Expression of new prognostic markers, peripheral-type benzodiazepine receptor and carbonic anhydrase IX, in human breast and ovarian carcinoma cell lines

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Peripheral benzodiazepine receptor (PBR), a mitochondrial protein involved in cell proliferation and differentiation, and carbonic anhydrase IX (CA IX), an intrinsic marker of hypoxia, have been studied in the panel of human breast (MCF-7, BT-20, MDA-MB-453, MDA-MB-231) and ovarian (A2780, A2780/CP, A2780/ADR, CH1, SKOV-3) carcinoma cell lines that differ by malignant progression. The expression of both antigens was detected by staining with the PBR-specific 8D7 and CA IX-specific M75 monoclonal antibodies and quantitated by flow cytometry. PBR was related to mitochondrial mass and CA IX to the cell density.

Breast carcinoma cell lines showed higher relative fluorescence intensity of PBR expression than ovarian cell lines, with the exception of A2780/CP cisplatin-resistant subline that was comparable to highly invasive MDA-MB-231 breast line. Among the breast cell lines, PBR expression increased with their invasive potential. The ovarian cell lines showed greater variability in fluorescence intensities and the expression of PBR did not correlate with the amount of mitochondria. Mitochondrial PBR density disclosed significant difference between cisplatin-sensitive (low PBR density) and -resistant (high PBR density) ovarian cell lines.

MTT test showed higher sensitivity of 2 breast cell lines MCF-7 and MDA-MB-231 (IC_{50} < 75 μM) to PBR ligand PK 11195 than all examined ovarian cell lines (IC_{50} > 90 μM, in chemo- and radio- resistant lines IC_{50} > 110 μM). Growth inhibitory effect of *PK 11195* did not correlate with the amount of PBR and was mediated probably by another, PBR-independent mechanisms.

The expression of CA IX was only marginal in majority of tested cell lines in subconfluent conditions and was inducible by high cell density. More than 5% of positive cells in sparse culture have been found in MDA-MB-231 and MDA-MB-453 breast cell lines while more than 15% of A2780/ADR adriamycin-resistant ovarian cells were positive for CA IX expression under the same conditions.

Our data indicate that PBR expression in breast and ovarian carcinoma cell lines is not proportional to the amount of mitochondria and should be expressed relatively to the cell mitochondrial mass. This assessment allows establishing high PBR density as a measure of aggressiveness (invasion in breast and resistance in ovarian cancer). Observation of relatively high CA IX expression in A2780/ADR cells evokes the assumption that multidrug resistance might be connected with selection advantage towards CA IX expressing cells.

Key words: breast and ovarian carcinoma, peripheral-type benzodiazepine receptor (PBR), carbonic anhydrase isozyme IX (CA IX), PBR inhibitor PK 11195, cisplatin resistance

The peripheral-type benzodiazepine receptor, recently presented as translocator protein [1] is an 18 kDa protein located mainly in the outer mitochondrial membrane, as a component of the mitochondrial permeability transition pore (MPTP) together with the voltage-dependent anion channel (VDAC) and the nucleotide adenine transporter (ANT) [2, 3]. It was reported to be involved in steroid biosynthesis [4, 5], ion transport [6], porphyrin transport and heme biosynthesis [7, 8], cellular proliferation and differentiation [9], immunomodulation [10], apoptosis [11], regulation of mitochondrial metabolism and functions [5, 10], cellular respiration and oxidative processes [12]. Its potential functions in pathological conditions were described in ischemia-

reperfusion injury [4], brain injury [13], epilepsy [14], neurodegenerative diseases [10, 13], peripheral neuropathy [15], psychiatric disorders [16] and cancer [9, 17-19]. Numerous studies implicate a role for cholesterol in the mechanisms underlying cell proliferation and cancer progression and PBR-mediated cholesterol transport into the nucleus can be involved in human breast cancer cell proliferation and aggressive phenotype expression [20, 21]. In spite of considerable knowledge on PBR expression and function in breast cancer, we lack similar information on ovarian cancer. The purpose of this study is to broaden our knowledge towards ovarian carcinomas, using a battery of human ovarian cancer cell lines.

Carbonic anhydrases are ubiquitous metalloenzymes involved in basic physiological processes as acid-base regulation, respiration, digestion, calcification and others [22]. In mammals, they are present in at least 15 different isoforms from which the 12 catalytically active isoforms play important physiological and pathophysiological functions. The carbonic anhydrase isozyme IX (CA IX) is a hypoxia-inducible enzyme, associated with neoplastic growth. It becomes an interesting pharmacological target due to its overexpression in cancer and its absence in normal tissue [23, 24]. In various tumors, expression of CA IX correlates with poor prognosis suggesting its potential utility for prediction of tumor expansion and therapeutic outcome [25, 26].

Both PBR and CA IX have recently been studied in various types of tumors and were proved to be promising prognostic markers [9, 17, 18, 23, 27-30].

Determination of PBR and CA IX expression on broad panel of breast and ovarian cancer cell lines with different invasiveness (breast) or chemo- and radio-sensitivity (ovarian), carried out in this study, aspires to enlighten context of possible associations of these proteins with tumor aggressiveness and/or response to therapy.

Materials and Methods

Reagents. PBR-specific monoclonal antibody 8D7 was kindly provided by P. Casellas (Sanofi-Synthelabo Recherche, Montpellier, France) [31]. Monoclonal antibody M75 specific for human carbonic anhydrase IX , directed to an epitope in the N-terminal proteoglycan (PG)-like region, was described elsewhere [32]. Ethyl dimethyl sulfoxide (DMSO), propidium iodide (PI) and 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma Chemical Co. (St. Louis, MO). Saponin and PK 11195 were purchased from Lambda life, a.s. (Bratislava, Slovakia). Fetal calf serum, L-Glutamine, Penicillin and Streptomycin were acquired from Biocom (Slovakia).

Cell culture. The human ovarian carcinoma cell line A2780, its cisplatin-resistant variant A2780/CP and A2780/ADR (multidrug-resistant, MDR-1 gene-coded Pgp/p170 overexpressing subline), cisplatin-sensitive line CH1, an intrinsically cisplatin resistant line SKOV-3 and breast carcinoma cell lines (MCF-7, BT-20, MDA-MB-453, MDA-MB-231) differing in their invasive and metastatic abilities [20, 33] were routinely cultured in RPMI supplemented with 10% fetal calf serum, 2mM L-glutamine, 100 μg/ml penicillin and 50 μg/ml streptomycin in 5% CO_2 at 37 °C. The cultures were passaged twice a week after reaching cell density of 0.8 – 1.0 x 10^6 cells/ml.

For CA IX determination, 2 types of cell density conditions were used: 2.7 x 10^6 cells/well (dense cultures) and 0.7 x 10^6 cells/well (sparse cultures) in TC-plate 6 well system.

Immunocytochemical staining. Cells were harvested by trypsinization, adjusted to 10^6 cells/sample, and washed in PBS.

For PBR staining, cells were fixed in 0.5% paraformaldehyde in PBS for 5 min. After permeabilization with saponin solution (0.2% saponin in PBS containing 0.1% BSA), cells were labeled for 30 min at room temperature with 1 mg/mL – of the anti-PBR 8D7 antibody. Thereafter, cells were washed twice in PBS/0.1%BSA and resuspended in FITC-conjugated goat F(ab')2 antibody to mouse IgG (Beckman Coulter) diluted 100-fold in PBS/0.1%BSA. Control samples were prepared without primary monoclonal antibody 8D7.

For CA IX staining, mouse monoclonal anti-human CAIX antibody M75 (50 μl of hybridoma medium) was added to tube containing a 50 μl aliquot of cells. Isotype –matched non-reactive anti-CD45 (Bra 55) monoclonal antibody was used as a negative control. Cells were incubated at room temperature for 30 minutes. FITC-conjugated goat anti-mouse F(ab')2 antibody was added after washing with PBS/0.1% BSA solution for another 30-minute incubation.

Finally, the cells were analyzed using a Coulter Epics Altra flow cytometer. PBR subcellular localization was checked using Olympus fluorescence microscope equipped by computerized image analysis software (Komet 5.5, Kinetic Imaging, Ltd Liverpool, UK).

Flow cytometry measurements and data analysis. Coulter Epics Altra flow cytometer was equipped with 488 nm excitation laser and fluorescence emission was measured using bandpass (525, 575 nm) filters with FL1, FL2 (respective) photomultipliers required for utilized fluorochromes: FITC (FL1), PI (FL2). Forward/side light scatter characteristic and/or PI positivity was used to exclude the cell debris and dead cells from the analysis. For each analysis, at least 1×10^4 cells were collected in a list mode. Data were analyzed with WinMDI version 2.8 software (J. Trotter, Scripps Research Institute, La Jolla, CA) and FCS Express version 3.0 (De Novo Software, Ontario, Canada). The relative fluorescence intensity (RFI) was expressed as multiple of fluorescence intensity of control samples.

Cytotoxicity assay. Effect of PBR inhibitor PK 11195 on survival of cells was determined by MTT assay [34]. Cells were seeded at (1-2) x 10^3 cell density in 96-well culture plates. Each dose of PK 11195 (added in the volume of 50 μl) was tested in triplicate or quadruplicate, and cytotoxicity curve was constructed from at least seven different concentrations. After 5 days, the cells were incubated with 50 μl of MTT (1 mg/ml) and left in the dark at 37 °C for an additional 4 h. Thereafter, medium was removed, the formazan crystals were

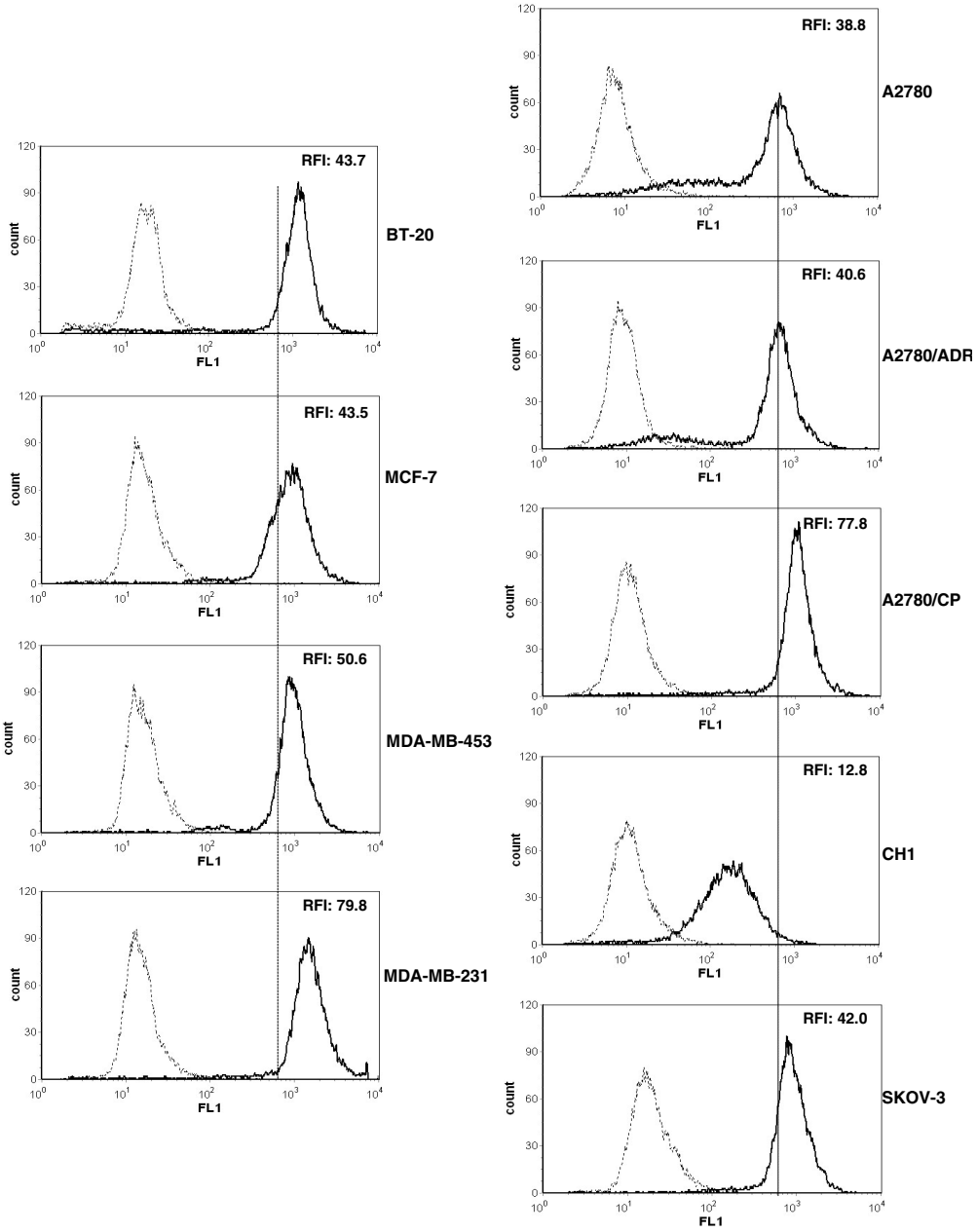

Figure 1. PBR expression in different human breast and ovarian cancer cell lines. Dashed histograms represent control samples and bold histograms represent 8D7 labeled cells. RFI was expressed as multiple of fluorescence intensity of control samples. Dotted vertical line marks medium ovarian RFI. Histograms of one representative experiment out of 3-8 are shown.

dissolved in 200 µl of DMSO, and the absorbance was measured at 540 nm and 690 nm in Microplate reader (Dynatech Lab Inc., Chantilly, VA, USA). The concentration of drug that inhibited cell survival to 50% (IC_{50}) was determined by Calcusyn software (version 1.1, Biosoft).

Mitochondrial mass determination. Mitochondria were stained with MitoTracker Green FM (MTG, Invitrogen, USA), which is preferentially accumulated in mitochondria regardless of mitochondrial membrane potential. However**,** MTG is known as a P-glycoprotein substrate and its staining profile is dependent on the activity of this protein. Thus we pre-incubated cells with MDR modulator Verapamil (10 µM, 10 min) to block P-glycoprotein activity first. Cells were then exposed to a 0.2 µM MitoTracker Green FM solution for 15 min at 37°C. The resulting fluorescent signal was detected by flow cytometry. PI was used to distinguish viable cells.

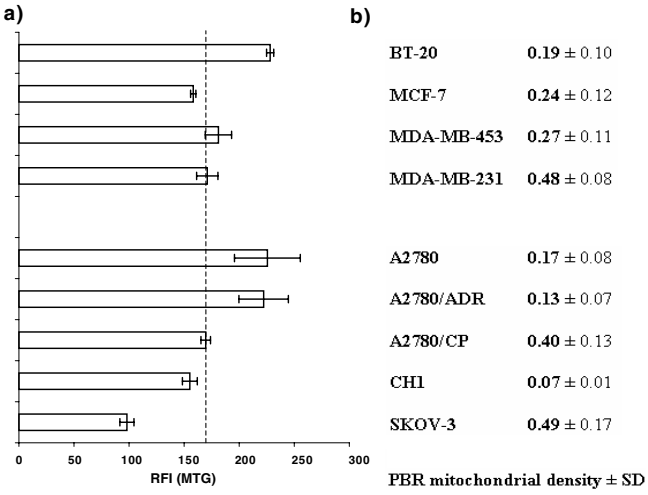

Figure 2. Amount of mitochondria (a) and PBR mitochondrial density (b). Dashed line represents medium MTG RFI (mitochondrial mass). PBR mitochondrial density was calculated as a ratio of RFI corresponding to PBR expression and MTG RFI. Averages of at least three independent experiments ± S.D. are shown.

Results

PBR expression in different human breast and ovarian cancer cell lines. All tested carcinoma cell lines showed high and mitochondrial PBR expression (more than 90% of positive cells), although they differed in relative fluorescence intensity (RFI) reflecting the amount of PBR (Fig.1). Generally, there was higher variability observed among the studied ovarian cell lines, ranging from cisplatin-sensitive CH1 cells with the lowest RFI to cisplatin-resistant A2780/CP with high RFI comparable to the aggressive breast MDA-MB-231 cell line. Among the breast carcinoma cell lines, we confirmed that PBR expression was the highest in highly invasive MDA-MB-231 cells, intermediate in MDA-MB-453 cells with intermediate invasiveness and the lowest in weakly invasive MCF-7 and BT-20 cells. Nevertheless, this relatively low RFI in breast MCF 7 and BT-20 cell lines was similar to the medium value of RFI in ovarian cells (A2780, A2780/ADR, SKOV-3), although majority of examined cell lines had comparable doubling time in our culture conditions (data not shown). In fact, we did not find any correlation between PBR expression and cell doubling time in tissue culture conditions.

Amount of mitochondria. MTG fluorescence has been used as a measure of mitochondrial mass independent of mitochondrial membrane potential. There were no significant differences in the mitochondrial mass of majority of tested cell lines, with the exception of SKOV-3 with extremely low MTG fluorescence and BT-20, A2780 and A2780/ADR with the high amount of mitochondria (Fig. 2a).

PBR mitochondrial density. Comparison of PBR expression with the amount of mitochondria revealed the lack of correlation between these two parameters. To quantify the amount of PBR in relation to the amount of mitochondria, we calculated PBR mitochondrial density as a ratio of RFI corresponding to 8D7 binding and RFI of MTG reflecting the mitochondrial mass (Fig. 2b). In this way we could distinguish 3 cell lines with high PBR density (highly invasive breast MDA-MB-231 and 2 cisplatin resistant ovarian cell lines A2780/CP and SKOV-3) and one ovarian cell line with extremely low PBR density (CH1) from the others. Thus PBR density seems to be significantly higher in highly invasive, ER negative breast carcinoma MDA-MB-231 cells than in medium or weakly invasive breast cell lines, independently on their ER status. Analogically, among the tested ovarian lines we recognized significant difference between the cisplatin-sensitive (low PBR density) and cisplatin-resistant (high PBR density) cell lines.

Effect of PK 11195 inhibitor. In spite of variable PBR densities observed in ovarian cell lines, MTT test did not show any significant inhibition of cell proliferation by PK 11195 up to 75 μM concentration, although extremely high concentration of this inhibitor (100 μM) reduced the cell number more than 50% in majority of examined ovarian cell lines. However, SKOV-3, intrinsically chemo- and radio- resistant cells remained resistant even to this high concentration of PBR ligand (IC_{50}>100 μM). Only 2 radio- and chemo-sensitive cell lines (A2780 and CH1) had IC_{50}<100 μM (Fig. 3a).

On the other hand, breast carcinoma cell lines tested were more sensitive to this inhibitor with the exception of BT-20 cells (IC_{50}>100 μM) with the following IC_{50} values: 52.3 μM for MCF-7, 67 μM for MDA-MB-231, 94.5 μM for MDA-MB-453 (Fig. 3b).

Based on our MTT results, we can conclude that the inhibitory effect of PBR ligand *PK 11195* is not dependent on the amount of PBR and can be mediated by non-specific, PBR-independent mechanisms.

CA IX expression. Almost all tested cell lines showed only marginal expression of CA IX in subconfluent cultures, with exception of breast MDA-MB-231 (high invasiveness), MDA-MB-453 (medium invasiveness) and multidrug-resistant ovarian A2780/ADR cell line. These 3 cell lines expressed low amount of CA IX (6-16% of positive cells) already in sparse cell density and this was not inducible (MDA-MB-453, MDA-MB-231) or only weakly- inducible (A2780/ADR) in dense cultures. However, in sufficiently dense cultures (~3 x 10^5 cells/cm^2) which are generally characterized by pericellular hypoxia, all the other cell lines significantly upregulated CA IX expression, except of parental ovarian A2780 cell line. Principally, weakly invasive breast cell lines (MCF-7, BT-20) showed stronger up-regulation (over 10 % of positive cells with 3,6 and 11-fold induction) in comparison with the tested ovarian cell lines, where only the cisplatin-resistant A2780/CP subline reached ~10% positivity and showed more than 3-fold induction (Fig. 4). These data suggest that in majority of human ovarian and breast cell lines CA IX is inducible by high cell density and the exceptions in breast carcinoma cell

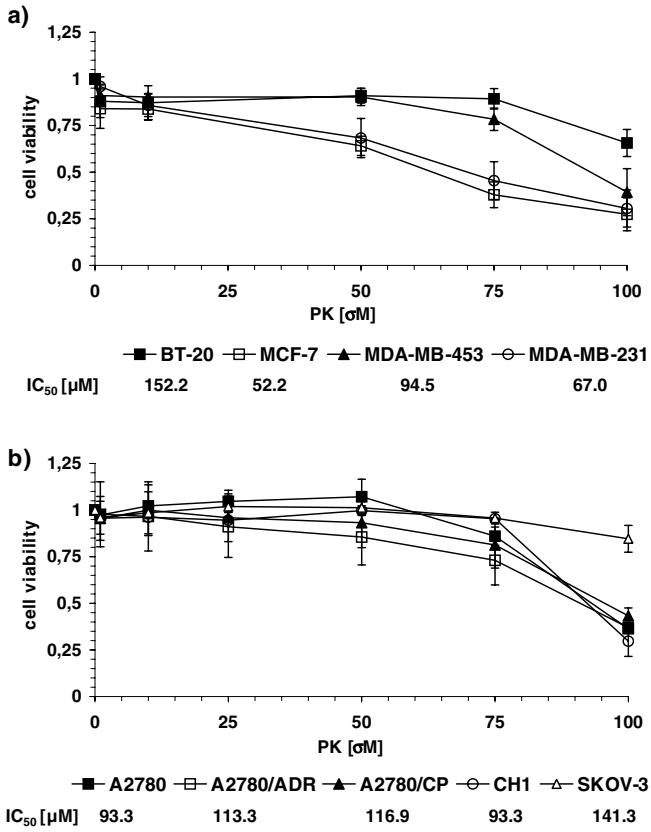

Figure 3. Effect of PBR inhibitor PK11195 on survival of human breast (a) and ovarian (b) cancer cell lines, determined by MTT assay. Averages of at least three independent experiments ± S.D. are shown.

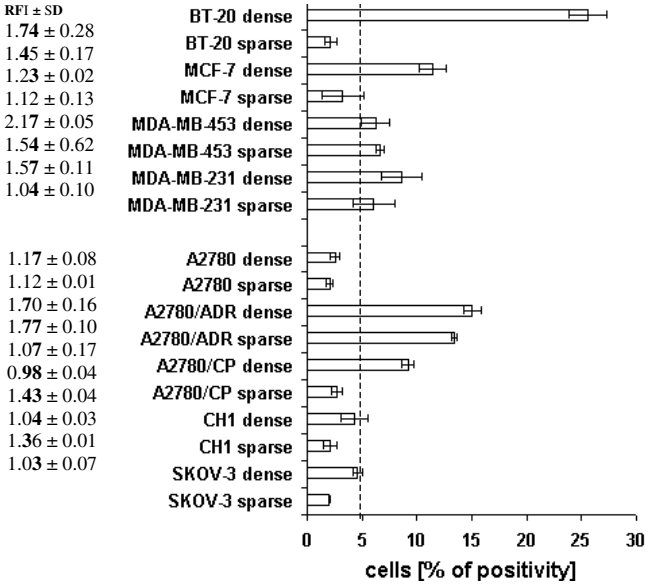

Figure 4. CA IX expression in human breast and ovarian cancer cell lines. Isotype –matched non-reactive monoclonal antibody was used as a control. Dense – confluent, sparse – subconfluent. Dashed line refers to 5 % of CA IX positive cells Averages of at least three independent experiments ± S.D. are shown.

lines (MDA-MB-231, MDA-MB-453) may be connected with disturbances in the hypoxic pathway regulation. Relatively high CA IX expression in the multidrug-resistant ovarian cell line A2780/ADR implies its association with the multidrug-resistant phenotype.

Discussion

High levels of peripheral-type benzodiazepine receptor are part of the aggressive human breast cancer cell phenotype in vitro [1, 5, 10]. However, elevated PBR expression is not a common feature of aggressive tumors [17], but may be limited to certain cancers, such as breast, colorectal and prostate cancers. We pursued this question with regard to ovarian cancer. As frequent emergence of drug resistance is the major concern in ovarian cancer progression, we preferred focusing on this aspect for ovarian cell lines. Applying a panel of human breast and ovarian cancer cell lines, we confirmed the previous reports on PBR expression in breast cancer cell lines reflecting their invasiveness and we found new association of high PBR density with cisplatin-resistance in ovarian cancer.

As previous data have shown that PBR is highly expressed in tissues involved in steroid synthesis [2, 21, 35], our findings of its high expression in breast and ovarian cancer cell lines are not surprising. Using the MAb 8D7, we detected PBR expression exclusively in association with mitochondria, we did not find any nuclear localization of this protein, neither in breast nor in ovarian cancer cell lines. This is in accord with other studies that utilized the same monoclonal antibody for its detection in various human tissues [36] and we can join the authors in their argumentation to explain the discrepancy with those findings, which indicated the expression of PBR in and around the nucleus in breast cancer cells [20], using different detection system. This may be related to a difference in the C-terminal epitope of PBR in MDA-MD-231 cells or to a different signaling involved in the regulation of PBR subcellular localization [36].

Although PBR expression in ovarian carcinoma cell lines was generally lower than in breast cancer cell lines, we noted higher variability among observed ovarian cell lines with two extremes (from the lowest RFI for cisplatin-sensitive CH1 cells reaching to the highest RFI for A2780/CP resistant cells).

Our finding that PBR expression does not correlate with amount of mitochondria, is new for ovarian cancer and contradictory to previous report of their positive correlation in breast cancer cell lines [37]. However, that study, performed in 2000, used NAO dye for mitochondrial mass determination, which was later shown not to be suitable as an assay of

mitochondrial mass in living cells because this staining is dependent upon the mitochondrial membrane potential [38]. Thus our study provides the only data of this type obtained by the appropriate mitochondrial mass determination technique.

There is growing evidence of direct action of cisplatin on mitochondria [39, 40], showing its preferential binding to mitochondrial DNA and voltage-dependent anion channel protein or reporting mitochondrial DNA resistance to cisplatin and oxidative stress response in resistant cells [41]. Cellular density of mitochondria was presented as the key factor for the determination of the cellular cisplatin sensitivity [42]. Our detection of extremely low amount of mitochondria in SKOV-3 cell line, known by its resistance to platinum-based regimens, complements these results. Moreover, high mitochondrial PBR density observed in both cisplatin resistant cell lines used in our study suggests that PBR may be involved in protection of cells to cisplatin-induced cell death probably by affecting cisplatin interaction with mitochondrial membrane proteins, particularly VDAC.

Although the tested carcinoma cell lines had different PBR expression (RFI), their sensitivity to PBR ligand PK 11195 did not reflect the same pattern. Especially ovarian cell lines were relatively insensitive to its cytostatic effect in comparison with breast cell lines, exhibiting IC_{50}<100 μM only for two radio- and chemo-sensitive cell lines (A2780 and CH1). Among the breast cancer cell lines, PK 11195 inhibited effectively the proliferation of one cell line with the highest (MDA-MB-231) and another with the lowest (MCF-7) amount of PBR. Thus antiproliferative effect of PK 11195 seems to be PBR-independent and related rather to the other cellular mechanisms inducible by this ligand as already observed by others [43]. This is also supporting several other works dealing with PK 11195 effects independent of PBR binding [44, 45].

Hypoxic cells become resistant to apoptosis and are more likely to migrate to less hypoxic areas of the body (metastasis). Recent studies demonstrated hypoxia-induced chemoresistance to cisplatin and doxorubicin in lung cancer cells to be affected by HIF pathway [46]. In the previous report the expression of HIF-1a was shown to up-regulate the expression and activity of multidrug resistance gene-encoded P-glycoprotein in human lung adenocarcinoma A549 cell line [47], suggesting that hypoxia-induced chemoresistance of tumor cells is mediated by P-glycoprotein. CA IX transcription is regulated by several cross-talking pathways where a hypoxia-regulated HIF-1 plays a major role.

It is known that under standard cultivation conditions, CA IX is expressed in various tumor-derived cell lines depending on the cell density. It is usually absent in subconfluent cultures and upregulated with high cell density associated with pericellular hypoxia in cell culture [48]. Our data match these findings, however we observed significant CA IX expression in MDA-MB-231, MDA-MB-453 and A2780/ADR already in sparse culture conditions. Relatively high expression of CA IX in A2780/ADR is interesting as their parental cells showed not only negligible CA IX expression but also its weak induction by high cell density. We therefore utilized the hypoxia mimetic desferrioxamine (DFO, 100 μM, 24h) to observe CA IX upregulation in both cell lines and proved its weak induction in parental A2780 cell line (2-fold increase in RFI and reaching 18% of positive cells) contrasting with huge upregulation (more than 10-fold increase in RFI and 70% percentage of positive cells) in its multidrug resistant subline. We hypothesize that multidrug resistance phenotype may be associated with selection advantage towards CA IX expressing cells in ovarian cancer. All examined ovarian cell lines exhibited weaker CA IX upregulation by high cell density than breast cell lines that did not show CA IX positivity in sparse conditions. On the other side, breast cell lines with CA IX positivity in sparse conditions did not show any significant upregulation by high density, nor by DFO. Similar observation was made in some other types of tumor cells lines, including malignant cervical and colorectal carcinoma cells [49].

This may suggest impairment in hypoxic pathway along the epithelial-mesenchymal transition (EMT) in progressing cancer. However, more complex study, principally including transcriptional complex HIF-1, is necessary to address this question. Although direct involvement of CA IX in tumor invasiveness was not reported, CA IX has been associated with increased malignancy and poor response to chemotherapy in numerous clinical studies [50]. We therefore speculate that higher expression of CA IX in MDA-MB-231, MDA-MB-453 can contribute to their higher aggressiveness by its ability to enhance the extracellular acidification, which may have implications for tumor progression [51].

To summarize, our study supplemented previous studies on PBR expression in breast cancer and revealed new association of PBR mitochondrial density with cisplatin resistance in ovarian cancer. Higher expression of CA IX in multidrug resistant ovarian cells as well as its possible deregulation in more invasive breast cancer cells requires further studies.

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