Effect of New Oxicam Derivatives on Efflux Pumps Overexpressed in Resistant a Human Colorectal Adenocarcinoma Cell Line

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Abstract. *Background: Oxicams are non-steroidal anti-inflammatory drugs (NSAIDs). Antitumor potential of NSAIDs has often been reported in literature. We studied antitumor activity of newly synthesized oxicam derivatives (PR17 and PR18) against doxorubicin-sensitive and resistant human colorectal adenocarcinoma cells (LoVo and LoVo/Dx). Materials and Methods: The cytotoxicity of oxicam derivatives alone and in combination with doxorubicin was assessed. Inhibition of P-glycoprotein (ABCB1) transport activity was monitored by flow cytometry. Expression of ABCB1 gene was analyzed by semi-quantitative reverse transcription PCR, while ABCB1 protein expression was assessed by western blotting. Results: Oxicam derivative PR18 was more cytotoxic to cancer cells than PR17. PR18 was observed to sensitize LoVo/Dx cells to doxorubicin and was identified as an effective multidrug resistance modulator. Additionally, ABCB1 expression was reduced in the presence of PR18. Conclusion: PR18 was identified as an effective modulator in LoVo/Dx resistant human colorectal adenocarcinoma cells which overexpressed ABCB1 efflux pump.*

The development of safe and effective cancer therapy is a continuing to be a scientific aim. Despite the existence of new technologies, the fight against cancer is too often ineffective. The major risk in cancer chemotherapy is that the cancer may develop resistance to the used drug. Cancer cells exposed to toxic compounds can develop resistance by a number of mechanisms. The most important among them are altered drug activation or degradation (1), as well as altered membrane transport either by decreased drug uptake or by increased drug efflux (2). The development of resistance to multiple chemotherapeutic agents by cancer cells often results from elevated expression of ATP-binding cassette (ABC) drug transporters in the cell membrane and constitutes a major factor responsible for the failure of chemotherapy. Many ABC proteins play a key role in pharmacokinetics affecting entry and extrusion of drugs into and out of cells. In particular, overexpression of P-glycoprotein (ABCB1) plays a significant role in chemoresistance. This protein is believed to act as an energy-dependent efflux-pump for a variety of structurally-diverse chemotherapeutic agents, thereby decreasing their intracellular accumulation (3). Consequently, cancer cells can evade the cytotoxic effect of anticancer drugs. New data on the antitumor potential of commonly used non-oncological drugs, such as non-steroidal anti-inflammatory drugs (NSAIDs) have recently appeared in literature (4, 5), and the challenge is to obtain new, more active compounds possessing anticancer or multidrug resistance-reversing activity. Many studies have indicated that NSAIDs may reduce the risk of cancer incidence, including of breast, colon, lung, and stomach cancer (5). Piroxicam is a NSAID of the oxicam class used as an analgesic and to relieve the symptoms of rheumatoid and osteoarthritis, primary dysmenorrhea, and postoperative pain. NSAIDs are inhibitors of the cyclooxygenases (COXs) a family of enzymes which catalyze the rate-limiting step of prostaglandin biosynthesis. COX2 was described as modulating cell proliferation and apoptosis, mainly in solid tumors, that is, colorectal, breast, and prostate cancer, and, more recently, in hematological malignancies (6-8).

Immunohistochemical analyses of human breast tumor specimens revealed a strong correlation between expression of COX2 and P-glycoprotein (9). Surowiak *et al.* (9) mentioned that COX2 might be the factor responsible for the regulation of expression not only of P-gp but also of two

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Key Words: Multidrug resistance (MDR), P-glycoprotein, non-steroidal anti-inflammatory drugs (NSAIDs), cyclooxygenase 2 (COX2).

Table I. *The sequences of primers and sizes of amplification products.*

Gene	Sequence of primers	Amplification product (bp)
ABCB1	Forward: 5'-AAGCTTAGTACC AAAGAGGCTCTG-3'	
	Reverse:5'-GGCTAGAAACAATAGTG AAAACAA-3'	243
GUS	Forward: 5'-CTGCCGCAGTTCTT CAACAACG-3'	
	Reverse: 5'-CGTCGGTGACTGTT CAGTCATG-3'	558

other ABC transporters: the multidrug resistance-associated protein 1 (MRP1, ABCC1) and the breast cancer resistance protein (BCRP, ABCG2). It has been suggested that COX inhibitors can sensitize cancer to chemotherapeutic drugs by inhibiting P-glycoprotein, MRP1 and BCRP, thus enhancing the cytotoxicity of anticancer drugs (10, 11). These effects of NSAIDs have been demonstrated in several different malignancies (12, 13). Moreover, cancer cells with the multidrug resistance (MDR) phenotype often display other properties, such as genomic instability, loss of checkpoint control or resistance to apoptosis, which also hamper successful chemotherapy (14). Pre-clinical and clinical studies suggest that COX2 inhibitors are highly promising agents not only for the treatment of pain and inflammation, but also for the prevention of cancer (15). Treatment with COX2-specific inhibitors results in a wide range of cellular effects, including induction of apoptosis, reduction of cell proliferation and enhanced cytotoxicity of anticancer drugs (16, 17).

It is well-known that pharmacological actions of drugs may be a consequence of their direct interaction with proteins (*e.g.* transporters) or their influence on biophysical properties of biological membranes which, in turn, may affect protein conformation. The therapeutic action of NSAIDs may also be the result of the indirect inhibition of COX due to their disruptive effect on membrane properties.

The traditional methods of searching for the relationship between chemical structure and pharmacological activity of a drug are now supported by the use of molecular modeling techniques. Structure–activity relationship studies open up the possibility of better understanding of the properties of biological systems based on the molecular structure of the interacting molecules. Quantitative methods for study of the structure-activity relationship (QSAR) allow for electronic, structural and topological parameters to be specified, as well as the hydrophobicity of tested compounds, and correlation of these to the effects of compounds on specific intracellular targets (*e.g.* proteins, membrane lipids). Maniewska *et al.* used QSAR methods to describe the oxicam derivatives studied here (PR17 and PR18) and to correlate these compounds' properties with their ability to affect phospholipid bilayers (18). The calorimetric measurements obtained by Maniewska *et al.* revealed that PR17 and PR18 interact with the phosphatidylcholine bilayer and change the lipid phase (18). Additionally, the presence of a carbonyl group increased the ability of PR18 to interact with model membranes.

The aim of the present study was to determine the anticancer properties of the two, newly-synthesized oxicam analogs, PR17 and PR18 (Figure 1) against doxorubicin-sensitive and -resistant human colon adenocarcinoma cell lines, LoVo and Lovo/Dx. Expression of MDR-related transporters in both cell lines was previously characterized by reverse transcription-polymerase chain reaction (RT-PCR) and immunochemical methods and elevated expression of P-glycoprotein was accepted to be responsible for doxorubicin-resistance in LoVo/Dx cells (19). The effect of the studied compounds on transport function of P-glycoprotein as well as on P-glycoprotein expression was also investigated.

Materials and Methods

Oxicam derivatives. PR17 [(4-chlorophenyl)-{2-[3-[4-(2-fluorophenyl) piperazin-1-yl]propyl]-4-hydroxy-1,1-dioxo-1,2-benzothiazin-3-yl}methanone] and PR18 (2-[3-(4-chlorobenzoyl)-4-hydroxy-1,1-dioxo-1,2-benzothiazin-2-yl]-1-[4-(fluorophenyl)piperazin-1-yl]ethanone) were synthesized as previously described (18) (see Figure 1). In PR18 1,2-benzothiazine ring is connected to 2-fluorophenylpiperazine by a methylene carbonyl linker, while PR17 possesses a propyl linker, which is one carbon atom longer. The compounds were dissolved in dimethylsulfoxide (DMSO).

Cell culture. Sensitive (LoVo) and doxorubicin-resistant human colon adenocarcinoma cell line (LoVo/Dx) were obtained from the Institute of Immunology and Experimental Therapy (Polish Academy of Sciences, Wroclaw, Poland). Both cell lines were grown in Dulbecco's medium supplemented with 10% fetal bovine serum, L-glutamine and antibiotics. LoVo/Dx cells were grown in the presence of doxorubicin (Sigma-Aldrich, Poznan, Poland) (100 ng/ml). Cells were grown at 37˚C in a humidified atmosphere containing 5% CO_2. In log-phase of growth, they were removed from the flask surface with non-enzymatic cell dissociation solution (Sigma-Aldrich, Poznan, Poland) . The density of cells in suspension was determined using Burker hemocytometer and was generally about 10^6/ml.

Sulphorhodamine B (SRB) assay. Cell viability was determined using SRB assay (20) with minor modifications. Sulphorhodamine is a protein-binding dye that binds to the basic amino acids of cellular macromolecules and it is used in the end-point determination of cell growth. Cells (4×10^4 cells per well) were seeded into 96-well plates in 75 μl of medium and were allowed to attach for 60 min at 37˚C. Medium containing 5, 10, 20, 50, or 100 μM of PR17 or PR18 was added and the cells were incubated for another 48 h. In the experiments on doxorubicin cytotoxicity, doxorubicin was used at 0.5-10 μg/ml and oxicam derivatives at 5 μM. The concentration of DMSO in the medium did not exceed 0.5%. Subsequently, the cells were incubated with 50 μl of ice-cold 50% trichloroacetic acid for 1 h at 4˚C. After washing with distilled water 10 times, the cells were

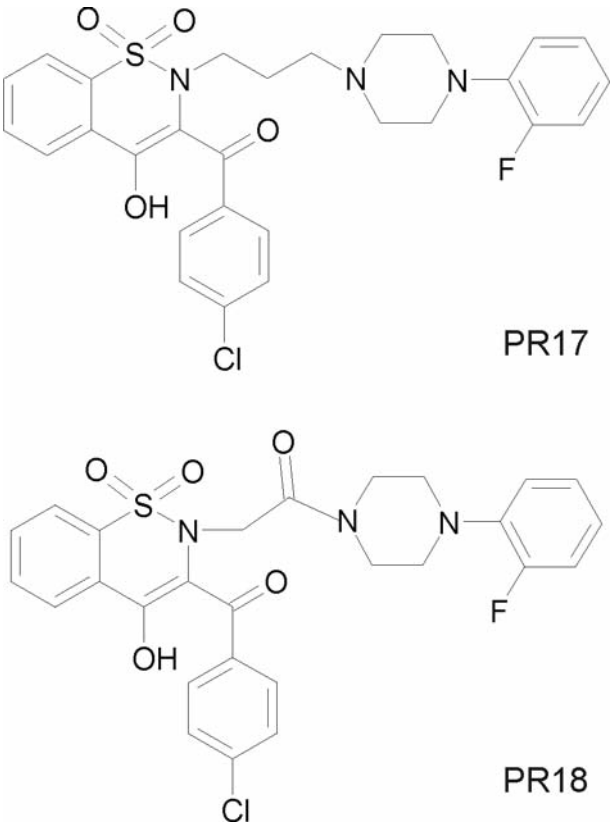

Figure 1. *Chemical structures of the studied oxicam derivatives.*

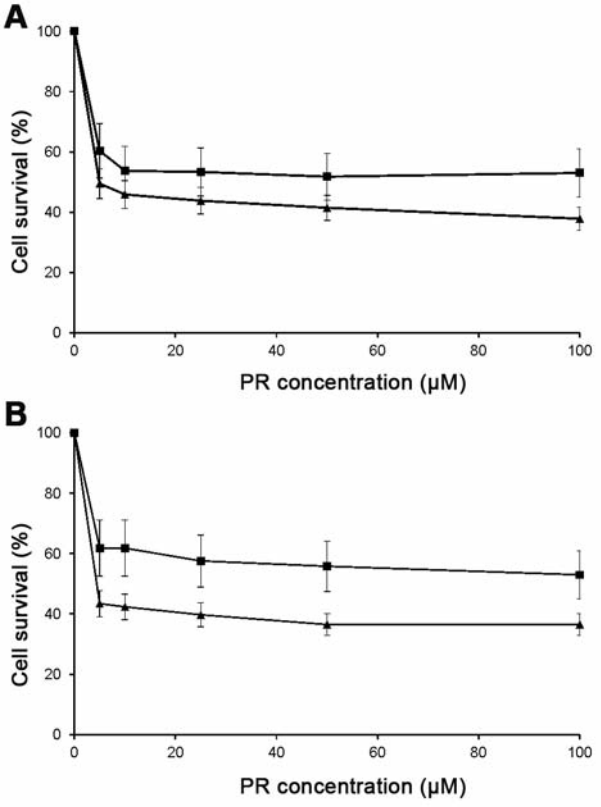

Figure 2. *Cytotoxicity of PR17 (squares) and PR18 (triangles) to LoVo (A) and LoVo/Dx cells (B). Means±SD of three independent experiments are presented.*

stained with 50 μl SRB solution (0.4%) at room temperature for 30 min in darkness. The unbound dye was removed by several washes with 1% acetic acid and each well of the plate was dried. To solubilize the bound SRB, 150 μl of 10 mM Tris base (pH=10.5) was added. The absorbance was measured at 492 nm using a scanning spectrophotometer (Labsystem Multiscan MS Type 352 Microplate Reader, Thermo, Helsinki, Finland). The experiments were repeated three times. The optical density (OD) of SRB in the well was directly proportional to the cell number hence absorbance values were plotted against concentration.

Accumulation of rhodamine 123 in cancer cells. Rhodamine 123 is commonly used in flow cytometry as a functional reporter for P-glycoprotein. Harvested LoVo or LoVo/Dx cells (3×10^5/ml in serum-free medium) were incubated with PR17 or PR18 at an appropriate concentration for 15 min at room temperature. Rhodamine 123 was then added (final concentration 2 μM) and the cells were incubated for 60 min at 37˚C. After incubation with the dye, cells were washed and resuspended in phosphate buffered saline for analysis. The fluorescence of the cell population was measured by BD FACS Control, Canto II, Becton Dickinson, USA Fluorescence was recorded using 530/30 nm band pass filter and 488 nm excitation wave. A total of 5,000 events were registered and analyzed with the use of Cell Quest® software, Becton Dickinson and Company, USA. The influence of DMSO (maximal concentration in samples 0.8%) on the cells was also monitored. The fluorescence intensity ratio (FIR) was calculated from the following equation on the basis of measured fluorescence intensity values (FL).

$$FIR = \frac{(FL_{LoVo/Dx\ treated}/FL_{LoVo/Dx\ control})}{(FL_{LoVo\ treated}/FL_{LoVo\ control})}$$

Control samples were treated with medium only (without modulator). Experiments were performed in triplicate.

Gene expression studies. Ability of oxicam derivatives to change the level of gene expression of multidrug transporter gene *ABCB1* was examined. The changes of mRNA expression induced by PR17 or PR18 were analyzed by semiquantitive RT-PCR method. Cells were seeded onto 6-well plates and incubated at 37˚C for 24 hours in order to be adsorbed onto the plate surface. Subsequently, PR17 and PR18 were added to obtain a final concentration of 100 μM. Cells were incubated in the presence of oxicam derivatives at 37˚C for 48 h.

RNA extraction. RNA extractions were carried out with the E.Z.N.A Total RNA Kit I (Omega Bio-Tek, Georgia, USA), according to the manufacturer's instructions. Harvested cells were

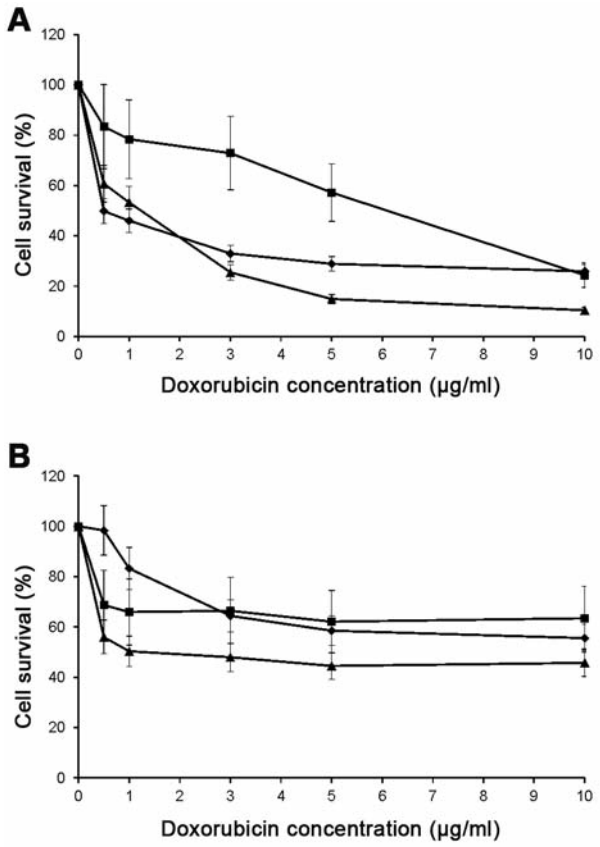

Figure 3. *Cytotoxicity of doxorubicin alone (diamond) and in combination with PR17 (squares, 5 μM), and PR18 (triangles, 5 μM) towards LoVo (A) and LoVo/Dx (B) cells. Means±SD from three independent experiments are presented.*

suspended in TRK Lysis Buffer containing β-mercaptoethanol. Samples were homogenized using syringes and needles and then an equal volume of 70% ethanol was added. Lysates were applied to the HiBind RNA spin columns and centrifugedfor 1 min at 10,000 ×g at 25˚C. Extracts adsorbed on the columns were purified with RNA Wash Buffer I and then with RNA Wash Buffer II. Subsequently, RNA was eluted by adding diethylpyrocarbonate-treated water. The concentration of RNA was determined by measuring the optical density at 260 nm and purity was estimated using the 260/280 nm absorption ratio, which was consistently >1.8 (NanoDrop® ND-1000; Thermo Scientific, Wilmington, USA).

Reverse transcription. For cDNA synthesis, the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Warsaw, Poland) was used. RNA (300 ng) was reverse transcribed in the presence of 1× RT Buffer, 1× RT Random Primers, 2 mM dNTP, 1.25 U MultiScribe Reverse Transciptase and 0.5 U RNase Inhibitor (Lab Empire, Warsaw, Poland). The reverse transcription processes were performed using BIO-RAD MJMini Personal Thermal Cycler, CA, USA using the program, including the 4 steps: at 25˚C for 10 min, 37˚C for 120 min, followed by denaturation at 85˚C for 5 minutes and then by cooling to 4˚C.

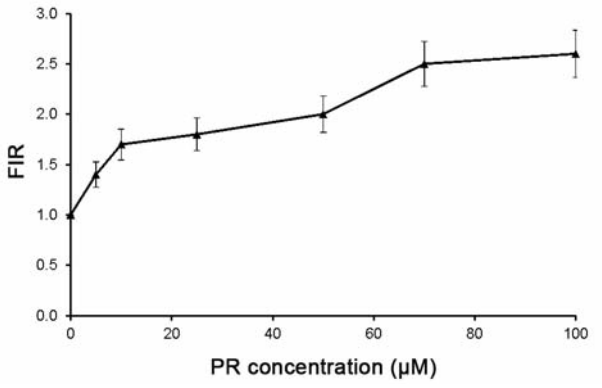

Figure 4. *Influence of PR18 on rhodamine 123 accumulation in LoVo/Dx cells.*

PCR. The primers used in the experiments were selected manually and synthesized at the Institute of Biochemistry and Biophysics Polish Academy of Science. The sequences were designed in such a way that the 5' and 3' end of each comprising different exones. All primers were compared with GenBank using the program Blast (www.ncbi.nlm.nih.gov) in order to determine their specificity. Semiquantitive PCR were performed using β-glucuronidase (*GUS*) as a reference gene. The sequences applied in our experiments and the sizes of amplification products are shown in Table I. PCR amplifications were performed in 1×Taq Buffer with KCl, 0.9 mM $MgCl_2$, 140 μM dNTP mix, 0.18 μM of each primer, along with 1 U Taq DNA Polymerase LC (Thermoscientific, Wilmington, USA) and 1×, 50× and 100× diluted cDNA in a final volume of 25 μl. The reactions were carried out under following conditions: initial denaturation at 95˚C for 2 min, 35 cycles of denaturation at 95˚C for 1 min, annealing at 60˚C for 1 min and extension at 72˚C for 1 min, followed by final elongation at 72˚C for 1 min and by cooling to 4˚C. The amplified fragments were separated by 2.5% agarose gel electrophoresis. For DNA staining, Simply Safe reagent (EURx, Gdansk, Poland) was used.

Western blot analysis of P-glycoprotein expression. Cell lysates used for western blot analysis were prepared from crude cell membranes following established procedures (21). Briefly, cells were harvested, washed, and centrifuged. The pellet was resuspended in hypotonic lysis buffer and centrifuged twice for 10 min at 4,000 ×g and 4˚C. The supernatant from the second centrifugation was retained and centrifuged again to obtain crude cell membranes that were then resuspended in sucrose buffer and used as lysates for western blot. Protein concentration in the cell lysates was determined with the Bradford assay (22), and equal amounts of isolated membrane proteins were loaded onto gels. Cell lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The blot was then probed with primary monoclonal antibody to P-glycoprotein (human) (C494, dilution 1:1000; Alexis Enzolifesciences, New York, USA) followed by reaction with horseradish peroxidase-conjugated secondary antibody (Pierce Rabbit Anti-Mouse IgG; Thermo Scientific, Wilmington, USA).

Statistical Analysis. All experiments were performed in triplicate. Data were described as means±SD and analyzed by the Student's *t* test. *p*-Values below 0.05 were considered to be statistically significant.

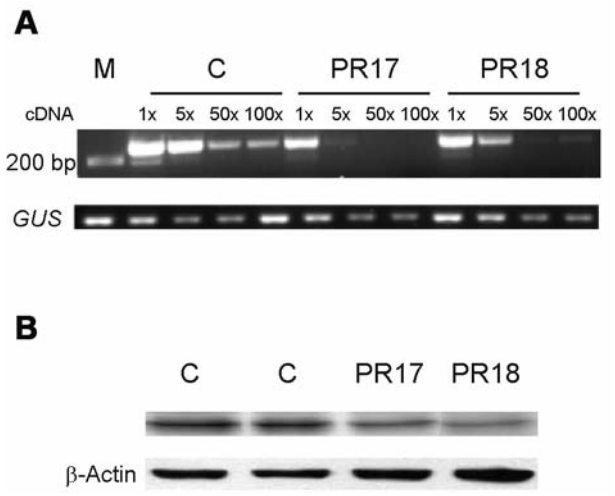

Figure 5. *Analysis of (ABCB1) (243 bp) gene expression in LoVo/Dx by RT-PCR (A) and western blot (B). M: Marker (Range Low –Ruler Fast); C: cancer cells not treated with oxicam derivatives; PR17 and PR18: cancer cells treated with 100 μM of PR17 or PR18, respectively.*

Results

Newly-synthesized oxicam derivatives exert their cell growth-inhibitory action by multiple pathways. Firstly, the cytotoxicity of PR17 and PR18 to doxorubicin-sensitive and -resistant human adenocarcinoma cells was determined using the SRB assay (Figure 2). The oxicam derivatives PR17 and PR18 were both cytotoxic to LoVo and LoVo/Dx cells, however PR18 was more cytotoxic than PR17 to both cell lines. Additionally, the effect of oxicam derivatives at 5 μM on doxorubicin cytotoxicity to LoVo and LoVo/Dx cells was investigated. PR18 was observed to sensitize LoVo/Dx cells to doxorubicin by increasing the cytotoxicity of the drug (Figure 3). Generally, PR18, with an additional carbonyl group, was found to be more cytotoxic to human colonic cancer cells than PR17, whose linker between 1,2-benzothiazine ring and 2-fluorophenylpiperazine is uncarbonylated and one carbon atom longer. The anticancer activity of other compounds with substituents and as a carbonyl group was also observed by other groups (23, 24). Moreover, PR18 has been identified as an effective MDR modulator since it increased doxorubicin cytotoxicity in doxorubicin-resistant LoVo/Dx cells

In order to confirm the putative role of PR18 as an MDR modulator, a flow cytometric test based on the measurement of the intracellular accumulation of rhodamine 123 (fluorescent substrate analog for P-glycoprotein) was applied. The P-glycoprotein inhibitory effect of PR18 was much more pronounced in LoVo/Dx cells than in LoVo, as shown by calculation of FIR values (Figure 4). It should be mentioned that specific inhibitors of BCRP and MRP1 (fumitremorgin C, novobiocin, MK571) did not change the MDR-reversal ability of PR18 in resistant LoVo/Dx cells (data not shown).

As shown in Figure 5A, both PR17 and PR18 reduced mRNA expression of P-glycoprotein multidrug transporter in the LoVo/Dx subline. P-Glycoprotein expression in MDR LoVo/Dx cells was also checked using western blot method. It was demonstrated that the level of this protein was also reduced in the presence of PR18 (Figure 5B).

Discussion

NSAIDs are mainly used to treat pain and/or inflammation. In cell line models they exhibit characteristics of compounds having anticancer potential. They can inhibit proliferation, induce apoptosis in a COX-2-dependent or -independent manner. These drugs have the ability to sensitize cancer cells to cytotoxic drugs by modulating ABC transporter activity. And this was demonstrated in several different malignancies.

Awara *et al*. suggested that inhibition of P-glycoprotein activity by NSAIDs is the main mechanism responsible for the observed enhanced antitumor effect of doxorubicin in the presence of these drugs (25). It was confirmed by a reduction of rhodamine 123. Ponthan *et al*. (2007) mentioned that celecoxib in combination with doxorubicin is very effective in the inhibition of cancer cell proliferation. In our studies PR18 in combination with doxorubicin enhanced the anti-tumour effect of doxorubicin (26). Anticancer activity of PR18 may also be a consequence of its interaction with biological membranes, which was confirmed by Maniewska *et al*. (18).

Similarly to other NSAIDs (such as indomethacin, sulindac, acemetacin), oxicams may also sensitize cancer cells to chemotherapy by introducing changes in the expression of ABC transporter proteins. P-Glycoprotein, BCRP and MRP1 are the three most commonly expressed transporters reported to contribute to the development of the MDR phenomenon. NSAIDs, particularly COX2 inhibitors, may suppress MDR not only by inhibition of the ABC transporter activity but also, as was suggested by Gibson *et al*. (27), by changes in ABC gene and protein expression. These effects of NSAIDs on the expression and function of P-glycoprotein were also reported in Caco-2 cells (28). Our findings using newly-synthesized derivatives of oxicame were in accordance with their theory.

Conclusion

PR18 was identified as an effective MDR modulator in MDR LoVo/Dx colonic cancer cells. The modulatory effect of this compound was manifested by increased doxorubicin accumulation in drug-resistant cells. This effect might be the result not only of reduced expression of P-glycoprotein but also by direct inhibition of its transport activity by PR18. These findings imply that this newly-obtained oxicam derivative can regulate P-glycoprotein activity and function. However, the exact elucidation of the mechanism underlying PR18 action in cancer cells requires further studies.

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