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Original article

Canine mammary carcinoma cell line are resistant to chemosensitizers: verapamil and cyclosporin A

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Abstract

Cancer chemotherapy can fail in many ways. One of the most significant is the development of multiple drug resistance (MDR), which constitutes a serious clinical problem. The development of MDR relates to the expression of a major membrane pump, P-glycoprotein (P-gp). Thus, currently one of the goals of experimental and clinical oncology is to decrease its activity. So far, many different P-gp inhibitors are available, but their efficacy is still questionable and requires further study.

The aim of our study was to assess an impact of classical P-gp inhibitors (verapamil and cyclosporin A) in the reversion of multidrug resistance in canine mammary cancer cells. We used two cell lines isolated from mammary tumors and two cell lines isolated from their lung metastases. All of them showed P-gp over-expression confirmed using Real-time rt-PCR, Skan^R screening station and confocal microscopy. The FACS analysis showed that in three of the examined cell lines, treatment with verpamil/cyclosporin A was ineffective to reverse cancer chemoresistance. However, more studies in this field are required.

Key words: multidrug resistance, canine mammary cancer, glycoprotein P

Introduction

Cancer chemotherapy can fail in many ways. One of the most significant is the development of multiple drug resistance (MDR), which is a serious clinical problem in some types of cancer (Król et al. 2010). Cytotoxic drugs can lead to the death of most tumor cells, but some of them can survive to grow again. These cells may be genetic variants that are more resistant to chemotherapy and, indeed, may be resistant to a number of different drugs, including active chemotherapeutic agents. MDR is frequently associated with the overexpression of transporters belonging to the ATP-binding cassette (ABC) protein super-

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family. The most important "efflux pump" seems to be P-glycoprotein (P-gp), which can actively pump out anticancer drugs from the cell at the expense of ATP hydrolysis (Perrotton et al. 2007). P-gp, the product of the *mdr-1* gene is expressed in the mammalian intestine, liver and other tissues. In cancer cell lines, P-gp is responsible for the drug-resistant phenotype (Lee et al. 1996, Pawłowski et al. 2013) which can lead to failure in therapy. For this reason currently one of the goals of experimental and clinical oncology is to reverse cancer MDR. So far, many different P-gp inhibitors are available, including calcium channel blockers such as verapamil, diltiazem, or niardipidine; calmodulin inhibitors such as quinine, quinidineor, reserpine, and other molecules such as cyclosporin A or new cyclosporine derivatives (Merlin et al. 1994, Palmeira et al. 2012).

The aim of this study was to assess the efficacy of P-gp inhibitors (verapamil and cyclosporin A) in the reversion of MDR in canine mammary cancer cells. Based on the results of our previous studies (Król et al. 2010a,b, Pawłowski et al. 2013) we used only cell lines that show the highest P-gp expression and, related to that, the highest resistance to vinblastine.

Materials and Methods

Cell lines and cell culture

The cell lines used for this study have been previously described (Król et al. 2010a, b, Majchrzak et al. 2012). Two adenocarcinoma cell lines isolated from canine mammary tumors (CMT-W1 and CMT-W2) and two cell lines isolated from their lung metastases (CMT-W1M and CMT-W2M) were kindly donated by Prof. Dr. Maciej Ugorski from Wroclaw University of Environmental and Life Sciences (Poland). P114 cell line (used as a control) was obtained from Dr. Gerard Rutteman from Utrecht University (The Netherlands). The cells were cultivated in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum (FBS), 50 U/ml penicillin, 50 μg/ml streptomycin, 2.5 μg/ml amphotericin B (reagents obtained from Sigma Aldrich Chemical Co., USA) and grown in tissue culture flasks (Nunc™, Denmark) in an atmosphere of 5% CO_2 and 95% humidified air at 37°C. They were routinely subcultured every second day.

Assessment of mitotic block due to vinblastine and PGP-inhibitor treatment

Exponentially growing cells were seeded on 6-chamber culture plates (Nunc Inc., USA) and cultured for 24 hrs. Vinblastine (Sigma, USA) was then added at concentrations of: 0.05; 0.5; 1.0; 5.0; 25.0 and 50.0 μg/ml and cells were incubated for: 1, 2, 3, 4 and 5 hrs (three replicates were performed). After removal with trypsin-EDTA, the cells were incubated with Ribonuclease A (Sigma, USA) and stained with Acridine orange (Invitrogen, USA) and the Mitotic Index was counted using FACS Vantage flow cytometer (Becton Dickinson Sunnyvale, CA). The coefficient of variation was <5%. A graph was obtained by plotting the logarithm of the mitotic index (1 + Mitotic Index) versus time of cell incubation in vinblastine.

To inhibit the PGP function, verapamil (Sigma, USA) was added to the culture medium at: 24 hrs, 12 hrs, 5 hrs, and 2 hrs before vinblastine, and given at the same time point with vinblastine). Doses of verapamil used in this study were: 1 μmol, 2.5 μmol, 5 μmol, 10 μmol, 25 μmol and 50 μmol. These doses were combined with all the vinblastine doses in all possible variants.

Doses of cyclosporin A (Sigma, USA) used in this study were: 1 μmol, 2.5 μmol, 5 μmol, 10 μmol, and 20 μmol. These doses were used with all the viblastine doses in all possible variants, as with verapamil.

Combinations of different doses and time of culture with vinblastine/verapamil and viblastine/cyclosporin A were used according to the published data (Limtrakul et al. 2004 and Nadir et al. 2005) and our own experience (Król et al. 2010a). As a control for this experiment, we used P114 cell line, which is sensitive to vinblastine treatment, probably due to lower P-gp expression than the examined cell lines (Król et al. 2010a, Pawłowski et al. 2013).

Apoptosis assay

Annexin V-FITC and propidium iodide (PI) dual staining were used for apoptosis analysis. Control cells and cells treated with vinblastine (50 μg/ml) and verapamil given at the highest dose (50 μmol) were harvested by trypsinization and together with the cells floated in medium (10% FBS containing RPMI 1640) were stained using an Annexin V Kit (Becton Dickinson, USA), according to the manufacturer's protocol. The cells were immediately (within 1 hr) analyzed by flow cytometry (BD FACS Aria II, Becton Dickinson, USA). Early apoptotic cells with exposed phosphatidylserine but intact cell membranes bound to Annexin V-FITC but excluded PI. Cells in late apoptotic stages were labeled with both Annexin V-FITC and PI, whereas necrotic cells were labeled with PI only. All samples were assayed in triplicate. The experiment was conducted twice. P114 cell line was used as a positive control.

Reverse-transcriptase qPCR

Total RNA was isolated using a Total RNA kit (A&A Biotechnology, Poland) according to the manufacturer's protocol. Isolated RNA samples were dissolved in RNase-free water. The quantity of isolated RNA was measured using NanoDrop (NanoDrop Technologies, USA). The mean concentration of RNA was 213 ng/fl, and the A260/280 ratio was between 1.8 and 2.0. Samples with adequate amounts of RNA were treated with DNaseI to eliminate DNA contamination. The samples were subsequently purified using RNeasy MiniElute Cleanup Kit (Qiagen). Finally, the RNA samples were analyzed using a BioAnalyzer (Agilent, USA) to measure final RNA quality and integrity. Only RNA with RIN (RNA Integrity Number) > 9 was used for further analyses. Primers used to detect the expression of multidrug resistance 1 (*mdr1*) gene were designed using PRIMER3 software (free on-line access) and checked using Oligo Calculator (free on-line access) and Primer-Blast (NCBI database). The sequences used were: GCTTAACACCCGGCTCACAGAC and TAAGAAAGCGGCACCAATAGAAAT (optimal annealing temperature: 72°C, optimal annealing time: 10 sec) (Pawłowski et al. 2013). Ribosomal Protein 19 (*rps19*) and hypoxanthine phosphoribosyltransferase (*hprt* genes were used as non-regulated references for the normalization of target gene expression (Brinkhof et al. 2006, Etschmann et al. 2006). Quantitative rt-PCR was performed using fluorogenic SYBR Green and the Sequence Detection System, Fast 7500 (Applied Biosystems). Data analysis was carried out using the 7500 Fast System SDS Software Version 1.4.0.25 (Applied Biosystems, USA). The results were analyzed using the comparative Ct method (Schmittgen & Livak, 2008). Relative transcript abundance of the gene equals ΔCt values ($\Delta Ct = Ct^{reference} - Ct^{target}$). Relative changes in transcript were calculated as ΔΔCt values ($\Delta\Delta Ct = 2^{-\Delta Ct}$). The experiment was carried out three times. Samples in which the cDNA was omitted were used as negative controls.

Immunofluorescence staining for cytometry and confocal microscopy

Anti-P-gp polyclonal rabbit antibodies (Molecular Probes, USA) and Alexa Fluor® 488 chicken-anti-rabbit antibodies (Molecular Probes, USA) were used to localize the P-gp in CMT-W1, CMT-W1M, CMT-W2, CMT-W2M cell lines. After incubation with antibodies, the cells were washed twice with PBS and then incubated with a 5 μg/ml solution of 7-aminoactinomycin D (7AAD) in PBS containing 2% FCS, 0.1% sodium azide, and 0.3% saponin for 10 min in the dark, to counterstain the DNA. Coverslips were mounted on microscope slides in ICN mounting medium (ICN Biomedicals Inc, USA).

Skan® analysis

The slides were examined using an Olympus Scan® screening station (Olympus Optical Co., Germany), a modular microscope-based imaging platform designed for fully automated image acquisition, and analyzed using Olympus Scan® software for screening applications (Olympus Optical Co., Germany). The mean fluorescence related to P-gp expression was calculated and visualized on histograms. Digital images were processed using Adobe Photoshop software.

Confocal microscopy

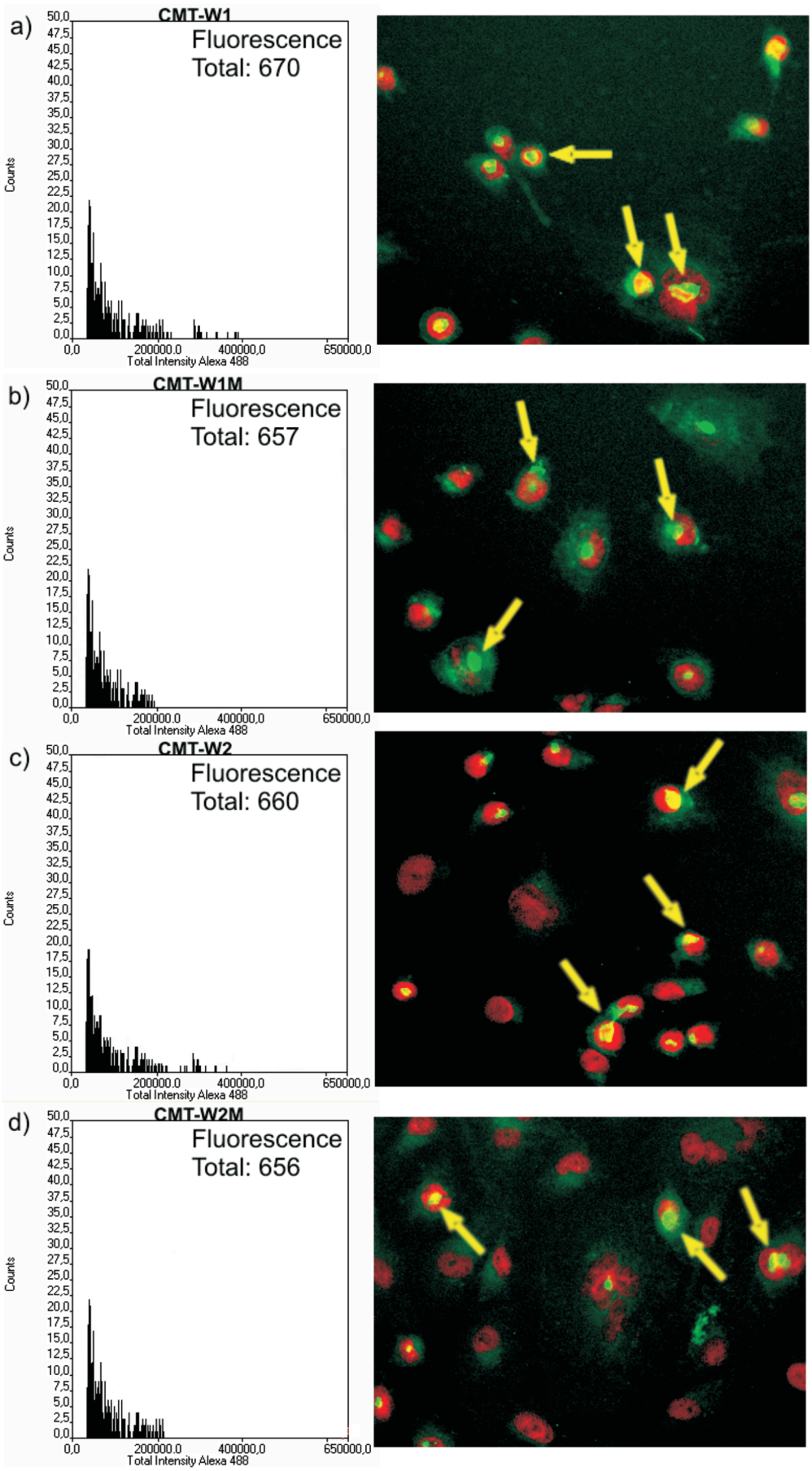
Cells were visualized using the confocal laser microscope FV-500 system (Olympus Optical Co, Germany). The combinations of excitation/emission were: Argon 488 nm laser with 505-525 nm filter for Alexa Fluor® 488 and HeNe 543 nm laser with 610 nm filter for 7AAD staining. The pictures were gathered separately for each fluorescence channel. The cells were examined using Fluoview software (Olympus Optical Co., Germany).

Statistical analysis

Analysis for statistical purposes was done using Prism version 5.00 software (GraphPad Software, USA). The one-way ANOVA and Tukey HSD (Honestly Significant Difference) post-hoc test were applied, as well as regression analysis. A p-value of <0.05 was recognized as significant, and p-value <0.01 and p-value <0.001 as highly significant. The data was expressed as means +/- S.D. For molarity calculations, the Molarity Calculator (GraphPad, USA) on-line platform was used.

Results

To assess the role of P-gp inhibitors *in vitro* in canine mammary cancer we used cell lines showing high expression of P-gp (Pawłowski et al. 2013). Real-time rt-PCR analysis revealed that relative *mdr1* expression in the CMT-W1 cell line was 17.85 and in CMT-W2 16.42 (Pawłowski et al. 2013), whereas in CMT-W1M and CMT-W2M it was 17.05 and 18.04,



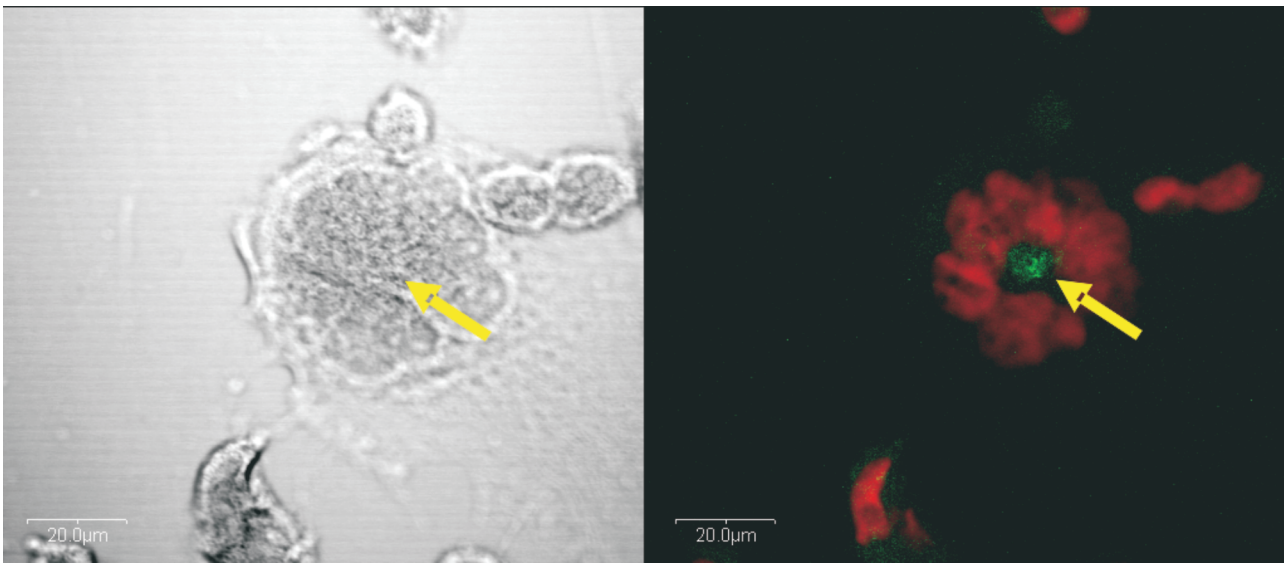


Fig 2. Pictures of canine mammary cancer cells showing P-gp localization (green color, indicated by arrows). Pictures were taken using confocal microscopy (Olympus, Germany).

respectively. Expression of the *mdr1* gene was significantly lower ($p<0.001$) in the control P114 cell line (Pawłowski et al. 2013). P-gp expression at protein level was confirmed using a Skan^R screening station (Fig. 1). The mean fluorescence of P-gp in CMT-W1, CMT-W1M, CMT-W2, and CMT-W2M was 670, 657, 660, and 656, respectively. Analysis of P-gp expression using confocal microscopy showed its cytoplasmic localization (Fig. 2).

The vinblastine concentration of 0.05 µg/ml has been described as optimal to block cells in the mitosis phase of the cell cycle (Darzynkiewicz et al. 1994). We also found this dose as effective in some canine mammary cancer cells (Król et al. 2009, 2010a). Those cells however, showed lower P-gp expression (Król et al. 2010, Pawłowski et al. 2013) than those examined in the present study. In CMT-W1, CMT-W2, CMT-W1M, and CMT-W2M a vinblastine concentration of 0.05 µg/ml was completely ineffective. In these cell lines, vinblastine given at even a 1000 times higher dose did not have any effect. The results of our previous study showed that the IC_{50} dose of vinblastine in these cell lines was also high and ranged between 8 000 and 10 000 nmol given for 48 hrs (these values are higher than maximal tolerable blood levels). In other cell lines with lower P-gp expression IC_{50} doses of vinblastine ranged between 1588 and 2 744 nmol given for 48 hrs (Pawłowski et al. 2013).

To assess the efficacy of P-gp inhibitors, two various response modifiers (RM) were added: verapamil and cyclosporin A (Colombo et al. 1994; Arboix et al. 1997) at various concentrations and time points (see Materials and Methods). Verapamil was effective only in the CMT-W2 cell line (the highest dose given 2 hrs before treatment with the highest dose of vinblastine) (Fig. 3), successfully reversing P-gp activity and causing mitotic block. In the other cell lines RMs given at different doses and time points failed in reversion of P-gp activity.

Moreover, treatment of three canine mammary cancer cell lines with neither vinblastine nor with vinblastine and RMs, caused no effect on apoptosis (Fig. 4). There were no significant changes between the number of apoptotic cells in control conditions or after the vinblastine or vinblastine and RMs treatment in CMT-W1, CMT-W1M and CMT-W2M cell lines. Only in the CMT-W2 cell line, the number of apoptotic cells increased significantly due to vinblastine and verapamil treatment (Fig. 4). In these conditions the number of apoptotic cells increased from 13.2% to 20.1% ($p<0.001$).

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Fig 1. Representative histograms showing mean P-gp related fluorescence (left panel) and pictures showing P-gp expression (green) (right panel) in (a) CMT-W1, (b) CMT-W1M, (c) CMT-W2 and (d) CMT-W2M cell lines. Pictures (x400) taken using Skan® screening station (Olympus, Germany).

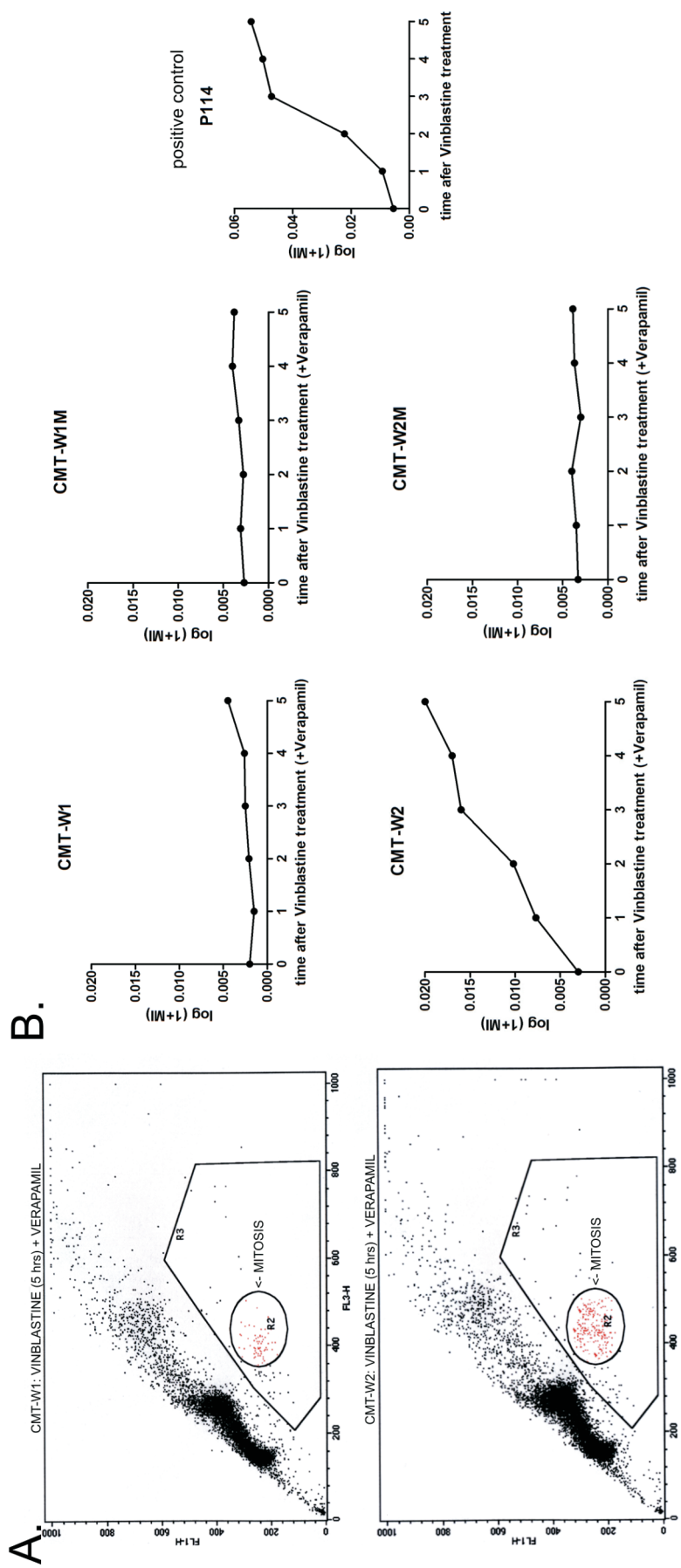

Fig 3. Cytograms of CMT-W1 and CMT-W2 cells after 5 hrs of incubation with vinblastine (50 µg/ml) and Verapamil (50 µmol). Cells were stained with Acridine Orange (Invitrogen, USA). Region R2 shows mitotic cells. Analysis was performed using BD FACS Ventage (Becton Dickinson, USA).
A. Graphs of Mitotic Index (MI) in CMT-W1, CMT-W2, CMT-W1M and CMT-W2M cell lines in control conditions (0 hrs) and after 1, 2, 3, 4, and 5 hrs after vinblastine treatment (50 µg/ml) and Verapamil (50 µmol).

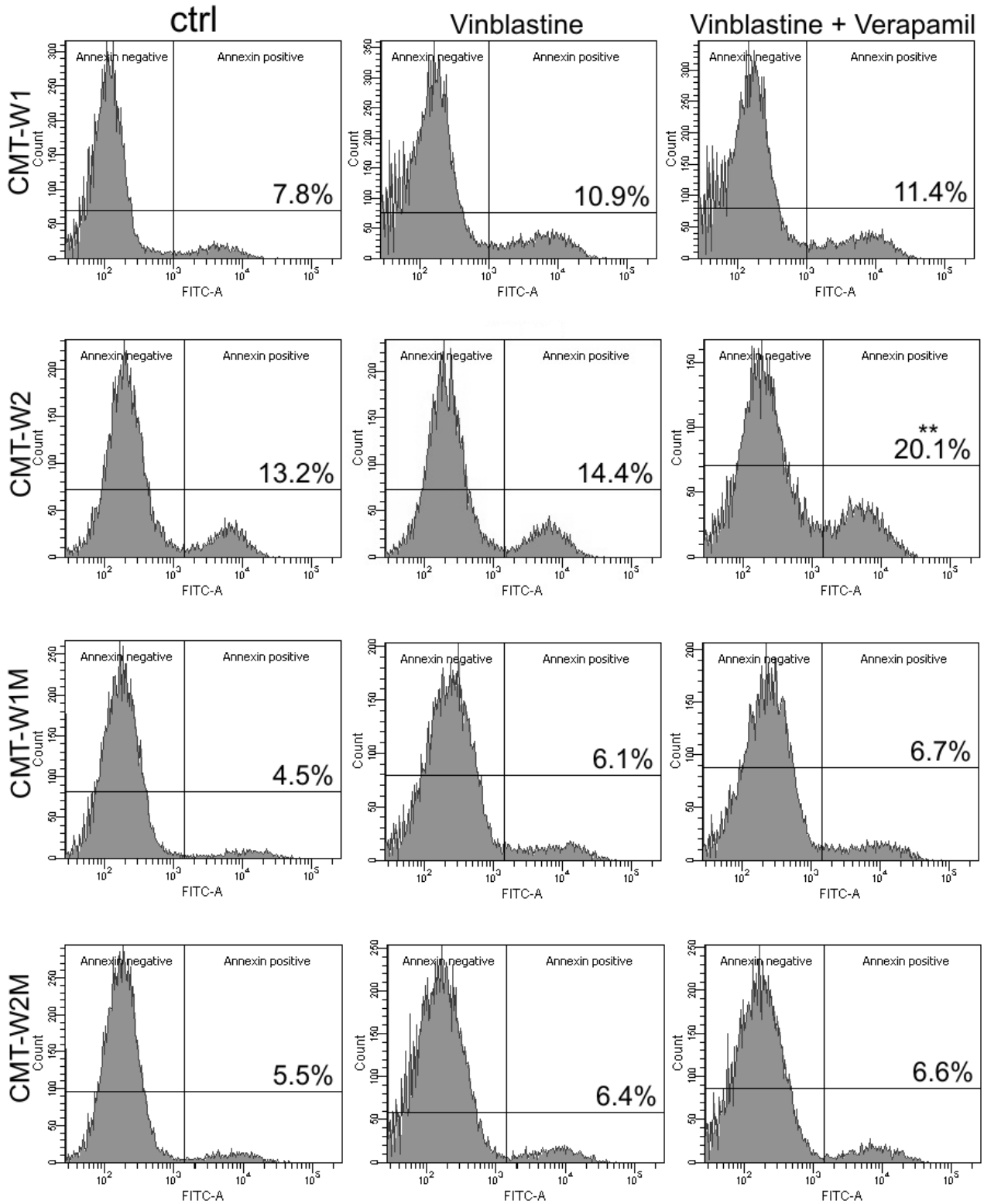

Fig 4. The representative histograms showing number of apoptotic cells represented as a percentage of Annexin-V-positive cells (±SD) obtained using FACS Aria II (Becton Dickinson). Apoptosis significantly increased in the CMT-W2 cell line after vinblastine (50 μg/ml) and Verapamil (50 μmol) treatment. The experiment was conducted in triplicate, two repetitions were performed. One-way ANOVA followed by Tukey HSD post-hoc test were applied, $p<0.01$ is marked as **.

Discussion

Cancer cells retain the important mechanism of self-potection against chemotherapeutic agents through the activity of numerous membrane pumps. These pumps are responsible for the efflux of anticancer drugs from the cytoplasm (Pawłowski et al. 2013). The best known efflux pump is P-glycoprotein (P-gp). Its expression in tumor cells has a prognostic value in patients with primary breast cancer and is likely to be a marker of a more malignant and invasive phenotype (Linn et al. 1995). Similarly, its higher expression has been found in the invasive canine mammary cancer cell lines (Król et al. 2010a, Pawłowski et al. 2013). These results are in accordance with the findings that highly invasive cancer cells are usually more resistant to chemotherapy (Morrison et al. 2013).

In 1981 the reversal of P-gp activity by verapamil was discovered. Since then, efflux pump inhibitors have been intensively studied as potential reversers of multidrug resistance (Palmeira et al. 2012).

The aim of our study was therefore to investigate the efficacy of the P-gp inhibitors verapamil and cyclosporin A in canine mammary cancer cell lines treated with vinblastine. Vinblastine inhibits the exchange of tubulin at the ends of *in vitro* reassembled microtubules in a significant manner. Thus, vinblastine causes mitosis block of cycling cells, followed by cell death (Jordan et al. 1992). In the present study we assessed mitotic block and apoptosis related to vinblastine treatment (given as a single agent or as a combined therapy with P-gp inhibitors: verapamil or cyclosporin A) in cell lines showing high expression of P-gp (Pawłowski et al. 2013).

Our results showed that partial reversal of P-gp activity was possible only in one of the four examined cell lines. However, the mitotic block obtained was much lower than in control cells expressing in control conditions a significantly lower level of P-gp.

These results indicate that achievement of P-gp inhibition using classic RMs may be very difficult in canine mammary cancer, especially in highly invasive tumors. Further studies in this field are thus required in order to improve cancer therapy.

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