Chemotherapy Sensitivity Recovery of Prostate Cancer Cells by Functional Inhibition and Knock Down of Multidrug Resistance Proteins

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BACKGROUND. In several cancer types, expression of multidrug resistance (MDR) proteins has been associated with lack of chemotherapy response. In advanced prostate cancer (PCa) the use of chemotherapy is mainly palliative due to its high resistance. Previously, we described that MDR phenotype in PCa could be related with high basal and drug-induced expression of MDR proteins P-Glycoprotein (P-Gp), MRP1, and LRP.
METHODS. Using primary cell cultures from PCa patients, we evaluated the effect of function and expression inhibition of P-Gp, MRP1, and LRP, on cell survival after chemotherapy exposure. Cells were treated with specific MDR protein substrates (docetaxel and mitoxantrone for P-Gp, methotrexate for MRP1 and cisplatin for LRP) and pharmacological inhibitors (cyclosporine A, genistein and 3-aminobenzamide), and cell survival was evaluated trough 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and cell cycle analysis. MRP1 activity was evaluated by FACS using the specific inhibitor MK571. Cells were transfected with MDR proteins siRNAs and treated with the corresponding substrates.
RESULTS. PCa cell resistance to MDR protein substrates was partially reversed, decreasing cell survival in around 20%, by treating primary cell cultures with specific pharmacological inhibitors. PCa cells transfected with siRNAs against MDR proteins decreased cell survival when treated with the corresponding drugs. Docetaxel was the most effective chemotherapeutic drug to induce cell death and decrease survival.
CONCLUSION. Low chemotherapy response in PCa could be explained, in part, by over-expression of functional MDR proteins. Expression and function of these proteins should be evaluated to enhance efficacy of docetaxel-based therapies of patients with hormone-resistant PCa. *Prostate 71: 1810–1817, 2011.*

KEY WORDS: **chemotherapeutic drugs; multidrug resistance proteins; primary cell culture; prostate cancer; siRNA**

INTRODUCTION

Prostate cancer (PCa) is the most frequently diagnosed cancer and the second-leading cause of cancer death in men over the world [1,2]. Advanced stages of PCa are related with metastasis and low response to surgery and radiotherapy, and most patients progress to a hormone refractory (HR) phase showing no response to these treatments [3]. Chemotherapy for HR-PCa is used as a palliative treatment with no reports of efficacy increase when used in earlier stages [4]. Several clinical trials using

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chemotherapeutic drugs to increase HR-PCa patient survival showed that only docetaxel-based chemotherapy improved median survival in a few months respect to other drug combinations [5,6]. Pre-operative adjuvant treatment with docetaxel and mitoxantrone in high-risk PCa patients increased 5-year survival [7]. Docetaxel treatment can increase survival benefits by combination with other drugs, and several novel drugs targeting specific pathways involved in cell signaling, proliferation, angiogenesis, apoptosis, and immune modulation are currently under investigation [8]. Previously, we reported that PCa primary cell cultures are more resistant to docetaxel and mitoxantrone than commercial PCa cell lines. This resistance may be related with the expression of multidrug resistance (MDR) proteins P-Glycoprotein (P-Gp), MRP1, and LRP. These proteins show higher expression levels when compared with PCa cell lines. Moreover, MDR proteins level is related with previous exposure to chemotherapeutic drugs and with the tumor Gleason grade [9]. MDR proteins in normal tissues are responsible for ATP-dependent transport of a variety of xenobiotics, including drugs, lipids, and metabolic products across the plasma and intracellular membranes. MDR proteins are overexpressed in several cancer types and have been associated with tumor progression, cell migration, invasion and metastasis, and transport of cancer-promoting substrates and tumor associated metabolites [10].

MATERIAL AND METHODS

Cell Cultures

Primary cell cultures were established as described previously [11] from PCa samples delivered from our Institutional Hospital. Primary cultures and PC3 cell line (ATCC) were maintained in DMEM-F12 medium supplemented with 7% and 10% of fetal bovine serum (Gibco, Invitrogen) respectively, in a 5% CO_2 atmosphere at 37°C.

Ethical Considerations

This study has been approved by the Ethics Committee of the Clinical Hospital of the University of Chile and the Ethics Committee for Research in Humans of the Faculty of Medicine of the University of Chile.

Chemotherapeutic Drugs

Mitoxantrone, docetaxel, methotrexate, cisplatin, cyclosporine A, genistein, and 3-aminobenzamine were obtained from Sigma Co, and dissolved in DMSO.

Cell-Survival Assay

Cells cultured in 96-well plates were treated for 72 hr with chemotherapeutic drugs and MDR proteins specific inhibitors (1 μM) and MTT assayed according to previous report [9].

Functional Assay of MDR Proteins

The fluorescent dyes 3,3′-diethyloxacarbocyanine iodide ($DiOC_2(3)$), 20 ng/ml, and Carboxifluorescein diacetate (CFDA) 0.1 μM, (Sigma, Co.) were used as substrates for P-Gp and MRP1, respectively. Cyclosporine A, 10 μM and MK571, 100 μM (Cayman Chemicals) were used as inhibitors for P-Gp and MRP1, respectively. 10^5 cells/well seeded on six-well plates were incubated in presence or absence of inhibitors for 60 min at 37°C, followed by a second incubation adding either $DiOC_2(3)$ or CFDA. Later, cells were detached, collected, and centrifuged. The pellet was re-suspended in cold propidium iodide (PI), 10 μg/ml and kept in ice. Intracellular fluorescence accumulation was measured by flow cytometry (FACS Scan, Beckton Dickinson). Data was analyzed using FlowJo 8.7 software (Tree Star, Inc.). Dead cells (PI positives) were excluded from analysis. All assays were carried out in triplicate.

Small Interference RNA

Invitrogen siRNAs were used according to manufacturer protocol. Transfections were performed with 100 nM of siRNA and 100 nM of a fluorescent oligonucleotide (Block-iT Fluorescent Oligo, Invitrogen) as control. siRNA sequences were: *Gp-P*: 5′-GAG UGG GCA CAA ACC AGA UAA UAU U-3′ and 5′-AAU AUU AUC UGG UUU GUG CCC ACU C-3′. *MRP1*: 5′-CCG GUC UAU UCC CAU UUC AAC GAG A-3′ and 5′-UCU CGU UGA AAU GGG AAU AGA CCG G-3′. *LRP*: 5′-GCA GGA CAA UGA GAG GGU ACU GUU U-3′ and 5′-AAA CAG UAC CCU CUC AUU GUC CUG C-3′. After 4 hr, transfection medium was replaced for regular culture medium and efficiency evaluated by fluorescence microscopy after 48 hr. mRNA expression was measured considering the times of minimal expression (48, 96, and 72 hr post-transfection for P-Gp, MRP1, and LRP, respectively). Blockade efficiency was assessed at 48–96 hr measuring corresponding mRNAs and proteins. Transfected cells were treated for 72 hr with chemotherapeutic drugs before MTT cell survival assay.

Western Blot

Whole cell lysates (50 μg) were electrophoresed on SDS–polyacrylamide gels, transferred to nitrocellulose membranes and probed with primary antibodies

against P-Gp (Santa Cruz Biotechnology), MRP1 (Novocastra), LRP (Novocastra), and actin (Immuno, MP Biomedicals). Goat anti-rabbit or goat anti-mouse IgG conjugated to horseradish peroxidase (HRP, Jackson ImmunoResearch) were used as secondary antibodies. Quantitative evaluation was performed by densitometry using UN-SCAN-IT gel software (Silk Scientific Corporation, UT) and intensities were normalized to actin.

ReverseTranscription-Polymerase Chain Reaction (RT-PCR)

Total RNA (2 μg) from treated cells was reverse transcribed, and PCR carried out as described previously [9]. Amplificates were visualized by electrophoresis in 2% agarose gels. Quantitative analysis was performed by densitometry using UN-SCAN-IT gel software (Silk Scientific Corporation). The intensities were normalized to GAPDH and expressed as arbitrary units.

Flow Cytometry for Cell Cycle and Apoptosis Analyses

Cells were treated with specific concentrations of drugs and MDR protein inhibitors for 72 hr, trypsinized and fixed in 75% cold ethanol. Later, cells were washed in cold PBS and incubated with PI, 40 mg/ml and RNAse 1 mg/ml (Sigma, Co.) in PBS, for 30 min in darkness at 37°C. Fluorescence was measured by a FACScan™ flow cytometer and cell percentage in each cell cycle stage was analyzed (G1, S, and G2). Cells with sub-G1 amount of DNA were considered as apoptotic.

Statistic Analysis

Data evaluation was performed using non-parametric test of Kruskal–Wallis followed by Dunn's post-test. Statistic significance was considered for $P < 0.05$. All experiments were repeated at least three to four times and results were expressed as mean ± SEM.

RESULTS

Effect of Pharmacologic Inhibitors of MDR Proteins in Survival of PCa CellsTreated With Chemotherapeutic Drugs

Previously, we described low response to chemotherapeutic drugs and high expression of P-Gp, MRP1, and LRP in PCa primary cell cultures [9]. To evaluate the role of these proteins in drug resistance, the effect pharmacological specific inhibitors was analyzed. Cell were treated with P-Gp substrates mitoxantrone (250 and 500 nM) and docetaxel (5 and 20 nM) in the presence or absence of the P-Gp inhibitor cyclosporine A (1 μM), with the MRP1 substrate methotrexate (0.5 and 1 μM) and the inhibitor genistein (1 μM), and finally, with the LRP substrate cisplatin (100 and 200 μM) and the inhibitor 3-aminobenzamide (3-ABZ, 1 μM). At that concentrations use of inhibitors alone showed no effect on cell survival (Fig. 1).

PCa cells were more sensitive to low doses of P-Gp substrates docetaxel and mitoxantrone when compared to methotrexate (MRP1) and cisplatin (LRP). All combinations of MDR protein substrates and corresponding inhibitors were able to significantly increase the effect of chemotherapeutic drugs on PCa cell survival (Fig. 2). PCa primary cell cultures recovered, in part, their sensitivity to chemotherapy when MDR proteins were inhibited. This effect was higher when the P-Gp inhibitor cyclosporine A was used (Fig. 2A,B). High concentrations of methotrexate and cisplatin, probably out of the range used for cancer treatment, were able to reduce cell survival.

Effect of Pharmacologic Inhibitors of MDR Proteins on Cell Cycle and Apoptosis of PCa CellsTreated With Chemotherapeutic Drugs

We previously showed that the main effect of chemotherapeutic drugs on survival of PCa primary cell cultures was the apoptosis induction [9]. Accordingly, the addition of MDR protein inhibitors to drug treatments increased apoptosis compared to drug alone (Fig. 3). Increased apoptosis was observed when P-Gp and LRP function were inhibited, but no effect was found with the MRP1 inhibitor genistein (Fig. 3C).

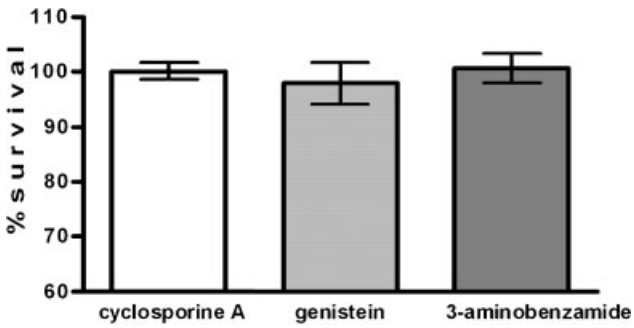

Fig. 1. Effect of pharmacological inhibitors of MDR proteins on cell survival of primary cell cultures of PCa. Cell cultures were treated with cyclosporine A, genistein, and 3-aminobenzamide (1 μM) and cell survival was assessed by MTT after 72 hr of treatment, n = 3.

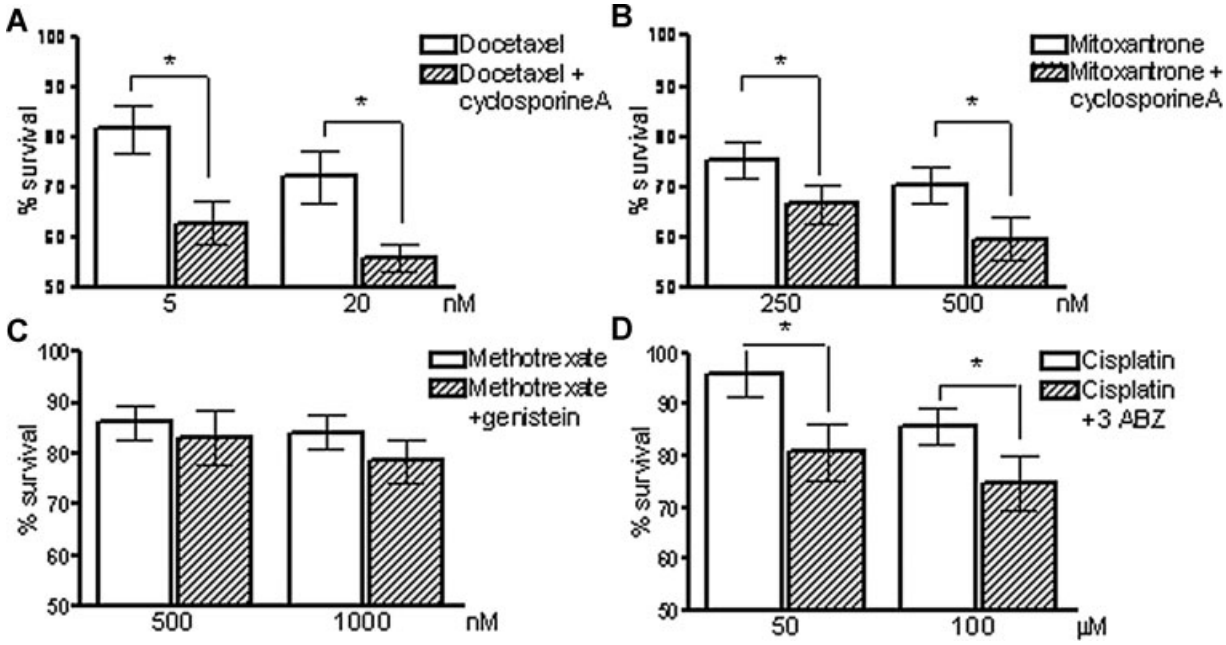

Fig. 2. Effect of pharmacological inhibitors of MDR proteins on cell survival of primary cell cultures of PCa after 72 hr of treatment with chemotherapeutic drugs assessed by MTT. (**A**) Treatment with docetaxel and cyclosporine A (1 μM), (**B**) Treatment with mitoxantrone and ciclosporin-A (1 μM), (**C**) Treatment with methotrexate and genistein (1 μM), (**D**) Treatment with cisplatin and 3-aminobenzamide (3 ABZ, 1 μM). $^{*}P < 0.05$, n = 12. All bars presented $P < 0.05$ with respect to the untreated cells (100%).

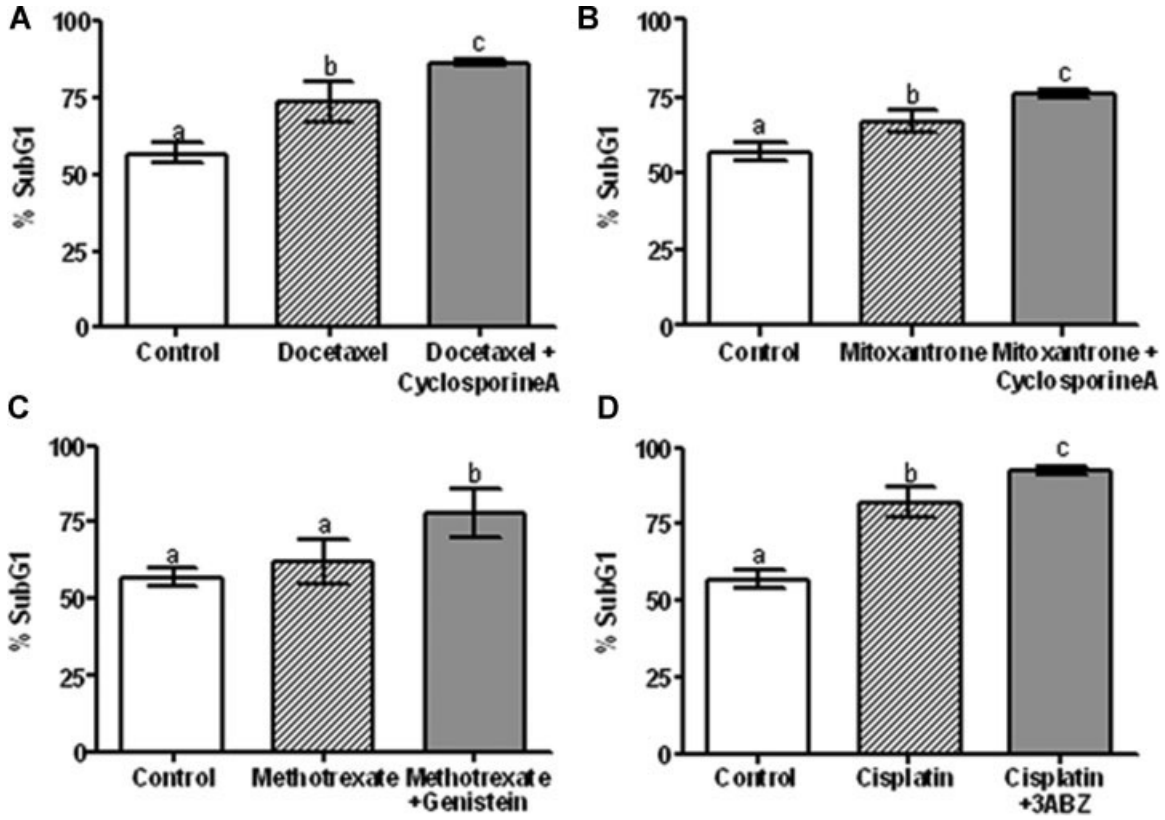

Fig. 3. Percent of subG$_1$ events (apoptotic cells) after 72 hr of treatment with chemotherapeutic drugs and inhibitors of MDR proteins of primary cell cultures from PCa, evaluated through flow cytometry. (**A**) Treatment with docetaxel (5 nM) and cyclosporine A (1 μM), (**B**) Treatment with mitoxantrone (250 nM) and cyclosporine A (1 μM), (**C**) Treatment with methotrexate (500 nM) and genistein (1 μM), (**D**) Treatment with cisplatin (50 μM) and 3-aminobenzamide (3 ABZ, 1 μM), n = 4. Different letters correspond to $P < 0.05$.

Evaluation of Pharmacological Inhibition of MDR Proteins on Efflux Activity

To evaluate MDR proteins activity in PCa cells, we performed a functional assay of P-Gp and MRP1. Cells expressing low level of MDR proteins accumulate more fluorescent dyes compared to cells showing high level of these proteins. PC3 cells treated with the $DiOC_2(3)$ and cyclosporine A, showed no difference compared to untreated control. This finding is in agreement with the absence of P-Gp in PC3 (Fig. 4A). However, the evaluation of MRP1 in PC3 cells showed a 100% fluorescence increase in cells treated with MK571 respect to untreated cells (Fig. 4B). Under MK571 treatment, PCa primary cultures showed lower accumulation of CFDA than PC3 cells (Fig. 4C). This difference can be explained for an increase in cellular MRP1 function associated with higher MRP1 expression in primary cell cultures respect to PC3 [9].

Effect of Knock Down of MDR Proteins by siRNA in Cell Survival of PCa Cell Cultures

To determine the effect of MDR proteins on PCa cell drug-sensitivity, these proteins were knocked-down by specific siRNAs. MRP1 and LRP inhibition was evaluated in PC3 cell line and P-Gp inhibition in PCa primary cell cultures due to, as previously reported, PC3 cells do not express P-Gp [9]. Gp-P mRNA expression decreased over 80%, while MRP1 and LRP decreased almost 60% respect to untreated cells (Fig. 5A). The knockdown effect on protein expression was higher in MRP1 (88%) than LRP (36%) (Fig. 5B).

Inhibition of P-Gp increased sensibility to docetaxel around 10%, but also decreased resistance to cisplatin in PCa primary cell cultures (Fig. 6A). In PC3, siRNA for MRP1 increased sensibility to methotrexate. In addition, this knockdown also increased

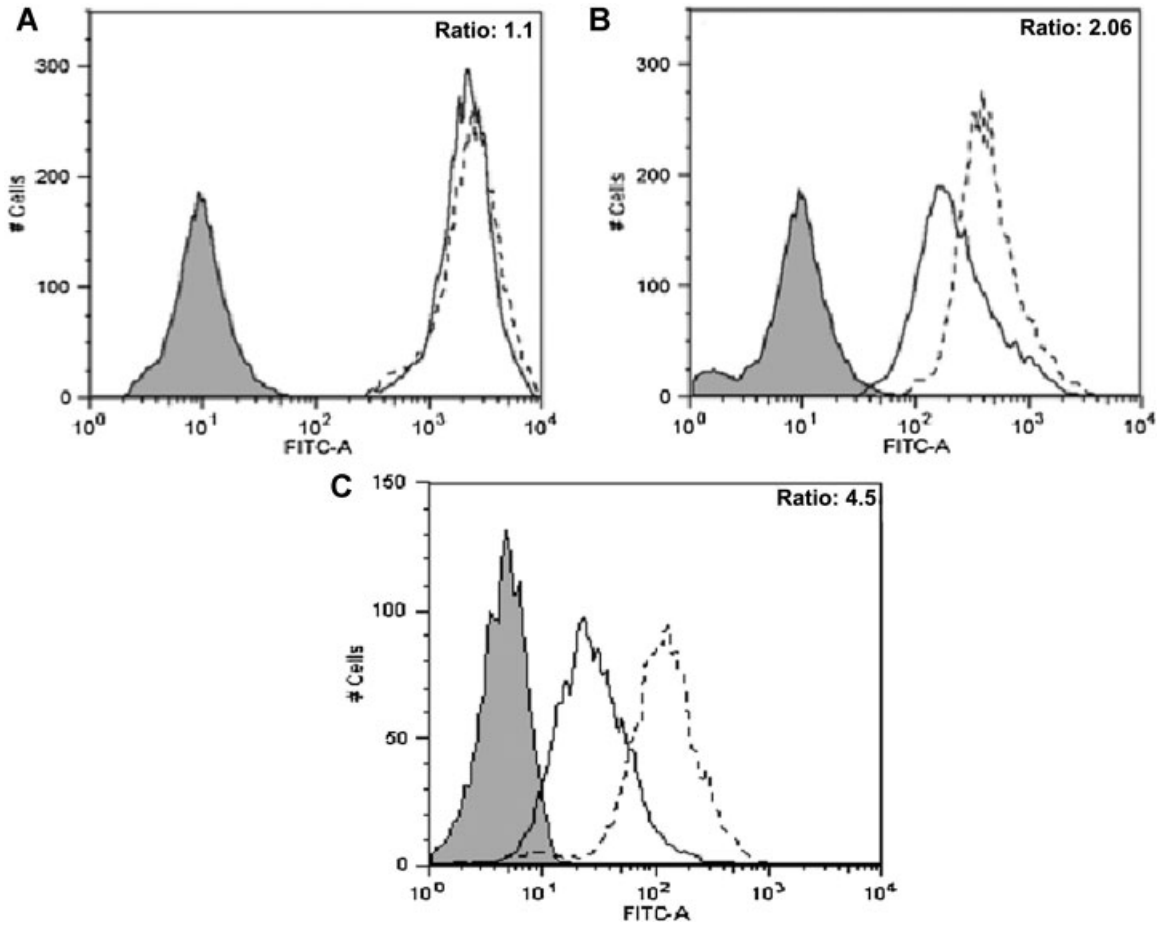

Fig. 4. Examples of functional assay of MDR proteins evaluated by accumulation of fluorescent dyes inside PCa cells measured by flow cytometry. (**A**) Evaluation of P-Gp activity in PC3. (**B**) Evaluation of MRPI function in PC3 and (**C**) primary cell cultures of PCa. Grey: non-treated cells; white, continuous line: cells loaded with fluorescent dye ($DiOC_2(3)$ for P-Gp or CFDA for MRPI); White, discontinuous line: cells loaded with fluorescent dye and MDR specific inhibitors (cyclosporine A for P-Gp or MK571 for MRPI). Ratio = fluorescence median intensity of cells treated with fluorescent dye and MDR inhibitor versus cells treated with fluorescent dye alone.

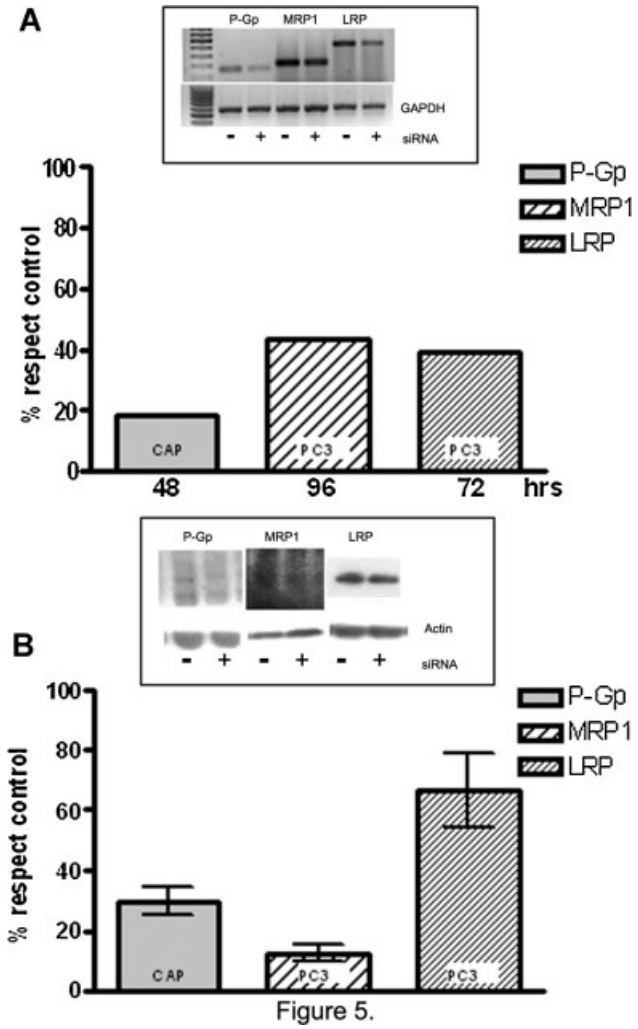

Figure 5.

Fig. 5. Effect of siRNA transfection for MDR proteins in their expression in prostate cell cultures. (**A**) mRNA levels of MDR proteins evaluated at different hours, through RT-PCR respect to the non-treated cells (100%); in the insert are included representatives RT-PCRs. (**B**) Levels of MDR proteins respect to the control considered as 100% in transfected cells; in the insert are included representatives Western blots. P-Gp was evaluated in primary cell cultures from PCa; MRPl and LRP were evaluated in PC3 cell line, n = 4.

sensibility to docetaxel and mitoxantrone in about 5% (Fig. 6B). LRP inhibition decreased resistance to cisplatin in 15%, but also increased the effect of docetaxel and methotrexate (Fig. 6C).

DISCUSSION

Several studies have been focused on molecular patterns of PCa progression [12], commonly classified according to Gleason score [13]. Previously, we found a positive relationship between several molecular markers and PCa malignancy [14]. MDR proteins, P-Gp, MRP1, and LRP, are related with tumor malignancy as their expression increase with Gleason score and chemotherapy exposure [9], suggesting that PCa develops intrinsic and acquired MDR phenotypes.

Docetaxel is the most effective drug in terms of overall survival, life-quality, pain, and PSA decline [15]. Analysis of P-Gp expression could predict overall survival and the effective docetaxel dose. Also, the simultaneous use of P-Gp modulators might diminish secondary effects. In patients with acute myeloid leukemia, P-Gp is used as a prognosis marker and can be rapidly detected before therapy initiation [16].

The MDR protein expression in PCa is not only related with chemotherapy response, as P-Gp can transport bicalutamide and may be responsible for resistance in long-term androgen-deprivation response [17]. MDR proteins inhibition has been evaluated to increase chemotherapy sensitivity in different cancer types. In our work, we found an increased sensibility in PCa primary cell cultures after administration of drugs in combination with MDR protein modulators cyclosporine A, genistein, and 3-aminobenzamide. These effects were independent on the blocked protein, suggesting a multifactor nature of MDR phenotype. The major effect was found with docetaxel and cyclosporine A, demonstrating a major P-Gp role in PCa MDR phenotype. Sensitivity increase was mainly due to apoptosis, and this effect may diminish the selection of tumor cells overexpressing MDR proteins. High concentrations of MDR protein inhibitors have direct effect on cell survival, mainly inducing apoptotic pathways [18,19]. However, treatments with those doses result toxic for most patients. For this reason, we used lower doses of MDR protein modulators avoiding direct effects on apoptosis. When combined with docetaxel and cisplatin, genistein increased PC3 drug-sensibility, mainly through MRP1 inhibition (PC3 cells lack P-Gp) [20]. These data and our results, suggest that docetaxel resistance is related to P-Gp and MRP1.

Toxicity associated with MDR protein inhibitors has decreased with the development of second and third-generation P-Gp modulators [21]. The lack of better results with these modulators indicates that some important aspects should be considered. As we have described, MDR phenotype is a redundant mechanism related to over-expression of several MDR proteins probably affecting clinical trials results [21]. Also, pharmacokinetic interactions resulting from the P-Gp inhibition in normal tissues such as gastrointestinal tract and kidney can generate decreased clearance of anticancer agents, as well as cytochrome P450 inhibition [19,22]. It is necessary to consider that MDR proteins modulators can decrease

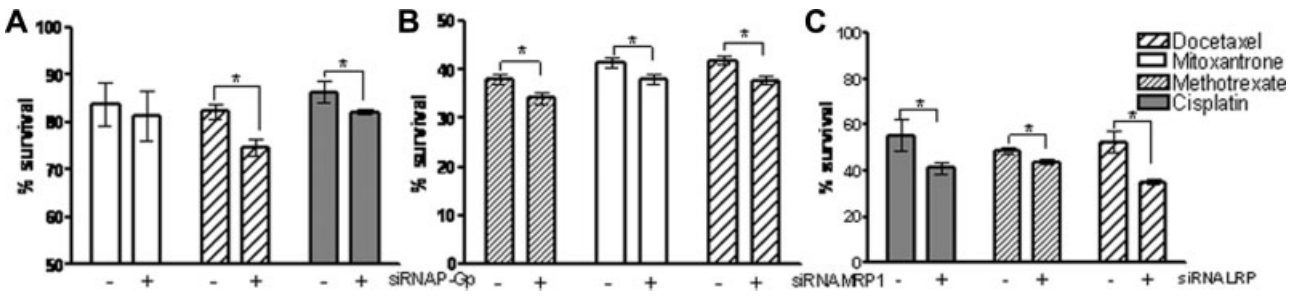

Fig. 6. Effect of siRNA of MDR proteins on cell survival of PCa cells treated with different chemotherapeutic drugs during 72 hr evaluated through MTT. (**A**) P-Gp inhibition in primary cell cultures (**B**) MRPI inhibition in PC3 cells. (**C**) LRP inhibition in PC3 cells. Survival of non-treated and non-transfected cells was considered as 100%. $^{*}P < 0.05$, n = 4.

the drug amount needed to achieve the same effect [19].

Modulators cytoxicity led us to evaluate MDR phenotype reversal through other methodology. Using siRNA technology, we found that P-Gp expression inhibition increased PCa cell sensibility to chemotherapeutic drugs. Other studies combining docetaxel with topoisomerase inhibitors have shown that P-Gp down-regulation increases drug cytotoxicity [23]. Survival of siRNA-transfected cells was significantly lower than non-transfected cells, showing the highest sensitivity in presence of docetaxel and the inhibition of the three proteins under study. Drug-sensitivity was not specific for all substrates, confirming the complexity of MDR phenotype. Knocking-down MDR proteins to increase PCa cell sensibility is a novel strategy that might be used to improve chemotherapy. siRNAs have been used in different clinical trials, for the treatment of degenerative diseases and viral infections with relative success [24]. In animal models of PCa, promising results with systemic delivery of siRNA for androgen receptor have been obtained [25]. In vivo studies have shown decreased P-Gp expression using shRNA plasmids systemically administered [26]. Recently, tissue specific delivery of siRNA to PCa cells has been achieved using siRNA-aptamers conjugates against prostate-specific membrane antigen [27].

Studies on therapy-associated biomarkers are arising to improve diagnosis and overcome focalized therapies [28]. Patient heterogeneity in molecular profiles and biological tumor behavior should be taken in account when selecting therapeutic targets. In addition, a profile of drug-response biomarkers for every patient should be considered for more efficient therapies [29].

CONCLUSIONS

The presence of MDR proteins is related with the low chemotherapy response observed in PCa. MDR proteins present in PCa cells are functional and their blockade increases cell sensitivity to chemotherapy, including docetaxel therapy. MDR proteins may represent biomarkers to predict drug response in PCa patients. Expression and function of these proteins should be evaluated to enhance efficacy of docetaxel-based therapies and increase survival of patients with hormone-resistant PCa.

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