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P-glycoprotein mediates the collateral sensitivity of multidrug resistant cells to steroid hormones

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ABSTRACT

The overexpression of P-glycoprotein (P-gp, ABCB1) in cancer cells often leads to multidrug resistance (MDR) through reduced drug accumulation. However, certain P-gp-positive cells display hypersensitivity, or collateral sensitivity, to certain compounds that are believed to induce Pgp-dependent oxidative stress. We have previously reported that MDR P-gp-positive CHO cells are collaterally sensitive to verapamil (VRP; Laberge et al. (2009) [1]). In this report we extend our previous findings and show that drug resistant CHO cells are also collaterally sensitive to physiologic levels of progesterone (PRO) and deoxycorticosterone (DOC). Both PRO and DOC collateral sensitivities in CH^RC5 cells are dependent on P-gp-expression and ATPase, as knockdown of P-gp expression with siRNA or inhibition of P-gp-ATPase with PSC833 reverses PRO- and DOC-induced collateral sensitivity. Moreover, the mitochondrial complexes I and III inhibitors (antimycin-A and rotenone, respectively) synergize with PRO and DOC-induced collateral sensitivity. We also show that VRP inhibits PRO and DOC collateral sensitivity, consistent with earlier findings relating to the VRP's modulation of PRO and DOC-stimulation of P-gp ATPase. The findings of this study demonstrate a P-gp-dependent collateral sensitivity of MDR cells in the presence of physiologically achievable concentrations of progesterone and deoxycorticosterone.

1. Introduction

Drug resistance is a major cause of treatment failure in clinical oncology [2]. Cancerous tumor cells display intrinsic or acquired resistance towards most chemotherapeutic drugs in cancer treatment. Cancer cells treated with various chemotherapeutic drugs overexpress one of three ABC transporters; P-glycoprotein (ABCB1), multidrug resistance protein 1 (ABCC1) and/or the breast cancer resistance protein (ABCG2) [3]. All three ABC transporters have been shown to cause drug resistance via an ATP-dependent efflux of anti-cancer drugs [4]. Among the drug resistance causative ABC transporters, P-gp is the most studied biochemically and clinically. Normal physiologic expression of P-gp points to chemo-defense function, protecting normal tissues and organs from toxic drugs and xenobiotics [5]. In cancer cells, P-gp expression has been associated with poor clinical outcome in several cancers, including breast, neuroblastoma and acute promyelocytic leukemia (AML) [6]. However, efforts to overcome the drug resistance function of P-gp in the clinic using MDR-reversing drugs in combination with chemotherapeutic drugs did not show a significant improvement in clinical response [7]. The use of MDR-reversing drugs, although effective at inhibiting P-gp drug efflux function in cancer cells, caused unacceptable drug toxicity in normal tissues and organs that express P-gp [8]. Efforts to inhibit the growth of P-gp-positive cancer cells without inhibiting P-gp efflux functions have now focused on a class of drugs that elicit collateral sensitivity in P-gp-positive cells [9]. Collateral sensitivity was described more than three decades ago by Bech-Hansen et al. [10] as "*hypersensitivity*" of cells to various non-toxic drugs that included calcium channel blockers, steroid hormones and non-ionic detergents. In an earlier study by Karwatsky et al. [11], we described the mechanism of verapamil-induced collateral sensitivity in CH^RC5 P-gp-positive cells to be mediated by the activation of P-gp ATPase, leading to a rise in reactive oxygen species and oxidative stress-induced apoptosis.

In this study we show that physiologic concentrations of progesterone (PRO) and deoxycorticosterone (DOC) induce collateral sensitivity in CH^RC5 cells in a P-gp-dependent mechanism. Moreover, PRO or DOC-induced collateral sensitivity synergized with the mitochondrial electron transport chain inhibitors, rotenone or antimycin-A. In addition, verapamil inhibited both PRO and

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DOC-induced collateral sensitivity. The implications of these findings with respect to drug-induced collateral sensitivity in P-gp-positive cells are discussed.

2. Materials and methods

2.1. Cell culture and siRNA transfection

Parental, drug-sensitive (AuxB1) and -resistant (CH^RC5) cells were grown in α-minimal essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C in the presence of 5% CO_2 without or with 5 μg/ml colchicine (CH^RC5). For siRNA transfection, siRNA duplex sequences corresponding to the Chinese hamster *mdr1* sequence [5′AGAGAAGAAACCAGUGGU-C3′ (sense) and 5′GACCACUGGUUUCUUCUC-U3′ (antisense)] were synthesized by Invitrogen Life technology Inc. Cells in exponential phase at 50–60% confluency were transfected with 1 nmole siRNA complexed with lipofectamine 2000 (Invitrogen, Burlington, On, Canada) in serum-free α-MEM according to manufacturer's protocol, with some modification. Briefly, prior to transfection, cells were washed once with serum-free α-MEM and incubated with the transfection mix for 6 h. Cells were washed with serum-containing media and incubated with or without drugs for the rest of the experiment.

2.2. Cell growth assay

Cells transfected with siRNA sequences were seeded in triplicate into 96-well plates 48 h post-transfection. Following 24 h incubation, cells were exposed to increasing concentrations of drugs and allowed to grow for 72 h. For drug combinations, cells were preincubated 4 h with PSC833, rotenone or antimycin-A prior to the addition steroids or VRP. The assay was developed by the addition of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye to a final concentration of 0.5 mg/ml into each well and plates were incubated at 37 °C for 4 h. The MTT crystals were dissolved with 200 μL of DMSO. Cell growth was determined from the absorbance values measured at 570 nm relative to control or untreated cells. The data was analyzed using Prism 5.0 (GraphPad Software) to obtain the 50% inhibitory concentration (IC_{50}). All graphs shown represent the mean ± SD of three independent experiments done in triplicate.

2.3. Protein extraction and immunodetection of hamster cells

siRNA transfected cells (1×10^6) were harvested on the 48–72 h post-transfection. Cells were washed three times with PBS and lysed in 50 μl of lysis buffer (50 mM Tris–Cl pH 7.8, 150 mM NaCl,

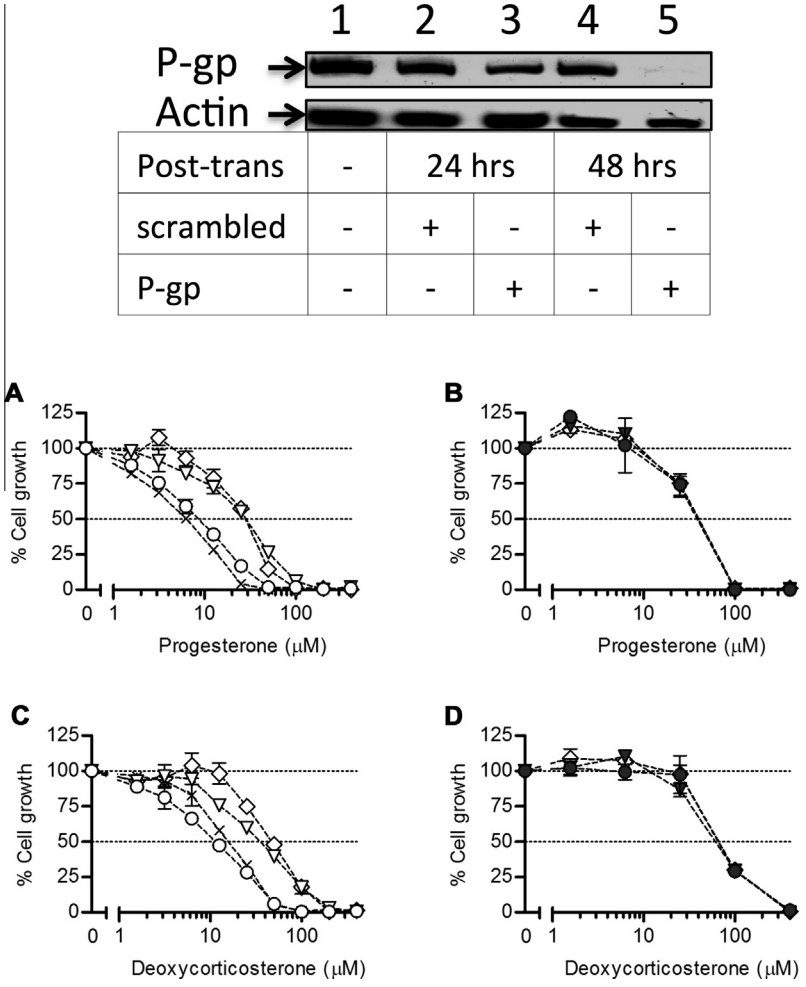

Fig. 1. Effects of P-gp knockdown on cell grown in increasing concentrations of progesterone or deoxycorticosterone. Total cell extracts from untransfected CH^RC5 cells (lane 1), P-gp-specific or scrambled siRNA transfected CH^RC5 cells, 24 h or 48 h post-transfection (lanes 3 and 2 or 5 and 4, respectively) were resolved on SDS–PAGE and Western blots probed with P-gp-specific mAb or anti-actin mAb. Panels A and B show drug-sensitive cells (AuxB1 cells; (◇)) and P-gp-positive drug resistant cells [CH^RC5 cells (×), P-gp-siRNA transfected CH^RC5 (○) and scrambled-siRNA transfected CH^RC5 (▽) grown in the presence of increasing concentrations of progesterone and deoxycorticosterone, respectively. Panels B and D cells show drug sensitive cells [AuxB1 (◇), P-gp-siRNA transfected AuxB1 (●) and scrambled siRNA transfected AuxB1 (▼)] grown in the presence of increasing concentrations of progesterone or deoxycorticosterone, respectively. Graphs show percent of cell growth in the presence of drugs 72 h following siRNA transfection. Error bars represent standard deviation from at three different experiments done in triplicate.

1% NP-40, containing 1% protease inhibitor cocktail). The cell lysate was centrifuged at 13,000 rpm for 20 min and the supernatant fraction was stored at −80 °C, if not used immediately. For immunodetection by Western blots, 25 μg samples of cell lysates were resolved on Fairbanks gels [12] and resolved proteins were transferred onto a nitrocellulose membrane [13]. The same nitrocellulose membrane was probed for P-gp and actin expression using a P-gp-specific monoclonal antibody (265/F4, Abcam, Cambridge MA) and actin specific polyclonal antibody (Sigma, Oakville, On, Canada) at 1:250 v/v dilution, respectively. HRP-conjugated goat α-mouse or α-rabbit IgGs (BioRad, Hercules, CA) were used at 1:3000 v/v dilution as secondary antibodies. HRP detection was performed using Immobilon Western detection kit (Millipore, Billerica, MA) according to manufacturer instructions.

2.4. Plasma membrane preparation and ATPase assay

Plasma membrane fractions from AuxB1 and CH^RC5 cells were prepared as previously described [11]. P-gp ATPase activity was determined by quantifying the release of inorganic phosphate from ATP as previously described [11]. P-gp-specific ATPase activity was determined in the presence of increasing concentrations of PRO or DOC as previously described [11]. Each condition represents experiment done three independent times with six replicates.

2.5. Statistics analyses

All graph and statistics were performed using Graphpad prism version 5. All statistics represent the student *t* test.

3. Results and discussion

3.1. P-gp knockdown reverses CH^RC5 cells collateral sensitivity to steroid hormones

Earlier reports have suggested that P-gp-positive MDR cells are collaterally sensitive to calcium channel blockers, non-ionic detergents and steriod hormones [10,14]. To determine if P-gp is directly responsible for steroid hormones induced collateral sensitivity, P-gp expression in CH^RC5 cells was knocked down with P-gp-specific siRNA and then examined for collateral sensitivity to steriod hormones. Fig. 1 shows P-gp knockdown in CH^RC5 cells with scrambled or P-gp-specific siRNA. The results in Fig. 1 (lanes 3 and 5) show diminished P-gp expression in CH^RC5 cells transfected

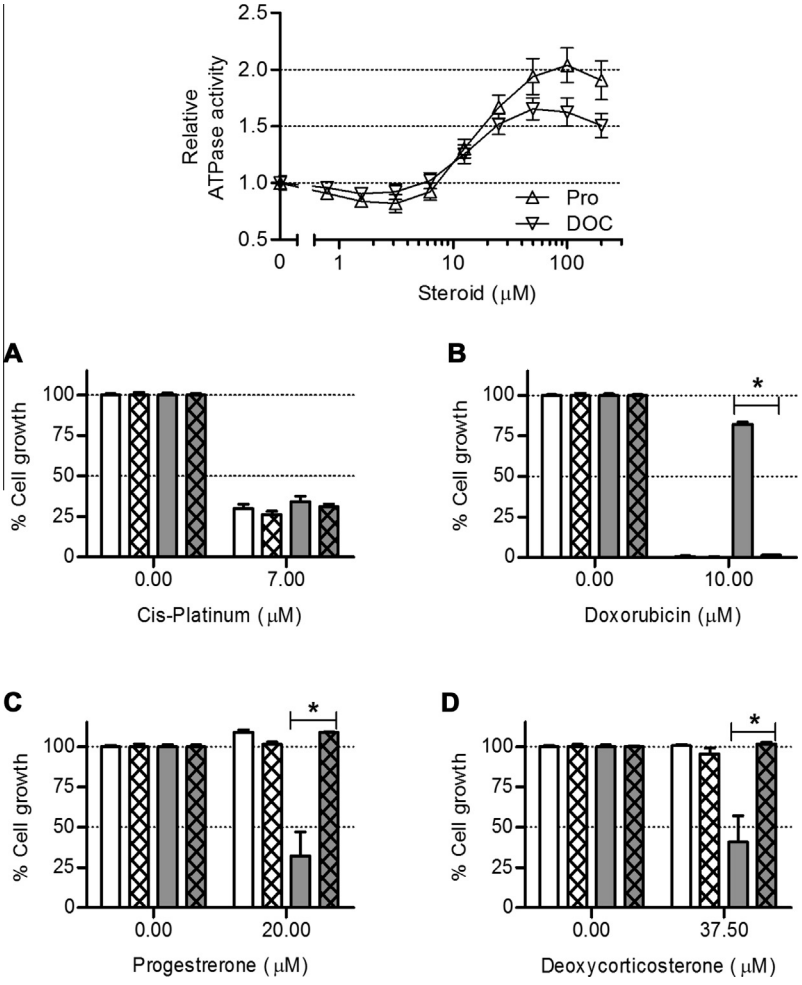

Fig. 2. Reversal of steroid-induced collateral sensitivity with P-gp ATPase inhibitor. P-gp ATPase was measured in purified CH^RC5 plasma membranes exposed to increasing concentrations of progesterone (triangle) or deoxycorticosterone (inverted triangle). ATPase activity is plotted relative to un-stimulated P-gp ATPase. To determine the effects of ATPase inhibitors on steriod hormones induced collateral sensitivity, drug-sensitive AuxB1 and -resistant CH^RC5 cells without PSC833 (solid white and gray bars, respectively) or with 2 μM PSC833 (crossed-white and gray bars, respectively) were grown in the absence and presence of 7 μM cis-platinum (panel A), 10 μM doxorubicin (panel B), 20 μM progesterone (panel C) or 37.5 μM deoxycorticosterone (panel D). The bar graphs represent relative growth of AuxB1 and CH^RC5 cells following 72 h exposure to drugs as determined by MTT assay. Error bars represent standard deviation from one representative of at least two experiments done in triplicate. * indicates, $P < 0.01$, statistically significant difference.

with P-gp-specific siRNA 24 and 48 h post-transfection compared to cells transfected with scrambles siRNA (Fig. 1, lanes 2 and 4). Having established specific P-gp knockdown in CH^{R}C5 cells, the effects of steroid hormones on P-gp-positive cells were compared to P-gp-negative drug-sensitive (AuxB1) and P-gp-siRNA or scrambled siRNA transfected CH^{R}C5 cells. Fig. 1A and C show that untransfected CH^{R}C5 are hypersensitive or collaterally sensitive to progesterone and deoxycorticosterone (IC_{50} of 5 μM and 12 μM, respectively) relative to AuxB1 (IC_{50} of 40 μM and 64 μM, respectively) cells. The results in Fig. 1A and C show that knockdown of P-gp expression with P-gp-siRNA, but not with scrambled siRNA, reversed the collateral sensitivity of CH^{R}C5 cells to progesterone and deoxycorticosterone to the same level as AuxB1 P-gp-negative drug sensitive cells (Fig. 1A and C). To rule out the possibility that the reversal of progesterone or deoxycorticosterone collateral sensitivity is due to off-target effects by P-gp siRNA, P-gp-negative cells (AuxB1) were transfected with P-gp-specific siRNA or scrambled siRNA and allowed to grow in the presence of increasing concentrations of progesterone and deoxycorticosterone (Fig. 1B and D, respectively). Consistent with the specificity of P-gp-siRNA, the results in Fig. 1B and D show that siRNA transfection did not change the sensitivity of AuxB1 cell to progesterone or deoxycorticosterone. Taken together, the above results demonstrate that P-gp is directly responsible for collateral sensitivity to progesterone and deoxycorticosterone in CH^{R}C5 cells. It is interesting that the expression of P-gp in CH^{R}C5 cells increased the sensitivity of these cells by 5–8-folds to steroid hormones relative to the parental P-gp-negative drug sensitive cells. These results are similar to our earlier reports with the calcium channel blocker verapamil, whereby knockdown of P-gp expression in CH^{R}C5 cells increased their sensitivity to verapamil by >30-folds [1], hence verapamil appears to be a more potent collateral sensitivity drug for these P-gp-positive MDR cells than steriod hormones.

3.2. Inhibition of P-gp ATPase reverses collateral sensitivity of CH^{R}C5 to steroid hormones

We have previously demonstrated that verapamil induced collateral sensitivity in CH^{R}C5 cells correlates with its stimulation of P-gp ATPase [11] and consequently have proposed that the capacity of a given drug to stimulate P-gp ATPase correlates with its ability to induce collateral sensitivity in CH^{R}C5 cells in P-gp-dependent manner. To determine if progesterone and deoxycorticosterone induce P-gp ATPase in CH^{R}C5 cells, Fig. 2 shows the relative increase in P-gp ATPase to increasing concentrations of progesterone and deoxycorticosterone relative to un-stimulated basal P-gp ATPase in CH^{R}C5 membrane fractions. The results in Fig. 2 show progesterone causes 2-fold stimulation of basal P-gp ATPase at 100 μM and a ½ max at 20 μM. Similarly, deoxycorticosterone causes 1.6-fold stimulation of P-gp ATPase at 50 μM max and ½ max at 12.5 μM. Although it is not clear how *in vitro* drug concentrations required for max P-gp ATPase stimulation relate to the same *in vivo* in CH^{R}C5, the results in Fig. 2 show progesterone to be more effective at increasing P-gp ATPase than deoxycorticosterone. Interestingly the latter is consistent with progesterone being

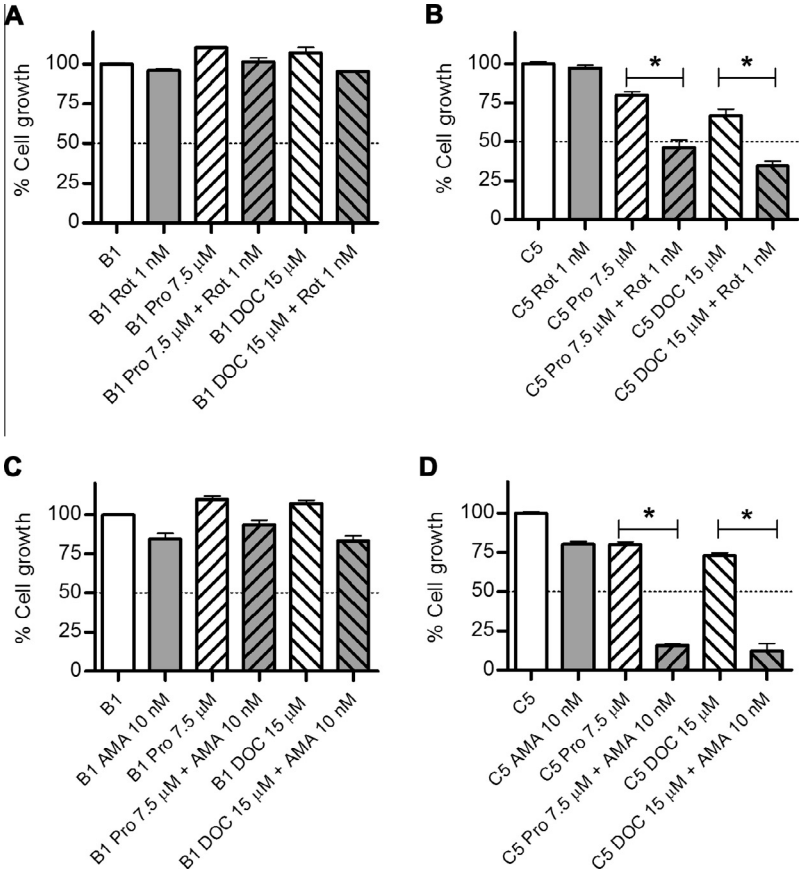

Fig. 3. Effects of mETC inhibitors on progesterone or deoxycorticosterone collateral sensitivity. AuxB1 (or B1) drug sensitive and CH^{R}C5 (C5) drug resistant cells incubated without and with rotenone (Rot) and antimycin-A (AMA) plus progesterone (Pro, Panels A and B, respectively), or plus deoxycorticosterone (DOC, Panels C and D, respectively). The bar graphs represent relative growth of AuxB1 and CH^{R}C5 cells following 72 h exposure to drugs as determined by MTT assay. Error bars represent standard deviation of one representative experiment repeated at least twice done in triplicate. * indicates, $P < 0.01$, statistically significant differences.

more effective than deoxycorticosterone at inducing collateral sensitivity in CH^{R}C5 cells (5 μM versus 12 μM, Fig. 1A and C).

The cyclosporine analog, PSC833, has been previously shown to specifically inhibit basal and drug stimulated P-gp ATPase [15]. Moreover, we have previously demonstrated that PSC833 inhibits verapamil stimulated P-gp ATPase and collateral sensitivity of CH^{R}C5 [11]. To determine if P-gp ATPase is required for progesterone and deoxycorticosterone induced collateral sensitivity, AuxB1 and CH^{R}C5 cells were grown in the absence and presence of progesterone and deoxycorticosterone, without and with 2 μM of PSC833 (Fig. 2C and D, respectively). As PSC833 is a cyclosporine analog, and as such may inhibit apoptosis, it was important to rule out the latter possibility. Fig. 2A and B show the growth of AuxB1 and CH^{R}C5 cells in the presence of cis-platinum or doxorubicin without and with 2 μM of PSC833. The results in Fig. 2A show that cis-platinum (7 μM) was equally toxic to AuxB1 and CH^{R}C5 cells in the absence or presence PSC833. The sensitivity of CH^{R}C5 cells to cis-platinum in the absence of PSC833, is consistent with the fact that cis-platinum is not a substrate for P-gp drug efflux mechanism. By contrast, the results in Fig. 2B show that CH^{R}C5 cells grown in the absence of 2 μM PSC833 are resistant to 10 μM of doxorubicin; while the combined presence of 10 μM doxorubicin with 2 μM of PSC833 shows no growth of CH^{R}C5 cells. These results confirm that PSC833 does not inhibit drug-induced apoptosis, but rather inhibits the P-gp ATPase and sensitize CH^{R}C5 cells to doxorubicin. Fig. 2C and D show that in the absence of PSC833, CH^{R}C5 cells are sensitive to progesterone (20 μM) and deoxycorticosterone (37.5 μM), but the presence of PSC833 (2 μM) reverses the sensitivity of CH^{R}C5 cells to both progesterone and deoxycorticosterone (from 30% to 105% and from 45% to 100% cell growth, respectively). These results suggest that P-gp ATPase is required for progesterone and deoxycorticosterone collateral sensitivity. Given that P-gp is an abundant membrane protein, it is conceivable that knockdown of P-gp expression with siRNA can modify the fluidity of the cell membrane and this in turn reverses the sensitivity of cells to progesterone and deoxycorticosterone. However, the above results show that the addition of P-gp ATPase inhibitor reverses CH^{R}C5 collateral sensitivity to progesterone and deoxycorticosterone negates the role of membrane fluidity in collateral sensitivity. Moreover, the collateral sensitivity of CH^{R}C5 cells to steroid hormones rules out a role for Ca^{2+} in verapamil induced collateral sensitivity in the same cell line as previously observed [1,11].

3.3. mETC inhibitors synergize with steroid hormones-induced collateral sensitivity

It is now believed that drug-induced collateral sensitivity in P-gp-positive cancer cells is due to the accumulation of reactive oxygen species (ROS) leading to oxidative stress-induced apoptosis [9]. Moreover, we have previously demonstrated that drugs that cause further increase in ROS, independent of P-gp activities, can synergize with stimulators of P-gp ATPase (e.g., verapamil) to induce collateral sensitivity [1]. The mitochondria is a major source of ROS, largely due to a small percentage of electron leak down the mitochondria electron transport chain (mETC, [16]). Consequently

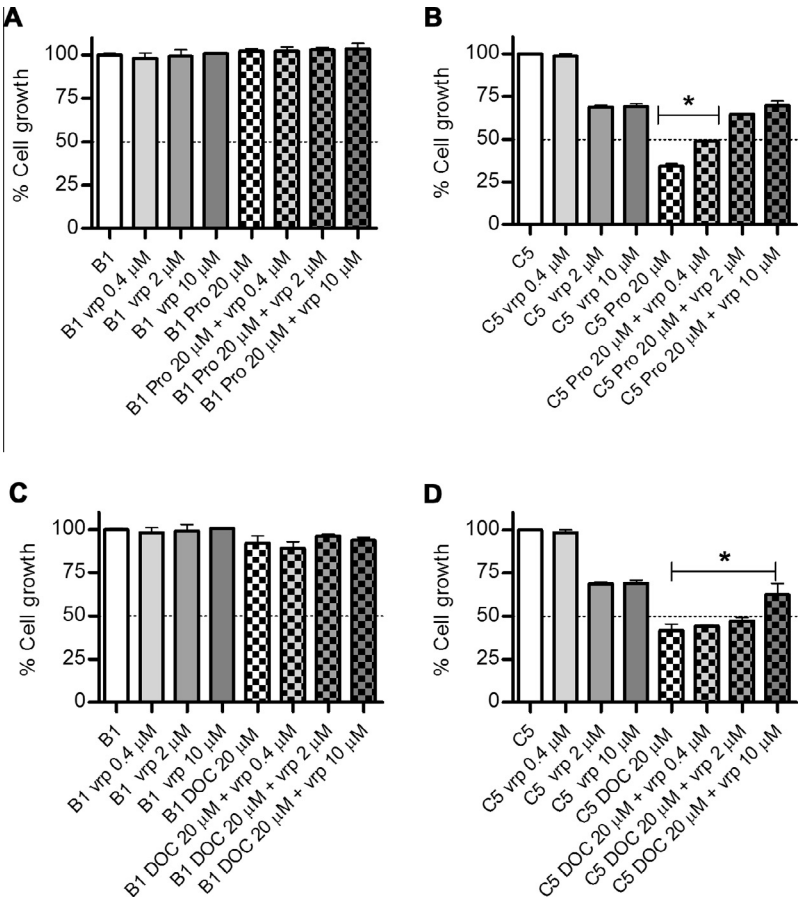

Fig. 4. Verapamil reverses steroid-induced collateral sensitivity. Panels A and C show AuxB1 (or B1) drug sensitive cells incubated with increasing concentrations of verapamil (vrp; 0.4, 2, and 10 μM) without and with 20 μM progesterone (Pro) or deoxycorticosterone (DOC). Panels B and D show CH^{R}C5 (or C5) cells grown in the presence of increasing concentrations of verapamil (vrp; 0.4, 2, and 10 μM) without or with 20 μM progesterone (Pro) or deoxycorticosterone (DOC). The bar graphs represent relative growth of AuxB1 and CH^{R}C5 cells following 72 h exposure to drugs as determined by MTT assay. Error bars represent standard deviation of one representative experiment repeated at three times in triplicate. * indicates, $P < 0.01$, statistically significant differences.

drugs that inhibit or interfere with the functions of some of the complexes of the mETC lead to higher percentage of leaked electrons and a subsequent rise in ROS. Two well established inhibitors of mETC, rotenone and antimycin-A, have been shown to inhibit complexes I and III with a consequent increase in ROS [17,18]. Using these inhibitors, we have previously shown their effects to synergize with verapamil to induce collateral sensitivity in CH^RC5 cells [1]. Given that both progesterone and deoxycorticosterone stimulate P-gp ATPase, it was of interest to determine if they synergize with rotenone and antimycin-A. Fig. 2 shows the effects of adding low concentrations rotenone (1 nM) and antimycin-A (10 nM) to AuxB1 or CH^RC5 cells in the absence and presence of progesterone (7.5 μM) or deoxycorticosterone (15 μM). The results in Fig. 3B and D show that the addition of each of the four drugs (1 nM rotenone, 10 nM antimycin-A, progesterone, or deoxycorticosterone) alone cause 0–20% reduction in the growth of CH^RC5 cells. In contrast, the presence of progesterone or deoxycorticosterone together with 1 nM rotenone or 10 nM antimycin-A caused 60–80% drop in CH^RC5 cell growth (Fig. 3B and D). As expected, neither drug combination caused a significant drop in the growth of AuxB1 cells (Fig. 3A and C). Together, these results suggest that the combined ROS generated by P-gp ATPase stimulating drugs and mitochondria decoupling drugs synergize to induce collateral sensitivity in CH^RC5 cells. It should be mentioned that direct measurements of intracellular ROS with various ROS-sensitive fluorescent probes, as previously demonstrated with ABCC1-expressing cells [19], is not possible for P-gp expressing cells as these reagents are effluxed by P-gp from CH^RC5 cells (unpublished results).

3.4. Verapamil modulates steroid hormones-induced collateral sensitivity

In an effort to induce higher levels of collateral sensitivity, we sought to combine two ATPase stimulating drugs, namely verapamil and progesterone or deoxycorticosterone. Fig. 4 shows the effects of verapamil in the absence or presence of fixed concentrations of progesterone or deoxycorticosterone on the growth of AuxB1 and CH^RC5 cells. The results in Fig. 4B and D show that the verapamil rescues, rather than increases the collateral sensitivity of, CH^RC5 cells from progesterone and deoxycorticosterone induced collateral sensitivity. The addition of increasing concentrations of verapamil (0.4, 2 or 10 μM), in combination with 20 μM of progesterone or deoxycorticosterone, reversed the inhibitory effect of steroid hormones alone. By contrast similar drug treatments of AuxB1 cells did not affect their growth profile (Fig. 4A and C). The reversal of progesterone and deoxycorticosterone-induced collateral sensitivity in CH^RC5 with verapamil is not entirely clear, but could negatively modulate P-gp ATPase, causing a drop in ATP consumption and subsequent drop in ROS. Indeed, earlier findings have demonstrated an inhibitory effect of steroid hormones (e.g., progesterone and deoxycorticosterone) on verapamil stimulated ATPase [20] suggesting that these drugs are mutually non-exclusive modulators of P-gp ATPase. Thus, the interaction between P-gp and its substrates or modulators and how such interactions modulate P-gp ATPase are complex and not entirely understood. Therefore the use of combination drugs to amplify the collateral sensitivity effects should be mindful of the drugs' overall effect on P-gp ATPase.

Conflict of interest

There are no conflicts of interest from any of the authors.

Acknowledgments

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