorders, including myelodysplasia or known exposure to carcinogens, were excluded from the analysis. All patients with relapsed or persistent AML had been pretreated with IDA and AraC.

**Treatment and response categories**

The AIDA induction therapy (IDA at a dose of 12 mg/m² administered on days 1–3 as a 30-min infusion, and of AraC at a dose of 100 mg/m² as an intravenous bolus injection on day 2, followed by continuous AraC infusion at a dose of 200 mg/m² on days 2-6) was used for all 21 patients with newly diagnosed AML (Berman et al. 1991; Vogler et al. 1992; Flasshove et al. 1992; Wierink et al. 1992). Of the 21 patients who had received the induction therapy, 14 obtained a complete remission (67%) and received a further two cycles of an AraC-based postremission consolidation therapy. Three out of these 14 patients achieved a complete remission only after two induction therapies; in the others complete remission was diagnosed after the first course. Granulocyte- or granulocyte-macrophage colony-stimulating factors were not routinely employed before, during or after induction or consolidation therapy. Blast persistence was defined as more than 25% bone marrow blasts following induction therapy including standard-dose AraC.

**Cell preparation**

Patient samples eligible for efflux analysis had to have at least 80%–90% leukemic blasts, as determined microscopically and/or by fluorescence-activated cell sorting analysis of surface markers. Before treatment, samples were collected in heparinized tubes and mononuclear cells were isolated within 2–3 h after sample acquisition by Ficoll-Hypaque density-gradient centrifugation (density < 1.077 g/ml; Pharmacia, Uppsala, Sweden).

**Cell lines**

The T-lymphoblastic leukemia cell line CEM-CCRF was obtained from the American Type Culture Collection (ATCC, Manassas, Va., USA). The CEM vinblastine-resistant cell line CEM-VBL was a generous gift from Dr. W. T. Beck, Memphis, Tenn. (Beck et al. 1979). CEM-VBL cells have been previously shown to express p170 on their surface membrane as determined by the monoclonal antibody HYB-241 (Meyers et al. 1989). The cell lines were grown in RPMI-1640 medium (Gibco; Karlsruhe, Germany) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS; Gibco) and 1% l-glutamine (Gibco), and incubated at 37°C with 5% CO₂ and 95% humidified air.

For RNA isolation, samples were lyzed in 4 M guanidine isothiocyanate immediately after aspiration and processed further for nucleic acid isolation as previously described (Chomczynski and Sacchi 1987). RNA obtained from either procedure was resuspended in 50 µl diethylpyrocarbonate-treated water containing 10 U RNAsin (Serva, Heidelberg, Germany) and immediately processed for further use or stored at −70°C.

For resistance induction the MDR-negative cell line CEM-CCRF was incubated with DNR, IDA, DNR-OL and IDA-OL in a concentration of 12.5–40 ng/ml for about 3 weeks. Medium was renewed every 3 days.

**Flow-cytometric determination of P-glycoprotein expression**

The measurement of P-glycoprotein expression was essentially performed as previously described (Müller et al. 1994). After preparation of a single-cell suspension, 2 × 10⁶ cells/tube were resuspended in 50 µl MRK16 antibody diluted to 50 µg/ml in phosphate-buffered saline containing 10% bovine serum albumin (PBS/BSA; test sample). As a control for nonspecific staining, cells were incubated with mouse myeloma protein-IgG2A (Sigma, Deisenhofen, Germany) diluted to 50 µg/ml (control sample). After incubation for 45 min at 4°C, cells were washed twice with PBS. Cells were then resuspended in 50 µl FITC-conjugated rabbit anti (mouse IgG) serum (Sigma, Deisenhofen, Germany) diluted 1:32 in PBS/BSA and incubated for 45 min at 4°C. After two washes with PBS, cells were incubated with 6 µg/ml Hoechst 33342 for 30 min at 37°C. Subsequently, 5 µM propidium iodide was added and cells were immediately analyzed on the flow cytometer.

**Cellular efflux studies**

The efflux of the anthracyclines was estimated by flow cytometry (FACS). For efflux studies, cells at a concentration of 1 × 10⁶/ml were incubated with either DNR, DNR-OL, IDA, IDA-OL or R123 at a concentration of 5 µg/ml with and without VER or DNIG. After 15 min of incubation at 37°C with 5% CO₂ and 95% humidified air, cells were washed and resuspended in RPMI medium with 10% FCS alone or with VER or DNIG. Drugs were diluted with RPMI medium containing 10% FCS before each experiment. Flow-cytometric analysis was performed after 15 min of incubation at 37°C with 5% CO₂ and 95% humidified air. Cells were washed and resuspended in ice-cold RPMI medium with 10% FCS to stop reaction. The P-glycoprotein function was quantified by the percentage decrease of the fluorescence signal after 15 min compared to the maximum fluorescence at the end of the influx.