

Received 20 May 2003
Accepted 24 July 2003

Short Communication

**THE INTERACTION OF DAUNORUBICIN AND MITOXANTRONE
WITH THE RED BLOOD CELLS OF ACUTE MYELOID LEUKEMIA
PATIENTS**

AGNIESZKA MARCZAK^{1*}, AGATA WRZESIEŃ-KUŚ², EUZEBIUSZ
KRYKOWSKI², TADEUSZ ROBAK² and ZOFIA JÓŹWIAK¹

¹Department of Thermobiology, University of Łódź, ul. Banacha 12/16, 90-237
Łódź Poland, ²Department of Hematology, Medical Academy, Łódź, Poland

Abstract: The effect of DNR and MIT on erythrocyte membrane structure was examined using Electron Spin Resonance spectroscopy and the fluorimetric technique. The results suggest that the *in vivo* interaction of the drugs with the RBCs of AML patients led to a perturbation in the structure of plasma membrane components. Differences between DNR and MIT were only noted in the interaction of the drugs with deeper regions of the lipid bilayer.

Key Words: Daunorubicin, Mitoxantrone, Erythrocytes, AML

INTRODUCTION

The anthracycline daunorubicin (DNR) is one of the oldest drugs in leukemia therapy and is still the preferred agent in the treatment of patients with acute myeloid leukemia. DNR cytotoxicity is generally believed to be the result of drug-induced DNA damage, the inhibition of topoisomerase II activity and the generation of reactive oxygen species [1].

Currently, a number of studies are utilizing alternative drugs that are more active against AML. Among the anthracycline-related drugs, mitoxantrone (MIT) is now recognized as a useful agent in first line therapy of acute myeloid leukemia [2].

* Corresponding author, E-mail: aszwar@biol.uni.lodz.pl Fax: (42) 6354473

Abbreviations used: DNR – daunorubicin; MIT – mitoxantrone; RBCs – red blood cells; AML – acute myeloid leukemia; ESR – Electron Spin Resonance; 5-DSA – 5-doxylstearic acid; 16-DSA – 16-doxylstearic acid; Maleimide – 4-maleimido tempo; TMA-DPH – 4'-trimethyl- ammonio-1,6-diphenyl-1,3,5-hexatriene; ROS – Reactive Oxygen Species.

MIT is an anthracenedione derivative with some structural and functional similarities to DNR. Like daunorubicin, MIT is an intercalating agent that causes DNA cleavage via the inhibition of topoisomerase II, gives rise to chromatin compaction and produces free radicals damaging [3, 4]. By contrast to anthracycline drugs, MIT does not undergo metabolic reduction. The biological activity of the drug is probably due to oxidative activation by the peroxidase/H₂O₂ system [5].

Other toxic effect-inducing interactions of DNR and MIT with cellular components are much less known. As both DNR and MIT are intravenously administered to AML patients and are easily absorbed into the circulation, these drugs may also interact with other blood components than leukemic cells. This study was designed to analyse red blood cells damage induced by intravenous injection of DNR and MIT to AML patients. Using electron spin resonance spectroscopy and the spectrofluorimetry technique, we investigated the alterations in the protein conformation and the fluidity of human erythrocytes in the period of 24 h after drug exposure.

MATERIALS AND METHODS

Patients and treatment protocols

Fourteen newly diagnosed AML patients were treated either with DNR (45 mg/m²) or with MIT (12 mg/m²) only. The groups consisted of 7 patients each, aged 47±13 and 63±6, respectively. Blood samples were collected for each patient before drug injection (to serve as controls) and at the indicated time after the administration of the drugs: 0.5, 1, 2 and 24 h.

Preparation of samples

Human peripheral blood was centrifuged at 600×g for 10 minutes. After removal of the plasma and buffy coat, the erythrocytes were washed three times in PBS, (5 mM sodium phosphate buffer, containing 0.15 M NaCl, pH 7.4) and suspended in the same medium. Erythrocyte membranes were obtained by hypotonic lysis according to the procedure of Dodge *et al.* [6], performed at 4°C. Protein concentration was estimated using the method of Lowry *et al.* [7] with bovine serum albumin as the standard.

Electron spin resonance (ESR) measurements

Erythrocytes suspended in PBS to a hematocrit of 50% (75×10⁸ cell/ml) were labeled with 5-DSA or 16-DSA and the membrane proteins were labeled with maleimide as described previously [8].

Fluorescence measurements

The cell suspensions (H=0.004%) were incubated with a TMA-DPH fluorescence probe (final concentration - 10⁻⁶M) at room temperature for 10 min. The measurements were taken at room temperature using a LS-5B Perkin Elmer

Spectrofluorimeter. Fluorescence anisotropy was determined using the method of Van der Meer [9]

Statistical analysis

Data were analyzed via the two-tailed Student's *t*-test of paired data.

RESULTS

As shown in Tab. 1, there were no significant differences in either the order parameter or the anisotropy values for the control and for the erythrocyte samples taken 0.5-24 h after DNR and MIT injection. These data suggest that the two drugs, intravenously administered to AML patients, did not affect lipid fluidity in the upper regions of erythrocyte membranes for up to 24 h.

Tab. 1. The effect of DNR and MIT on the fluidity of the cell membrane in the upper regions of the lipid bilayer in the erythrocytes of patients with acute myeloid leukemia. (n=7).

time [h]	Daunorubicin		Mitoxantrone	
	S	r	S	r
0	0.742±0.009	0.346±0.024	0.733±0.015	0.320±0.015
0.5	0.743±0.010	0.339±0.021	0.736±0.008	0.320±0.013
1	0.742±0.010	0.336±0.033	0.733±0.010	0.318±0.010
2	0.743±0.008	0.338±0.030	0.729±0.010	0.322±0.008
24	0.743±0.011	0.332±0.012	0.728±0.012	0.316±0.015

S – the order parameter derived from the ESR spectrum of 5-DSA, r – anisotropy values derived from the TMA-DPH fluorescent probe.

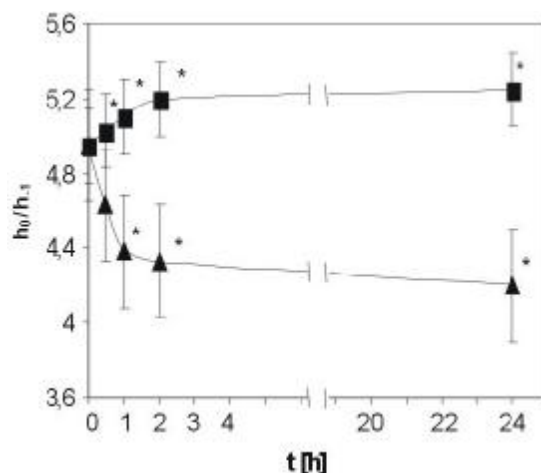


Fig. 1. The effect of DNR (-■-) and MIT (-▲-) on the h_0/h_1 parameter of membrane bound 16-DSA in the erythrocytes of patients with acute leukemia. Means \pm SD of seven experiments. (*) Statistically significant differences ($p < 0.01$).

Fig. 1 shows the results obtained by ESR study using 16-DSA. The analysis of these findings showed that DNR and MIT induced significant changes in the h_0/h_{-1} ratio. DNR caused a progressive increase in the h_0/h_{-1} ratio, while MIT induced a gradual and statistically significant diminution of this parameter with increasing post-treatment time.

Alterations in the conformation and organization of erythrocyte membrane proteins were determined in the presence of maleimide spin label. The W/S ratio used to characterize the spectrum of maleimide bound to the membrane proteins is presented in Tab. 2.

Tab. 2. The effect of DNR and MIT on the erythrocyte membrane proteins as monitored by the W/S ratio of maleimide spin label. (n=7) (*) Statistically significant differences ($p < 0.01$).

time [h]	Daunorubicin	Mitoxantrone
0	4.51±0.22	3.01±0.32
0.5	4.34±0.25*	2.52±0.35*
1	4.29±0.33*	2.40±0.27*
2	4.28±0.23*	2.48±0.17*
24	4.11±0.25*	2.49±0.19*

We revealed that DNR and MIT caused a marked decrease in the W/S ratio of maleimide bound to erythrocyte membrane proteins. Both drugs induced similar alterations in the conformation and organization of membrane skeletal proteins.

DISCUSSION

These studies were conducted in order to determine whether intravenously injected anticancer drugs to AML patients might induce perturbations in the structure of RBCs. Using ESR and fluorescent spectroscopy, we showed that both the examined drugs, DNR and MIT, significantly altered the lipid fluidity and protein conformation of the RBC plasma membrane.

Studies performed by Garnier-Suillerot and Gattegno [10] suggested that the interaction of anthracyclines with RBCs is electrostatic and occurs primarily at the level of the ionized phosphate groups. Anthracycline drugs, including DNR, bind to RBCs, inducing a number of the morphological and ultrastructural alterations [8, 11, 12]. The interaction of MIT with human erythrocyte membranes remains unclear. It has been found that the drug rapidly leaves the plasma compartment after intravenous administration [13]. Both *in vitro* and *in vivo* studies have shown that MIT accumulates in leukemic cells. Mitoxantrone concentration in leukemic cells is about 10 times higher than in the plasma, and it remained stable for up to 24 h [14]. *In vitro* studies suggested that MIT, like anthracyclines, induced conformational changes of plasma membrane proteins in human erythrocytes and caused disturbances of the lipid fluidity at hydrophobic parts of bilayer [15]. Another study demonstrated the electrostatic interaction of MIT with cellular membranes [4].

In this study, the new findings are related to the *in vivo* interactions of DNR and MIT with the RBCs of AML patients. Using ESR and fluorescent spectroscopy, we showed that the interactions between erythrocytes and drugs led to a perturbation in the structure of membrane lipids and proteins. Both drugs caused statistically significant alterations in the conformation of proteins and marked differences in membrane fluidity. Human red blood cells are anucleated, do not contain organelles and cannot synthesize DNA. Thus, drug-induced changes of RBCs could be generally explained by the generation of reactive oxygen species and their interaction with the plasma membrane. DNR and MIT produce free radicals by different mechanisms. Anthracyclines generate ROS by a reaction of one-electron reduction of a quinone moiety [1, 16]. By contrast to anthracyclines, mitoxantrone produces damaging radicals during oxidation of hydroquinone group [5].

Our results suggest that differences in the interaction of these drugs with RBCs may result not only from their ability to produce ROS, but also from changes in the binding capacity of membrane phospholipids [17, 18]. The different effect of DNR and MIT on the lipid fluidity in the upper region compared with the deeper regions of the erythrocyte membrane indicates that both drugs are predominantly intercalated into the hydrophobic core of the lipid bilayer. Anthracycline drugs, including DNR, bind to negatively charged phospholipids [14]. Specific affinities of MIT to membrane components are not well known. The opposed alterations in membrane fluidity induced by the two drugs in human erythrocytes suggest that MIT may interact with another group of phospholipids or with the negatively charged lipids by different processes than DNR.

In summary, our data confirmed that cancer chemotherapy remains largely nonspecific. Both DNR and the alternative drug, MIT, intravenously injected to AML patients, may interact also with RBCs producing significant perturbations in the organization of the plasma membrane.

REFERENCES

1. Fenster, E., Canadni, E. and Weiner, L.M. Dependence of nucleic acid degradation on *in situ* free radical production by adriamycin. **Biochemistry** 32 (1998) 13156-13161.
2. Wrzesien-Kus, A., Robak, T., Jamroziak, K., Wierzbowska, A., Dmoszynska, A., Adamczyk-Cioch, M., Kuliczowski, M., Mazur, G., Holowiecki, J., Konopka, L., Maj, S., Marianska, B. and Zawilska, K. The treatment of acute myeloid leukemia with mitoxantrone, etoposide and low-dose cytarabine in elderly patients - a report of Polish Acute Leukemia Group (PALG) phase II study. **Neoplasma** 49 (2002) 405-411.
3. Hande, K.R. Clinical application of anticancer drugs targeted to topoisomerase II. **Biochim. Biophys. Acta** 1400 (1998) 173-184.
4. Burns, C.P., Haugstad, B.N. and Mossmann, C.J. Membrane lipid alteration: Effect on cellular uptake of mitoxantrone. **Lipids** 23 (1988) 393-397.

5. Kołodziejczyk, P., Reszka, K. and Lown, J. Enzymatic oxidative activation on transformation of the antitumor agent mitoxantrone. **Free Radic. Biol. Med.** 5 (1988) 13-25.
6. Dodge, J.T, Mitchell, C. and Hanahan, D.J. The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. **Arch. Biochem. Biophys.** 100 (1963) 119-130.
7. Lowry, O.H., Rosenbrough, N.J, Farr, A.L. and Randal, R.J. Protein measurement with the Folin phenol reagent. **J. Biol. Chem.** 193 (1951) 265-275.
8. Szwarocka, A., Robak, T., Krykowski, E. and Józwiak, Z. Interactions of anthracyclines with erythrocytes at hyperthermic temperature. **Int. J. Pharmac.** 135 (1996) 167-176.
9. Van der Meer, B.W. Subcellular biochemistry. **Fluorescence Studies on Biological Membranes** (Hilderson HJ, Harris JR, ed.). Plenum Press. New York 13 (1988) 1.
10. Garnier-Suillerot, A. and Gattegno, L. Interaction of adriamycin with human erythrocyte membranes. Role of the negatively charged phospholipids. **Biochim. Biophys. Acta** 936 (1988) 50-60.
11. Arancia, G., Bordi, F., Calcabrini, A., Diociaiuti, M. and Molinari, A. Ultrastructural and spectroscopic methods in the study of anthracycline-membrane interaction. **Pharmacol. Res.** 32 (1995) 255-272.
12. Szwarocka, A., Fiet, I. and Józwiak, Z. Idarubicin - induced changes in human erythrocyte membranes. **Biomed. Letters** 56 (1997) 81-89.
13. Thomas, X. and Archimbaud, E. Mitoxantrone in the treatment of acute myelogenous leukemia: a review. **Hematol. Cell Ther.** 39 (1997) 163-174.
14. Mollgard, L., Tidefelt, U., Surudman-Engberg, B., Lofgren, Ch., Lehman, S. and Paul, Ch. High single dose of mitoxantrone and cytarabine in acute non-lymphocytic leukemia. A pharmacokinetic and clinical study. **Therapeut. Drugs Monit.** 20 (1998) 640-645.
15. Szwarocka, A., Fiet, I., Józwiak, Z., Krykowski, E. and Robak, T. Plasma membrane as a site of interaction of mitoxantrone in human erythrocytes. **Hypertherm. Oncol.** 2 (1996) 670-672.
16. Davies, K.J.A. and Doroshov, J.H. Redox cycling of anthracyclines by cardiac mitochondria. Anthracycline radical formation by NADH dehydrogenase. **J. Biol. Chem.** 261 (1986) 3060-3067.
17. Gallois, L., Fiallo, M. and Garnier-Suillerot, A. Comparison of the interaction of doxorubicin, daunorubicin, idarubicin and idarubicinol with large unilamellar vesicles. Circular dichroism study. **Biochim. Biophys. Acta** 1370 (1998) 31-40.
18. Faulds, D., Balfour, J.A., Chrisp, P. and Langtry, H.D. Mitoxantrone: A review of its pharmacodynamic and pharmacokinetic properties and therapeutic potential in the chemotherapy of cancer. **Drugs** 41 (1991) 400-449.