

Received 15 October 2002  
Accepted 13 February 2003

Short Communication

**THE NITROXIDES PIROLIN AND PIROLID PROTECT THE PLASMA  
MEMBRANES OF RAT CARDIOMYOCYTES AGAINST DAMAGE  
INDUCED BY ANTHRACYCLINES**

ANETA KOCEVA-CHYŁA<sup>1</sup>, ADAM SOKAL<sup>2</sup>, KATARZYNA KANIA<sup>1</sup>,  
KRZYSZTOF GWOŹDZIŃSKI<sup>3</sup> and ZOFIA JÓŹWIAK<sup>1</sup>

<sup>1</sup>Department of Thermobiology and <sup>3</sup>Department of Molecular Biophysics,  
University of Łódź, Łódź, Poland, <sup>2</sup>Silesian Centre of Heart Disease, Zabrze,  
Poland

**Abstract:** This study was performed to evaluate the protective effects of pyrroline and pyrrolidine nitroxides Pirolin, PL, and Pirolid, PD, on the plasma membranes of rat cardiomyocytes treated *in vitro* with anthracycline drugs aclarubicin (ACL) and doxorubicin (DOX). The influence of two concentrations of drugs (10 and 20  $\mu$ M) and nitroxides (0.1 and 1 mM) as well as their combinations (a drug and a nitroxide) on membrane fluidity was investigated. The plasma membranes of cardiomyocytes were labelled with a hydrophobic fluorescence probe 12-AS and membrane fluidity was estimated on the basis of the fluorescence anisotropy of the probe. We found that aclarubicin and doxorubicin induced a significant dose-dependent decrease in membrane fluidity, whereas the nitroxides (PL and PD) caused its increase. Preincubation of cardiomyocytes with Pirolin entirely protected plasma membranes of these cells against damage caused by DOX. In the same conditions no protective effect of Pirolid was observed. What is more, Pirolid in combination with DOX caused fluidisation of the plasma membranes of cardiomyocytes.

Both nitroxides at low concentration (0.1 mM) protected plasma membranes against rigidification induced by aclarubicin, while high concentration (1 mM) was ineffective and caused fluidisation of the plasma membranes of cardiomyocytes.

**Key Words:** Cardiomyocytes, Anthracyclines, Plasma Membrane Fluidity, 12-AS, Nitroxides

---

Abbreviations used: Pirolin, PL - 3-carbamoyl-2,2,5,5-tetramethylpyrroline-1-oxyl; Pirolid, PD - 3-carbamoyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl; ACL - aclarubicin; DOX - doxorubicin; 12-AS - 12-(9-anthroyloxy)-stearic acid; MOPS - 3-morpholinopropane-sulfonic acid; BDM - 2,3-butanedione monoxime; ROS - reactive oxygen species.

## INTRODUCTION

One of the most severe side effects of chemotherapy with the use of anthracyclines, especially DOX, is acute and delayed dose-dependent cardiotoxicity, developed by an appreciable number of patients treated with these drugs [1]. It has been shown that cardiotoxic effects of anthracyclines depend mainly on oxygen free radical-induced oxidative stress, generated during the formation of anthracycline-iron complexes and metabolic activation of the drugs inside the cell [2]. Heart muscle cells are relatively unprotected against oxidative stress due to the low level of the intracellular antioxidant enzymes [3]. An increase in reactive oxygen species (ROS) in the cell can impair its viability by damage to DNA, lipids and proteins [4,5]. The anthracycline antibiotics doxorubicin and aclarubicin interact with plasma membrane lipids and proteins [6,7]. These drugs target the cell membrane at the anionic phospholipid level and thus degradation of the structural plasma membrane lipids by anthracyclines is considered as an important factor in their cytotoxicity [8].

Nitroxides are cell permeable stable radicals with antioxidant activity that may contribute to protection of cells against oxidative stress [9]. These compounds were shown to scavenge effectively superoxide anions by mimicking superoxide dismutase activity [10] and to protect cells from lipid peroxidation [11] and cytotoxicity induced by hydrogen peroxide, superoxide and radiation [12-14]. In order to evaluate the ability of nitroxides to protect the plasma membranes of cardiomyocytes from the toxicity of anthracyclines, we investigated the influence of pyrroline and pyrrolidine nitroxides: Pirolin, PL and Pirolid, PD on the fluidity of the plasma membranes of rat cardiomyocytes treated *in vitro* with DOX or ACL.

## MATERIALS AND METHODS

### Cells

Myocytes were isolated from hearts of healthy male Wistar rats (180-220 g) after their anaesthesia with 10 % chloral hydrate. Cardiomyocytes were obtained as described previously [15] with some modifications. Hearts were removed, placed in medium I (composition: 120 mM NaCl; 1 mM MgCl<sub>2</sub>; 5.4 mM KCl; 0.33 mM NaH<sub>2</sub>PO<sub>4</sub>; 11 mM glucose; 30 mM MOPS, pH 7.4; 5 mM taurine, 2 mM pyruvate; 1.5 mM glutamine) supplemented with BDM, and then washed with the same solution through the aorta to stop beating. Isolated hearts were cannulated and perfused through the aorta with medium I (4-6 ml/min) for 5 minutes, and next with medium C (composition: medium I supplemented with 25 mM BDM, 1 μM CaCl<sub>2</sub>, 90 U/ml collagenase and 4.6 U/ml protease) for about 8 minutes, at 37°C. Afterwards, the hearts were removed from the cannula and single separated cells, after mincing, were obtained by incubation in 10 ml medium II (composition: medium I supplemented with 25 mM BDM and 200 μM CaCl<sub>2</sub>) at 37°C for 5 minutes with gentle shaking. The resulted suspensions were filtrated through a

nylon mesh, the cells were suspended in medium II and centrifuged at  $22 \times g$  for 3 minutes. Then the cells were washed twice with medium III (composition: medium I supplemented with 1.5 mM  $\text{CaCl}_2$ ), pooled and resuspended in medium III at the final concentration of  $4 \times 10^5$  cells/ml/sample for the plasma membrane fluidity assay.  $4\text{-}5 \times 10^6$  cells on average were isolated from one heart.

#### **Incubation of cardiomyocytes with drugs and nitroxides**

Cardiomyocytes were incubated with 10  $\mu\text{M}$  or 20  $\mu\text{M}$  of drugs (ACL, DOX) for 2 hours or with nitroxides (0.1 mM or 1 mM of Pirolin, Pirolid) for 3 hours, under culturing conditions (37°C). Cells treated both with a nitroxide and a drug were incubated with Pirolin or Pirolid for 1 hour, followed by 2-hour incubations with ACL or DOX in the same conditions. Afterwards, the cells were washed with PBS and resuspended in Tris-KCl buffer (50 mM Tris-HCl and 0.15 M KCl), pH 7.4, for anisotropy fluorescence measurements.

#### **Fluorescence spectroscopic analysis of plasma membrane fluidity**

The plasma membranes of cardiomyocytes were labelled with a hydrophobic fluorescent probe 12-AS, (final concentration of the probe  $10^{-6}$  M) for 10 minutes at  $22 \pm 10^\circ\text{C}$ , in the dark, and fluorescence anisotropy was measured using a Perkin-Elmer LS 50B spectrofluorimeter with  $\lambda_{\text{ex}}=360$  nm and  $\lambda_{\text{em}}=471$  nm, at the same temperature. Fluorescence anisotropy was calculated as described by Van der Meer [16].

#### **Statistical analysis**

All values were expressed as mean  $\pm$  standard deviation of three or more separate experiments in 3-5 repeats each. Statistical analyses were done using the STATISTICA software (2000) (StatSoft, Inc., Tulsa, OK, USA). Statistical differences were analysed by the one-way analysis of variance (ANOVA) and Tukey's test. A p values less than 0.01 were considered as statistically significant.

## **RESULTS AND DISCUSSION**

The anthracycline drugs aclarubicin and doxorubicin caused a significant dose-dependent decrease (an increase in 12-AS fluorescence anisotropy) in the plasma membrane fluidity of heart myocytes, more profound in cardiomyocytes treated with ACL (Figs. 1 and 2). This is in accordance with our previous findings showing that anthracyclines (aclarubicin, doxorubicin and danorubicin) significantly influenced the fluidity of plasma membranes of rodent and human cells [6,17].

Other authors reported changes in the fluidity and depolarisation of the plasma membranes of normal and chronic lymphatic leukaemia lymphocytes, breast cancer cells and rat cardiomyocytes exposed to DOX and idarubicin [18]. It has been suggested that interaction of DOX with the cell membrane is very important for both doxorubicin neoplastic effects and drug resistance.

A relationship between membrane fluidity and doxorubicin resistance has been reported for sarcoma cells [19].

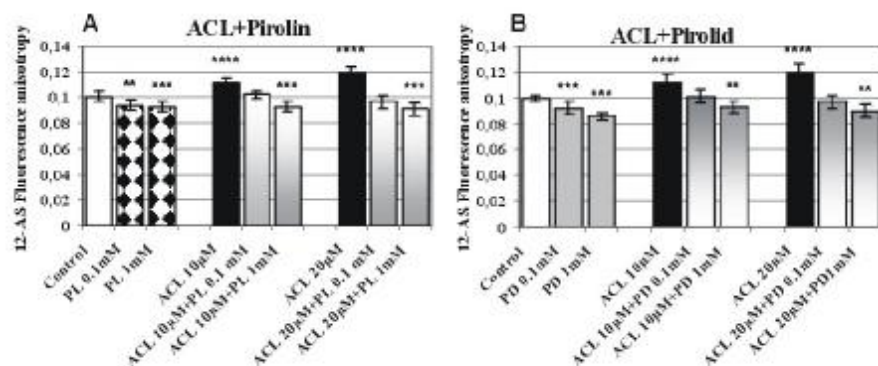


Fig. 1. The effects of aclarubicin and nitroxides Pirolin and Pirolid on fluorescence anisotropy of 12-AS. \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$  (statistically significant in relation to control).

We investigated the ability of pyrroline and pyrrolidine nitroxides: Pirolin and Pirolid to protect *in vitro* the plasma membranes of rat cardiomyocytes against damage caused by DOX and ACL. Surprisingly, both investigated nitroxides caused an increase in the plasma membrane fluidity, most notable in the cells treated with 1 mM Pirolid (Figs. 1 and 2). At the same time low concentrations of Pirolin and Pirolid (0.1 mM) entirely protected the plasma membranes of cardiomyocytes against rigidification induced by aclarubicin. Membrane fluidity of cardiomyocytes treated with 10  $\mu$ M or 20  $\mu$ M ACL and 0.1 mM Pirolin, Pirolid did not differ significantly from the membrane fluidity of control cells (untreated cardiomyocytes) (Fig. 1). Nonetheless, a higher concentration of the nitroxides (1 mM) in combination with aclarubicin resulted in fluidisation of the cardiomyocyte plasma membrane (Fig. 1), which suggests dose-dependency for the antioxidant activity of these nitroxides against aclarubicin toxicity.

Pirolin and Pirolid displayed a contradictory effect on cardiomyocytes treated with doxorubicin. Pirolin, irrespectively of its concentrations (0.1 and 1 mM), effectively protected the cell membrane from rigidification induced by DOX, whereas in the same conditions an increase in membrane fluidity was noted in cardiomyocytes pretreated with Pirolid (Fig.2). This indicates a modified antioxidant activity of Pirolid towards doxorubicin, compared to aclarubicin.

These findings imply that Pirolin similarly to Tempo and Tempol [20] can protect the plasma membranes of cardiomyocytes against rigidification induced by aclarubicin and doxorubicin. Pirolid displayed a protective effect on cardiomyocytes treated with aclarubicin, but was ineffective in the protection of the plasma membranes of these cells from the toxicity of doxorubicin. Similarly,

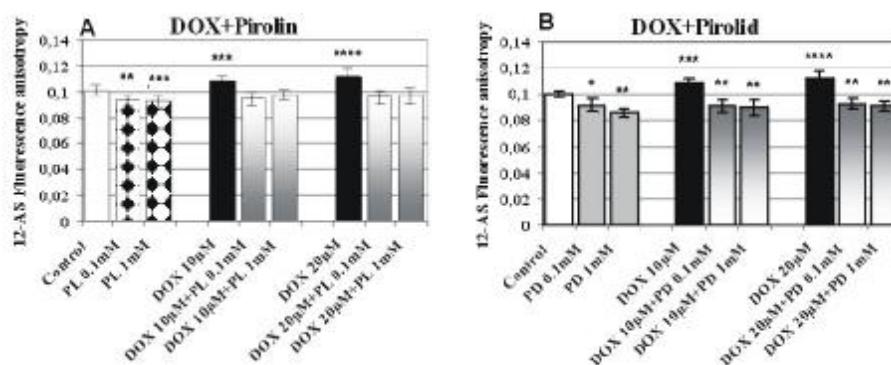


Fig. 2. The effects of doxorubicin and nitroxides Pirolin and Pirolid on fluorescence anisotropy of 12-AS. \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$  (statistically significant in relation to control).

we found different activities of these nitroxides against doxorubicin cardiotoxicity *in vivo* [21]. Pirolin exhibited an antioxidative effect and a significantly decreased level of lipid peroxidation in the heart tissue of rats treated with DOX, whereas in the same conditions structurally related Pirolid was ineffective and did not display any protective effect. These results confirm earlier findings indicating that the structure of the heterocyclic rings as well as its conformation and substituents are important and decisive for the antioxidant properties of nitroxides [22-24].

**Acknowledgements.** This work was supported by the Polish Committee for Scientific Research (KBN) Grant No. 4 PO5A 092 16.

## REFERENCES

1. Shan, K., Lincoff, A.M. and Young, J.B. Anthracycline-induced cardiotoxicity. **Ann. Int. Med.** 1 (1996) 47-58.
2. Horenstein, M.S., Heide, R.S.V. and L'Ecuyer, T.J. Molecular basis of anthracycline-induced cardiotoxicity and its prevention. **Mol. Genet. Metabol.** 71 (2000) 436-444.
3. Halliwell, B. and Gutteridge, J.M.C. (1989) **Free radicals in biology and medicine**. Oxford: Clarendon.
4. Kovacic, P. and Osuna, J.A. Jr. Mechanisms of anti-cancer agents: emphasis on oxidative stress and electron transfer. **Curr. Pharm. Des.** 6 (2000) 277-309.
5. Ling, Y-H., Priebe, W. and Perez-Soler, R. Apoptosis induced by anthracycline antibiotics in P388 parent and multidrug-resistant cells. **Cancer Res.** 53 (1993) 1845-1852

6. Jędrzejczak, M., Koceva-Chyła, A., Gwoździński, K. and Józwiak, Z. Changes in plasma membrane fluidity of immortal rodent cells induced by anticancer drugs doxorubicin, aclarubicin and mitoxantrone. **Cell Biol. Int.** 23 (1999) 497-506.
7. Szwarocka, A., Robak, T., Krykowski, E. and Józwiak, Z. Interaction of anthracyclines with human erythrocytes at hypothermic temperature. **Int. J. Pharm.** 135 (1996) 167-176.
8. DeWolf, F.A., Maliepaard, M., Van Dorsen, F., Berghuis, J., Nicolay, K. and Kruijff, B. Comparable interaction of doxorubicin with various acidic phospholipids results in changes of lipid order and dynamics. **Biochim. Biophys. Acta** 1096 (1990) 67-80.
9. Krishna, M.C. and Samuni, A. Nitroxides as antioxidants. **Meth. Enzymol.** 234 (1994) 580-589.
10. Samuni, A., Krishna, C.M., Riesz, P., Finkelstein, E. and Russo, A. A novel metal-free low molecular weight superoxide dismutase mimic. **J. Biol. Chem.** 263 (1988) 17921-17924.
11. Nilsson, U.A., Olsson, L.I., Carlin, G. and Bylund-Fellenius A.C. Inhibition of lipid peroxidation by spin labels: relationship between structure and function. **J. Biol. Chem.** 264 (1989) 11131-11135.
12. Samuni, A., Winkelsberg, L., Pinson, A., Hahn, S.M., Mitchell, J.B. and Russo, A. Nitroxide stable radicals protect beating cardiomyocytes against oxidative damage. **J. Clin. Invest.** 87 (1991) 1526-1530.
13. Samuni, A., Mitchell, J.B., Samuni, U., DeGraff, W.G., Krishna, C.M. and Russo, A. Nitroxide SOD-mimics: Modes of action. **Free Rad. Res. Commun.** 12-13 (1991) 187-194.
14. Hahn, S.M., Wilson, L., Krishna, C.M., Liebmann, J., DeGraff, W.G., Gamson, J., Samuni, A., Venzon, D. and Mitchell, J.B. Identification of nitroxide radioprotectors. **Radiat. Res.** 132 (1992) 87-93.
15. Chiesi, M., Wrzosek, A. and Grueningers, S. The role of sarcoplasmic reticulum in various types of cardiomyocytes. **Mol. Cell. Biochem.** 130 (1994) 159-171.
16. Van der Meer, B.W. Fluorescence studies on biological membranes. In: **Subcellular Biochemistry** (Hiderson, H.J. and Harris, J. R., Eds.) Plenum Press, New York. 13 (1988) 1-53.
17. Przybylska, M., Koceva-Chyła, A., Różga, B. and Józwiak, Z. Cytotoxicity of daunorubicin in trisomic (+21) human fibroblasts: relation to drug uptake and membrane fluidity. **Cell Biol. Int.** 25 (2001) 157-170.
18. Pacilio, C., Florio, S., Pagnini, U., Crispino, A., Claudio, P.P., Pacilio, G. and Pagnini, G. Modification of membrane fluidity and depolarization by some anthracyclines in different cell lines. **Anticancer Res.** 18 (1998) 4027-4034.
19. Siegfried, J.A., Kennedy, K.A., Sartorelli, A.C. and Tritton, T.R. The role of membranes in the mechanism of action of the antineoplastic agent of

- adriamycin. Spin-labeled studies with chronically hypoxic and drug-resistant tumor cells. **J. Biol. Chem.** 258 (1983) 339-343.
20. Koceva-Chyła, A., Sokal, A., Kania, K., Gwoździński, K and Józwiak, Z. Protective effect of nitroxides against damage to rat cardiomyocytes treated with doxorubicin. **Proceedings of the XIth Biennial Meeting of the Society for Free Radical Research International** (2002) *in press*.
  21. Koceva-Chyła, A., Gwoździński, K., Kochman, A., Stolarska, A. and Józwiak, Z. Effects of pyrroline and pyrrolidine nitroxides on lipid peroxidation in heart tissue of rats treated with doxorubicin. **Cell. Mol. Biol. Lett.** 8 (2002) 179-183.
  22. Krishna, M.C., DeGraff, W., Hankovszky, O.H., Sar, C.P., Kalai, T., Jeko J., Russo, A., Mitchell, J.B. and Hideg, K. Studies on structure-activity relationship of nitroxide free radicals and their precursors as modifiers against oxidative damage. **J. Med. Chem.** 41 (1988) 3477-3492.
  23. Koceva-Chyła, A., Kochman, A., Glebska, J., Gwoździński, K., Józwiak, Z. and Metodiewa, D. Tempicol-3, a novel piperidine-N-oxide stable radical and antioxidant, with low toxicity acts as apoptosis inducer and cell proliferation modifier of Yoshida sarcoma cells in vivo. **Anticancer Res.** 20 (2000) 4611-4618.
  24. Metodiewa, D., Skolimowski, J., Kochman, A. and Koceva-Chyła, A. The paradoxical apoptotic effects of novel nitroxide antioxidants on Yoshida sarcoma cells in vivo: a commentary. **Anticancer Res.** 20 (2000) 2593-2600.