

# The Effect of 5'-(N,N-Dimethyl)-amiloride on Cytotoxic Activity of Doxorubicin and Vincristine in CEM Cell Lines

I. RADVÁKOVÁ, A. MIROŠŠAY, J. MOJŽIŠ, L. MIROSSAY<sup>1</sup>

Department of Pharmacology, Faculty of Medicine, Šafárik University, Košice, Slovak Republic

Received March 3, 2000

Accepted October 20, 2000

---

## Summary

Intracellular pH ( $pH_i$ ) plays an important role in anticancer drug accumulation in cancer cells. Resistant cells often express membrane P-glycoprotein responsible for active drug extrusion and participating in increased  $pH_i$ . In the present paper, we report on the influence of  $Na^+/H^+$ -exchanger inhibitor, 5'-(N,N-dimethyl)-amiloride (AMI), on the cytotoxic effects of doxorubicin (DOXO) and vincristine (VCR) in the parental CEM, and resistant CEM/DNR and CEM/VCR cell lines. The obtained results revealed a potentiating effect of AMI to both anticancer drugs in parental CEM line. However, AMI did not significantly potentiate the effect of DOXO or VCR in resistant CEM cell lines. We conclude, that inhibition of  $Na^+/H^+$ -exchanger by AMI is not sufficient for reversal of drug resistance in the tested CEM/DNR and CEM/VCR cell lines and the possible change in  $pH_i$  does not affect the mechanisms of cell resistance.

---

## Key words

Amiloride • CEM cell lines • Anticancer drugs • Resistance • Intracellular pH

## Introduction

Intracellular pH seems to play an important role in the regulation of all cellular processes. In tumor cells, the interest was attracted by the observation that  $pH_i$  is in some correlation with cell responsiveness to chemotherapeutic drug effects. Multidrug resistance (MDR) of tumor cells is caused by a large scale of events and processes, but it is very often in close connection with changes in  $pH_i$ . Resistant cells as well as MDR transfectants are in general alkaline (Wei and Roepe 1994). Additionally, drug accumulation is enhanced by acidic shifts and reversed by alkaline shifts. Moreover,  $pH_i$  can be dose-dependently lowered by addition of multidrug-resistance modifiers, verapamil, tamoxifen and cyclosporine A (Hamilton *et al.* 1993). Therefore, it was

concluded that the alkaline shift observed in MDR is sufficient to prevent the accumulation of chemotherapeutic drugs. This prevention is independent of active drug efflux (Simon *et al.* 1994).

Furthermore, the correlation of MDR in tumor cells with an alkaline shift of cytosolic pH revealed that doxorubicin and daunomycin achieved the highest concentrations in the most acidic compartments, i.e. in lysosomes (Simon *et al.* 1994).

The aim of our study was to determine whether the  $Na^+/H^+$ -antiporter inhibitor 5'-(N,N-dimethyl)-amiloride (AMI) can influence the chemosensitivity of CEM cell lines to doxorubicin and vincristine. In our experiments, we employed sensitive CEM cells as well as two lines with acquired resistance to daunorubicin (CEM/DNR) and vincristine (CEM/VCR).

## Methods

### Cell cultures

Three human lymphoblastic cell lines CEM, CEM/DNR and CEM/VCR (parental sensitive cell line and two cell lines with acquired resistance to daunorubicin and vincristine, respectively) were obtained from Dr. M. Hajdúch (Olomouc, Czech Republic). Cells were maintained in RPMI 1640 (Gibco, GB) supplemented with 10 % fetal calf serum (FCS), 25 mg.100 ml<sup>-1</sup> glutamine, 0.5 mg.100 ml<sup>-1</sup> transferin, 40 I.U.ml<sup>-1</sup> insulin and penicillin/streptomycin (100 I.U. ml<sup>-1</sup> and 100 µg.ml<sup>-1</sup>, respectively).

### Drugs

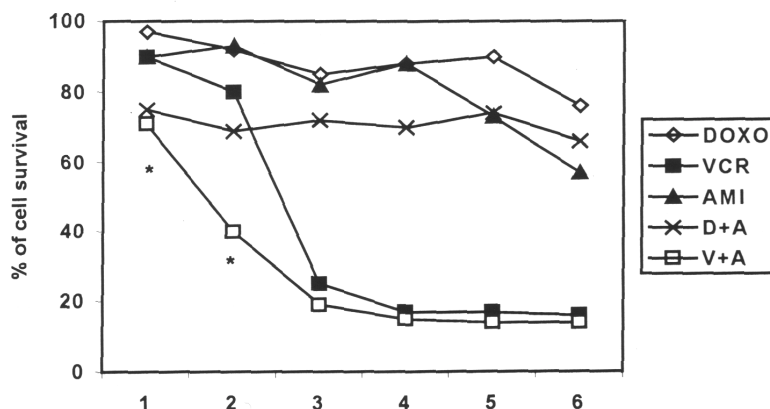
The antineoplastic agents used in experiments were diluted to final concentrations with RPMI 1640 that was usually used for the cell culture technique. Six tested concentrations were as follows: doxorubicin (DOXO) (Adriplastina, Farmitalia, Carlo Erba, I) in dilution 1:4 (2.0, 0.5, 0.125, 0.0312, 0.078, 0.0019 µg.ml<sup>-1</sup>), vincristine sulphate (VCR) (Richter RG) in dilution 1:4 (50.0, 12.5, 3.125, 0.782, 0.1953, 0.048 µg.ml<sup>-1</sup>).

The substance of 5'-(N,N-dimethyl)-amiloride (AMI, Sigma) was dissolved and diluted to six desired concentrations (10<sup>-5</sup>, 10<sup>-6</sup>, 10<sup>-7</sup>, 10<sup>-8</sup>, 10<sup>-9</sup>, 10<sup>-10</sup> mol.l<sup>-1</sup>) with Dulbecco's Modified Eagle's Medium (DMEM).

### Experimental procedure

The thiazolyl blue (MTT) method was used in all experiments for the assessment of the cytotoxic effect of the tested agents (Mosmann 1983, Mihál *et al.* 1995). The tetrazolium dye, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma, Germany), is reduced by living but not dead cells, and this reaction is used as the end point in a rapid drug-screening assay.

Briefly, cell suspensions containing 4x10<sup>4</sup> viable cells *per vial* were cultivated in 96-well tissue culture plates (Falcon Becton-Dickinson, USA) with or without (control) the tested substances in a final volume of 100 µl for three days. The cells were cultivated at 37 °C in a humidified incubator in an atmosphere of 95 % air and 5 % CO<sub>2</sub>. After 72 h of cultivation, MTT was added to each sample and cultivation was continued for additional 4-6 h. During this period the living cells produced blue insoluble formazan from the yellow soluble MTT. The reaction was stopped by addition of 100 µl of 10 % laurylsulfate in each well and the content of the wells was spontaneously dissolved within the following 12 h. The optic density of each well was measured spectrophotometrically at 540 nm on ELISA reader MRX Dynatech, GB. The obtained values (n=6) were calculated and expressed as the percentage of cell survival in comparison with the controls taken as 100 % survival. Student's t-test was applied to evaluate these growth experiments, p<0.05 was taken as significant.



**Fig. 1** Dose-response curves of doxorubicin (DOXO) and vincristine (VCR) administered in six different doses. Individual doses are shown at the x-axis as numbers from 1 to 6. The meaning for each number for DOXO was 2.0 (1), 0.5 (2), 0.125 (3), 0.031 (4), 0.078 (5) and 0.0019 (6) and for VCR 50.0 (1), 12.5 (2), 3.125 (3), 0.782 (4), 0.195 (5) and 0.048 (6) both in µg.ml<sup>-1</sup>. The same numbers (1-6) indicate six different concentrations 10<sup>-5</sup>, 10<sup>-6</sup>, 10<sup>-7</sup>, 10<sup>-8</sup>, 10<sup>-9</sup>, 10<sup>-10</sup> mol.l<sup>-1</sup> of 5'-(N,N-dimethyl)-amiloride (AMI) used alone. When testing the combination DOXO and AMI (D+A) or VCR and AMI (V+A) one selected dose of AMI (10<sup>-6</sup> mol.l<sup>-1</sup>) was used with each concentration of both cytotoxic drugs. All experiments were performed on CEM cell line. The results are expressed as mean values of six experiments. \* significantly different from VCR alone.

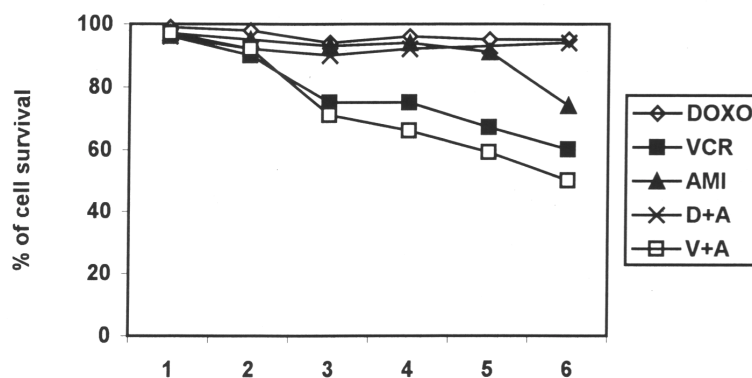
## Results

A number of drugs have been shown to sensitize parental and multidrug resistant cells to cytotoxic agents. In our experiments the sensitivity of three CEM cell lines to DOXO and VCR was measured. We also tested the effects of AMI, a known inhibitor of  $\text{Na}^+/\text{H}^+$ -exchanger, on the reversal of cell resistance.

DOXO alone did not exhibit significant cytotoxicity in the tested sensitive parental CEM cell line. About 20 % toxicity was achieved with the highest dose of DOXO. The addition of AMI in a concentration  $10^{-6}$  mol.l<sup>-1</sup> was not accompanied by increased cytotoxicity of

DOXO and the curve seemed to be shifted to lower cell survival for all concentrations of DOXO. However, this shift follows the mean of survival exerted with AMI alone (Fig. 1).

VCR significantly affected the growth of CEM cell line in a dose-dependent manner. The maximal effect was achieved between the third to sixth concentration and was equal to about 80 % inhibition of cell growth. The combination of VCR (from 50.0 to 12.5  $\mu\text{g}/\text{ml}$ ) with AMI ( $10^{-6}$  mol.l<sup>-1</sup>) resulted in dose-dependent curve with a similar shape as after VCR alone. However, AMI significantly potentiated ( $p < 0.05$ ) the effect of VCR at the first two lowest concentrations (Fig. 1).



**Fig. 2** Dose-response curves of doxorubicin (DOXO) and vincristine (VCR) administered in six different doses. Individual doses are shown at the x-axis as numbers from 1 to 6. The meaning for each number for DOXO was 2.0 (1), 0.5 (2), 0.125 (3), 0.0312 (4), 0.078 (5) and 0.0019 (6) and for VCR 50.0 (1), 12.5 (2), 3.125 (3), 0.782 (4), 0.195 (5) and 0.048 (6) both in  $\mu\text{g}\cdot\text{ml}^{-1}$ . The same numbers (1-6) indicate six different concentrations ( $10^{-5}$ ,  $10^{-6}$ ,  $10^{-7}$ ,  $10^{-8}$ ,  $10^{-9}$ ,  $10^{-10}$  mol.l<sup>-1</sup>) of 5'-(N,N-dimethyl)-amiloride (AMI) used alone. When testing the combination DOXO and AMI (D+A) or VCR and AMI (V+A) one selected dose of AMI ( $10^{-6}$  mol.l<sup>-1</sup>) was used with each concentration of both cytotoxic drugs. All experiments were performed on CEM/DNR cell line. The results are expressed as mean values of six experiments.

The effects of DOXO in two resistant CEM cell lines (CEM/DNR and CEM/VCR) are shown in Figures 2 and 3. The growth of both cell lines was not affected at all. The addition of AMI ( $10^{-6}$  mol.l<sup>-1</sup>) to all concentrations of DOXO did not exert any potentiating effect.

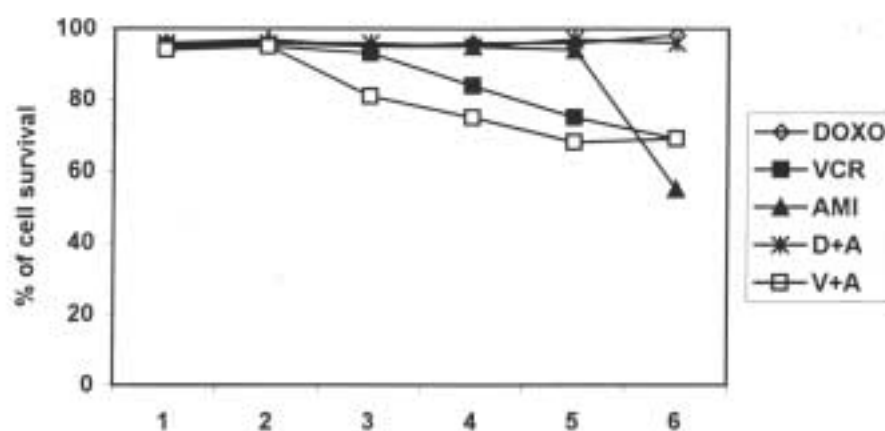
VCR exhibited dose-dependent inhibitory activity on CEM/DNR as well as CEM/VCR resistant cell lines. The combination with AMI ( $10^{-6}$  mol.l<sup>-1</sup>) resulted in an evident but insignificant increase of growth inhibitory effects (Figs. 2 and 3).

## Discussion

Cellular drug resistance is an important factor for the success or failure of chemotherapy. The mechanisms of resistance are highly variable and expressed at different levels of cell structure and metabolism. Several transport proteins are considered to be responsible for clinical drug resistance in malignant diseases. Proteins involved in multidrug resistance confer unresponsiveness to a number of unrelated small hydrophobic substances. The major protein linked to MDR is 170 kD drug efflux pump, P-glycoprotein (P-gp). Besides P-gp, different drug resistant cells overexpress a

variety of membrane proteins, including a vacuolar  $H^+$ -ATPase, multiple-resistance protein (MRP), which is homologous to the cystic fibrosis transmembrane conductance regulator (CFTR) and P-gp (Simon *et al.* 1994). One of the dominant functions of these proteins in cellular regulation is their influence on  $pH_i$ . These factors could serve as an additional mechanism of MDR, which alters the transmembrane partitioning or intracellular sequestration of drugs. Intracellular pH changes seem to be important in controlling the majority of cellular functions, including the cell cycle and the proliferative capacity of cells. Moreover, the binding of many anticancer drugs to cellular structures is pH dependent.

This is a fundamental principle of cancer chemotherapy as the pH of tumor cells is considerably more acidic than that of normal or MDR cells. In an attempt to increase the drug sensitivity of MDR tumor cells, agents were identified according to ability to inhibit P-gp-mediated drug efflux (Ford and Hait 1990). These resistance modifiers (verapamil, cyclosporine A, tamoxifen) induced cellular acidification, but they did not interfere with the function of major  $pH_i$ -regulating acid-base transporters,  $Na^+/H^+$ -antiporter and bicarbonate transporters (Hamilton *et al.* 1993).



**Fig. 3** Dose-response curves of doxorubicin (DOXO) and vincristine (VCR) administered in six different doses. Individual doses are shown at the x-axis as numbers from 1 to 6. The meaning for each number for DOXO was 2.0 (1), 0.5 (2), 0.125 (3), 0.0312 (4), 0.078 (5) and 0.0019 (6) and for VCR 50.0 (1), 12.5 (2), 3.125 (3), 0.782 (4), 0.1953 (5) and 0.048 (6) both in  $\mu\text{g}\cdot\text{ml}^{-1}$ . The same numbers (1-6) indicate six different concentrations ( $10^{-5}$ ,  $10^{-6}$ ,  $10^{-7}$ ,  $10^{-8}$ ,  $10^{-9}$ ,  $10^{-10}$   $\text{mol}\cdot\text{l}^{-1}$ ) of 5'-(N,N-dimethyl)-amiloride (AMI) used alone. When testing the combination DOXO and AMI (D+A) or VCR and AMI (V+A) one selected dose of AMI ( $10^{-6}$   $\text{mol}\cdot\text{l}^{-1}$ ) was used with each concentration of both cytotoxic drugs. All experiments were performed on CEM/VCR cell line. The results are expressed as mean values of six experiments.

In previous experiments, we studied the effect of AMI on cytotoxic effect of hypericin, a photosensitive agent. We tested one of its possible mechanisms of cytotoxic action, which was proposed to be mediated *via* direct  $pH_i$  lowering effect of hypericin. We found that AMI potentiate significantly the phototoxic effect of hypericin (Mirossay *et al.* 1999).

Therefore, the effect of  $Na^+/H^+$ -antiporter inhibitor AMI on the sensitive CEM cell line and two CEM cell lines with acquired resistance to daunorubicin and vincristine was investigated in our experiments. As is shown in the results, the most pronounced effect of AMI in combination with VCR was found in the parental

(sensitive) CEM cell line. The inhibition of  $Na^+/H^+$ -antiporter thus increased the sensitivity of this cell line to VCR. We did not find similar potentiating effect of AMI with DOXO. The decrease in cell survival was much less pronounced than in AMI with VCR. The results from the experiments with two resistant cell lines, CEM/DNR and CEM/VCR, revealed neither cytotoxic effect of DOXO alone, nor in combination with AMI. The cytotoxicity of VCR was dose-dependent but significantly smaller than in parental CEM cells. The addition of AMI resulted in nonsignificant potentiation of the VCR effect. These results are in agreement with the findings of Hamilton *et al.* (1993). These authors showed that changes of  $pH_i$  did

not correlate with the MDR-reversing activity of MDR modifiers.

Appropriate functioning of P-glycoprotein does not depend on metabolic modification of the substrate. Small hydrophobic molecules crossing the membrane by passive diffusion do not enter the cytoplasm in cells with increased expression of P-gp. The interaction between these molecules and P-gp takes place in the cell membrane and they are extruded extracellularly directly from the membrane (Vosseveld and Sonnenveld 1999). We therefore speculate that inhibition of  $\text{Na}^+/\text{H}^+$ -antiporter by AMI and a subsequent possible decrease in  $\text{pH}_i$  might have an effect on intracellular drug accumulation but it does not affect the function of P-gp. A similar finding was published a few years ago and supports our conclusion (Goda *et al.* 1996). Taken together, fully functional P-gp is then sufficient for

abrogation of the drug influx and the intracellular conditions even become more suitable for drug accumulation.

From these results, we conclude that the inhibition of  $\text{Na}^+/\text{H}^+$ -antiporter resulted in a potentiation of two cytotoxic drugs effects on the sensitive CEM cell line, but not in resistant CEM/DNR and CEM/VCR cell lines. It seems to be possible that the intracellular events following the effects of antiporter inhibitor, AMI, are not sufficient for effective sensitization of resistant cells to the tested anticancer drugs.

### Acknowledgements

Authors thank Dr. Marian Hajdúch for providing the CEM cells. This work was supported by an Internal grant (IG1/98 and IG2/99) of the Faculty of Medicine, University P. J. Šafárik, Košice, Slovak Republic.

### References

- FORD JM, HAIT WN: Pharmacology of drugs that alter multidrug resistance in cancer. *Pharmacol Rev* **42**: 155-199, 1990.
- GODA K, BALKAY L, MARIAN T, TRON L, ASZALOS A, SZABO G jr: Intracellular pH does not affect drug extrusion by P-glycoprotein. *J Photochem Photobiol B* **34**: 177-182, 1996.
- HAMILTON G, COSENTINI E, TELEKY B, KOPERNA T, ZACHERI J, RIEGLER M, FEIL W, SCHIESSEL R, WENZI E: The multidrug-resistance modifiers verapamil, cyclosporine A and tamoxifen induce an intracellular acidification in colon carcinoma cell lines in vitro. *Anticancer Res* **13**: 2059-2064, 1993.
- MIHÁL V, HAJDÚCH M, WEIGL E, VIDEMANOVÁ L, ŠAFÁŘOVÁ M: Our first experience with testing of leukaemic cells sensitivity to cytostatics in vitro. *Čs Pediatr* **50**: 387-392, 1995.
- MIROSSAY L, MIROŠŠAY A, KOČIŠOVÁ E, RADVÁKOVÁ I, MIŠKOVSKÝ P, MOJŽIŠ J: Hypericin-induced phototoxicity of human leukemic cell line HL-60 is potentiated by omeprazole, an inhibitor of  $\text{H}^+\text{K}^+$ -ATPase and 5'-(N,N-dimethyl)-amiloride, an inhibitor of  $\text{Na}^+/\text{H}^+$ exchanger. *Physiol Res* **48**: 135-141, 1999.
- MOSMANN T: Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxic assays. *J Immunol Meth* **65**: 55-63, 1983.
- SIMON S, ROY D, SCHINDLER M: Intracellular pH and the control of multidrug resistance. *Proc Natl Acad Sci USA* **91**: 1128-1132, 1994.
- VOSSEBELD PJM, SONNENVELD P: Reversal of multidrug resistance in hematological malignancies. *Blood Rev* **13**: 67-78, 1999.
- WEI LY, ROEPE PD: Low external pH and osmotic shock increase the expression of human MDR protein. *Biochemistry* **33**: 7229-7238, 1994.

### Reprint requests

Assoc. Prof. L. Mirossay, M.D. Ph.D., Department of Pharmacology, Faculty of Medicine, University of P. J. Šafárik, Tr. SNP 1, 040 66 Košice, Slovak Republic, fax: +421 95 6 428 524, e-mail: mirossay@central.medic.upjs.sk