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Biochimica et Biophysica Acta 1763 (2006) 93–100
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
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Molecular alterations of cells resistant to platinum drugs: Role of PKCα

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Received 23 June 2005; received in revised form 25 November 2005; accepted 12 December 2005
Available online 6 January 2006

Abstract

Development of resistance to platinum compounds may involve not only overexpression of defence mechanisms but also alterations in cellular response to the drug-induced genotoxic stress. To investigate the cellular bases of response to platinum compounds, we examined the profile of gene expression of ovarian carcinoma cells exhibiting sensitivity (A2780) or resistance (A2780/BBR3464) to platinum compounds. Using display PCR, we found that acquisition of resistance to the multinuclear platinum complex BBR3464 was associated with modulation of several transcripts, including up-regulation of the major substrate of protein kinase C (PKC), the myristoylated alanine-rich C kinase substrate (MARCKS). This feature was associated with PKCα down-regulation. To explore the role of PKCα in cellular sensitivity to platinum compounds, resistant cells were transfected with a PKCα-containing vector. PKCα-overexpressing resistant cells exhibited a decrease in sensitivity to cisplatin, whereas no significant change in sensitivity to BBR3464 was observed. A number of approaches designed to modulate the function or expression of PKCα support that the isoenzyme may play a role in determining resistance only to cisplatin but not to BBR3464, which is known to activate a different pathway of cell response. In conclusion, in spite of PKCα down-regulation in our model, its regulatory function was not apparently implicated in the development of resistance to platinum compounds and the present results do not support a general role of PKCα as a determinant of the resistance status.

Keywords: Cisplatin; Platinum drug; Resistance; Protein kinase C

1. Introduction

Platinum compounds are effective in the treatment of human solid tumors, including ovarian carcinomas [1]. Nevertheless, a major drawback in the clinical use of these cytotoxic agents is the frequent development of resistance [2–4]. Despite the recent advances in the cellular pharmacology of platinum compounds, the molecular context contributing to the sensitivity or resistance to the drugs has not been conclusively defined. Over the years, several mechanisms of resistance have been described. They include altered drug accumulation [5,6], reduced interaction with the intracellular target, enhanced DNA repair [4,7] and increased detoxification [8,9]. Reduced susceptibility to apoptosis has also been proposed as a mechanism of resistance to a variety of antitumor drugs including platinum compounds [10–13]. Moreover, alterations in the signal transduction pathways can affect cellular response to cytotoxic drugs [14,15]. In an attempt to identify critical determinants of cellular response to platinum compounds, we have performed a comparative study with display PCR and gene expression analysis in ovarian carcinoma cells sensitive to platinum drugs and in a subline selected for resistance to BBR3464, a multinuclear Pt complex characterized by a marked cytotoxic potency [16]. In the resistant cell line, we found up-regulation of the myristoylated alanine-rich C kinase substrate (MARCKS), the major and ubiquitous substrate of protein kinase C (PKC), but a down-regulation of PKCα. Since PKCα has been implicated in several biological processes including proliferation and drug resistance, using this cell model we investigated the role of PKCα as potential modulator of sensitivity to platinum compounds. The available results do not

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doi:10.1016/j.bbamcr.2005.12.007

support a general role of PKCα as determinant of resistance to Pt compounds.

2. Materials and methods

2.1. Drugs and chemicals

Cisplatin and BBR3464 (NO_3^- salt), were primarily dissolved in saline. All working dilutions were prepared in saline. Gö6976 (Calbiochem, San Diego, CA) was dissolved in dimethylsulfoxide and diluted in water. Phorbol 12,13-dibutyrate (PDBu; Sigma, St. Louis, MO, USA) was dissolved in dimethylsulfoxide and diluted in dimethylsulfoxide.

2.2. Cell lines and growth conditions

The human ovarian carcinoma A2780 cell line and the BBR3464-resistant A2780/BBR3464 subline were used in this study [17]. A2780/BBR3464 cells were generated by continuous exposure to increasing concentrations of drug. Resistance was stable up to 9 months when cells were grown in the absence of drug. The growth characteristics of sensitive and resistant cells were similar. Both cell lines were grown and maintained as monolayers in RPMI-1640 medium supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, CA).

2.3. Cellular sensitivity to drugs

Cellular sensitivity to platinum drugs or to Gö6976 was evaluated by colony-forming or growth-inhibition assays [18]. For colony-forming assays, cells in the logarithmic phase of growth were seeded in duplicates into 5 cm diameter dishes. Forty-eight h after seeding, the drug was added to the medium for 1 h. Cells were then washed with saline prior to addition of fresh medium. Seven days after treatment colonies of 30 or more cells were counted after fixing in ethanol and staining with crystal violet. IC_{50} is defined as the drug concentration causing a 50% reduction of colony number over that of untreated control.

For growth-inhibition assays, cells were seeded in duplicates into 6-well plates. Twenty-four h after seeding, the drug was added to the medium for 1 h and cells were incubated for 72 h. Gö6976 was added to the medium for 72 h before harvesting for counting. For combination with Gö6976, cells were treated for 1 h with cisplatin and then incubated for 72 h with 0.03 μM Gö6976. For combinations with PDBu, cells were treated with 1 μM PDBu for 15 min (short-term exposure) or 24 h (long-term exposure) before addition of cisplatin for 1 h. At the end of drug exposure (for 72 h treatment) or 72 h after drug treatment (for 1 h exposure) adherent cells were harvested using trypsin and counted with a cell counter.

2.4. Analysis of drug interaction

According to the method of Kern et al. [19], the expected value of cell growth (G_{exp}, defined as the product of the growth observed with drug A alone and the growth observed with drug B alone) and the actual growth observed (G_{obs}) for the combination of A and B were used to construct a synergistic index (R), as follows:

$$R = G_{exp}/G_{obs}$$

Synergy was defined as any value of R greater than unity. An R value of 1.0 (additive effect) or less indicated an absence of synergy.

2.5. Differential display assay

Cytoplasmic RNA from A2780 and A2780/BBR3464 was isolated by using the RNeasy Midi kit (Qiagen, Santa Clarita, CA) and fingerprinted by the Delta Differential Display kit (Clontech, Palo Alto, CA). Briefly, first-strand cDNA was synthesized using 2 μg of RNA, oligo(dT) as a primer, and Moloney murine leukemia virus reverse transcriptase. Two dilutions of each cDNA template (corresponding to 50 and 200 ng of reverse transcribed RNA) were amplified by PCR in the presence of 50 μM dNTPs, 1 μM primers, 50 nM [α-^{33}P]dATP (1000–3000 Ci/mmol; Amersham Pharmacia Biotech; Cologno Monzese, Italy), 1× Advantage KlenTaq polymerase and reaction buffer (Clontech). The PCR primers used were a pairwise combination of arbitrary "P" (P1–P2) and oligo(dT) "T" (T1–T4) primers. Amplification conditions were as follows: 94 °C for 5 min, 40 °C for 5 min, 68 °C for 5 min for 1 cycle; 94 °C for 2 min, 40 °C for 5 min, and 68 °C for 5 min for 2 cycles; 94 °C for 1 min, 60 °C for 1 min, and 68 °C for 2 min for the remaining 24 cycles. The last cycle was followed by a 10 min extension at 72 °C. PCR products were fractionated in a 6% polyacrylamide/8 M urea gel and visualized by autoradiography. The differentially expressed band in the A2780/BBR3464 was eluted from the gel, reamplified, blunt-ended, subcloned into the pMOSBlue vector using the pMOSBlue blunt ended cloning kit (Amersham Pharmacia Biotech) and automatically sequenced using the ABIPRISM Dye Terminator cycle sequencing ready reaction kit (PerkinElmer Corp., Brounchburg, NJ). Nucleotide sequence was subjected to BLAST searches for sequence homologies (National Center for Biotechnology Information).

2.6. Western blot analysis

Cell lysates from exponentially growing cells were prepared as previously described with minor modifications [20]. Samples (80 μg/lane) were fractionated by SDS-polyacrylamide gel electrophoresis and blotted on nitrocellulose sheets. Blots were preblocked for 1 h at room temperature in PBS containing 5% (w/v) dried nonfat milk. Filters were incubated overnight at 4 °C with monoclonal antibodies to PKC isoenzymes (Transduction Laboratories, Lexington, KY), to MARCKS (Upstate Biotechnology, Lake Placid, NY), to phospho-MARCKS (Calbiochem), to p53 (DakoCytomation, Glostrup, Denmark), to p21^{WAF1} (NeoMarkers, Fremont, CA), to Caspase-3 (Calbiochem) and to PKCζ (Upstate Biotechnology). A rabbit anti-actin antibody (Sigma) was used as control for loading. Antibody binding to the nitrocellulose blots was detected by enhanced chemiluminescence (Amersham Pharmacia Biotech).

2.7. PKC activity assay

The soluble and particulate fractions were prepared and fractions were tested for PKC activity as described [21,22], by measuring the amount of ^{32}P incorporated into histone IIIS (Sigma) from [γ-^{32}P]ATP (sp. act. 3000 Ci/mmol; Amersham) in the presence of phospholipids (PS and 1,2-dioleoylglycerol) and $CaCl_2$. Background activity was measured with EGTA in the absence of phospholipids.

2.8. Transfection of recipient cells

Transfections were carried out by lipofection as previously described with minor modifications [23]. Cells were seeded in 6-well plates and when they reached 70% confluency each well was incubated for 6 h with serum-free medium containing 30 μl of lipofectin (Invitrogen) and 3 μg of empty, PKCα- or MARCKS-containing vectors. Medium was replaced with serum-containing medium and 48 h later selection of stable clones was started by adding 200 μg/ml geneticin (Invitrogen). Protein expression was monitored by Western blot analysis. The plasmid containing the PKCα bovine sequence driven by pCMV was kindly provided by Dr. P.J. Parker (London, UK). The plasmid containing the human MARCKS cDNA [24] was kindly provided by Dr. P.J. Blackshear and Dr. D.J. Stumpo (Research Triangle Park, NC, USA).

2.9. Cell cycle analysis

Exponentially growing cells were seeded in 25 cm^2 flasks and 24 h later, they were exposed to drug for 1 h. Drug-containing medium was then replaced with fresh medium and cell cycle analysis was carried out after 24 h. Floating and adherent cells were harvested, fixed and stained with a propidium iodide (PI)-containing solution (30 μg/ml PI, 66 U/ml RNase A in PBS). The cell cycle

Table 1
Sensitivity of ovarian carcinoma cells to cisplatin and BBR3464[a]

	IC_{50} (μg/ml)	
	Cisplatin	BBR3464
A2780	0.72±0.1	0.046±0.004
A2780/BBR3464	3.19±0.9	1.47±0.3

[a] Cytotoxicity was assessed by colony-forming assay. Cells were exposed for 1 h to the drug. IC_{50} values (drug concentration inhibiting colony formation by 50%) represent the mean±S.D. of at least 3 independent experiments.

perturbations were measured by using a flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Ten thousand cells/sample were analyzed for DNA content and cell cycle distributions were calculated using WinMDI software.

3. Results

3.1. Analysis of gene expression

The A2780/BBR3464 subline was selected for resistance to BBR3464 and exhibited a cross-resistance to cisplatin (Table 1) [17]. Using display PCR the mRNA expression pattern of BBR3464-sensitive and -resistant cells was compared using different combinations of arbitrary and oligo-dt primers. We identified several bands that were reproducibly modulated in the A2780/BBR3464 cells. The bands were excised, re-amplified, cloned, sequenced and analyzed against the Genbank database. The isolated cDNA clones, which are shown in Table 2 included genes belonging to different pathways such as Cellular Apoptosis Susceptibility gene (CAS), beta-glucoronidase, S19 ribosomal protein, and acetoacetyl CoA tiolase. Among the modulated transcripts, we also found neurofilament low (NF-L), for which we have already documented lack of involvement in the resistant phenotype [17], and as shown in Fig. 1A, the mRNA encoding MARCKS, a ubiquitous substrate of PKC involved in different cellular responses including cell motility, secretion, cell cycle, transformation, regulation of actin cytoskeleton [25].

3.2. Validation of differential expression

Validation of differentially expressed genes was undertaken by Western blot or RT-PCR analysis. Some modulations were not confirmed as expected on the basis of the high rate of false positives reported for the display PCR approach [3,26]. Western blot analysis using an antibody against MARCKS indicated a

Table 2
List of the cDNA clones identified by differential display analysis

Accession number[a]	Matching[b]	High-scoring sequence	Function	Modulation[c]
D10522	1991–2496	Human mRNA for 80K-L protein	Substrate for PKC, localized to the plasma membrane; actin filament crosslinking protein. Involved in cell motility, phagocytosis, membrane trafficking and mitogenesis.	↑
X52142	877–1202	Human mRNA for CTP synthetase	It catalyzes the ATP-dependent amination of UTP to CTP with either L-glutamine or ammonia as the source of nitrogen.	↑
M81757	167–495	H. sapiens S19 ribosomal protein mRNA	Ribosomal protein	↓
X05608	4084–4408	Human gene for neurofilament subunit NF-L	Protein belonging to the intermediate filament family	↑
AH006432	2295–2635	H. sapiens brain cellular apoptosis susceptibility protein (CSE1/CAS) mRNA	Export receptor for importin alpha. It mediates importin-alpha reexport from the nucleus to the cytoplasm after import substrates have been released into the nucleoplasm. It plays a role in apoptosis and in cell proliferation.	↓
X77303	682–1020	H. sapiens CAK1 mRNA for cdk-activating kinase (CDK7)	Member of the cyclin-dependent protein kinase (CDK) family. Involved in transcription initiation and DNA repair. It is thought to serve as a direct link between the regulation of transcription and the cell cycle.	↓
AF161444	1321–1614	H. sapiens HSPC326 mRNA	Not known	↑
X55187	1627–1965	Human mRNA for alpha-actinin	F-actin cross-linking protein thought to anchor actin to intracellular structures. Bundling protein	↑
D90228	758–1102	Human mRNA for acetoacetyl-CoA thiolase	It plays a major role in ketone body metabolism	↓
M15182	1828–2178	Human beta-glucuronidase mRNA	Role in the degradation of dermatan and keratan sulfates	↓
U30827	893–1256	Human splicing factor Arg/ser-rich SRp40-3	Role in constitutive splicing and selection of alternative splice sites	↓

[a] Genbank accession number.
[b] bp (from–to) of the high-scoring sequence matching the sequenced fragment.
[c] Up (↑)- or down (↓)-modulation in A2780/BBR3464 versus A2780 cells.

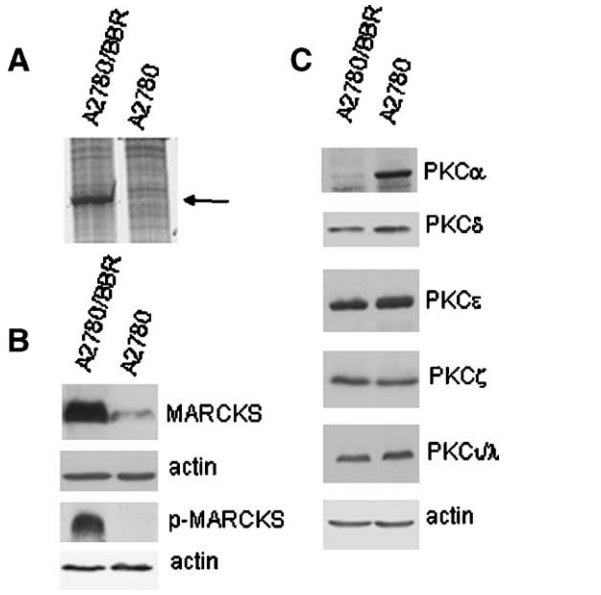

Fig. 1. Display PCR and Western blot analysis in A2780 and A2780/BBR3464 cells. (A) Display PCR; the arrow indicates the differentially expressed band corresponding to MARCKS. (B) Western blot analysis of MARCKS and p-MARCKS. Control loading is shown by actin. (C) Western blot analysis of different PKC isoforms. Control loading is shown by actin.

substantial up-regulation of the protein in the drug-resistant subline (Fig. 1B). Using a phospho-specific antibody, we found that MARCKS was phosphorylated in A2780/BBR cells (Fig. 1B).

3.3. Expression of PKC isoenzymes

As MARCKS is the major and ubiquitous substrate of PKC, which includes a family of at least 13 distinct serine/threonine kinases [27], we investigated the expression of PKC isoenzymes in BBR3464-sensitive and -resistant cells. Western blotting analysis indicated that PKC β, γ, η, and θ were not detectable in A2780 and A2780/BBR3464 cells (data not shown), whereas PKC δ, ε, ζ and ι/λ were markedly expressed at similar levels (Fig. 1C). An unexpected finding was a down-regulation of PKCα, which has been implicated in cell survival [28], in the resistant variant as compared with the parental cell line (Fig. 1C). Activity experiments of calcium-dependent protein kinases were consistent with differential expression as total activity was 4.7 pmol ^{32}P/min/10^3 cells in A2780 cells and 1.9 in A2780/BBR3464 cells.

3.4. Transfection of MARCKS into A2780 cells

To define whether MARCKS played a role in regulation of cell sensitivity to platinum drugs, we transfected A2780 cells with a plasmid containing the MARCKS full-length cDNA. Using Western blot analysis, we selected a clone and a polyclonal population overexpressing the protein (Fig. 2A). An analysis of cell sensitivity to cisplatin or BBR3464 (1 h exposure) with growth-inhibition assays indicated that modulation of cellular levels of MARCKS per se was not sufficient to change drug sensitivity. In fact, the IC_{50} values of A2780-MARCKS poly cells (2.37±0.13 μg/ml for cisplatin and 0.021±0.00035 μg/ml for BBR3464) or A2780-MARCKS clone (1.91±0.29 μg/ml for cisplatin and 0.022±0.003 μg/ml for BBR3464) were not significantly different from those of empty vector-transfected A2780 cells (2.8±0.8 μg/ml for cisplatin and 0.017±0.008 μg/ml for BBR3464).

3.5. Phenotype of A2780/BBR3464 cells overexpressing PKCα

To determine whether down-regulation of PKCα was related to the development of resistance in the BBR3464-resistant cells, A2780/BBR3464 cells were transfected with a plasmid expressing the PKCα full-length cDNA. Using Western blot analysis, we identified a clone overexpressing the protein (Fig. 2B). The sensitivity of the clone to the PKCα specific inhibitor Gö6976 was increased as compared to empty vector transfected

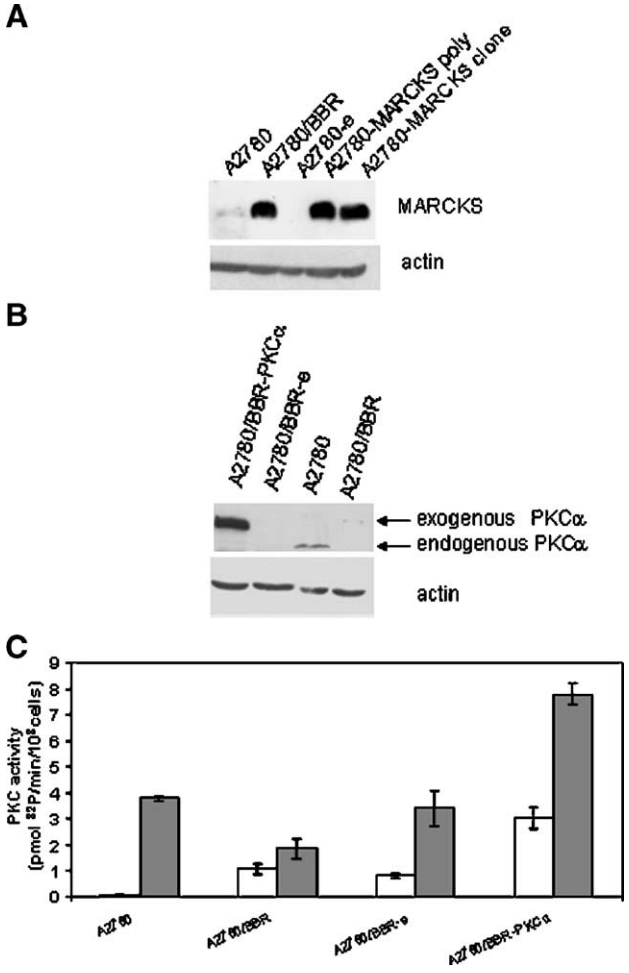

Fig. 2. MARCKS expression and PKC expression/activity in transfected cells. (A) Western blot analysis of MARCKS expression after transfection with MARCKS-containing vector (A2780-MARCKS poly and clone) and empty vector (A2780-e). Control loading is shown by actin. (B) Western blot analysis of PKCα expression after transfection with PKCα-containing vector (A2780/BBR-PKCα) and empty vector (A2780/BBR-e). Control loading is shown by actin. (B) Activity of calcium-dependent PKC. PKC activity in the soluble (open columns) and particulate (solid columns) fractions, expressed as enzyme activity pmol ^{32}P/min/10^3 cells. Each bar represents the mean value (±S.D.) of at least 3 independent experiments.

Table 3
Sensitivity of ovarian carcinoma PKCα-transfected cells to cisplatin and BBR3464[a]

	IC_{50} (μg/ml)	
	Cisplatin	BBR3464
A2780/BBR-e	2.89±0.38	2.34±0.61
A2780/BBR-PKCα	4.97±0.94	2.25±0.6

[a] Cell sensitivity was assessed by growth-inhibition assay on cells transfected with PKCα containing vector (A2780/BBR-PKCα) and empty vector (A2780/BBR-e). Cells were exposed for 1 h to the drug. IC_{50} values (drug concentration inhibiting colony formation by 50%) represent the mean±S.D. of at least 3 independent experiments.

cells, as shown by growth-inhibition assay after 72 h drug exposure. Indeed, IC_{50} was 1.6 μM in PKCα transfected cells and 10 μM in empty vector-transfected cells. The enzymatic activity of Ca^{2+} and lipid-dependent kinases (including PKCα) was assayed in the soluble and particulate fractions. We found that A2780/BBR-PKCα cells displayed an increased activity in both fractions (Fig. 2C).

Due to the interplay between PKC isoforms, overexpression of specific enzymes can result in changes in expression of other isoforms [29]. Thus, we investigated whether PKCα overexpression modulated expression of PKC isoenzymes. In our cell systems, no significant changes were revealed in A2780/BBR-PKCα cells versus cells transfected with empty vector (data not shown). Moreover, no modulation of MARCKS expression was observed in PKCα transfectants, suggesting that overexpression of the PKC substrate MARCKS in A2780/BBR3464 cells was not a homeostatic change resulting from reduced levels of the kinase (data not shown).

The model was further characterized to investigate the biological significance of specific alterations related to modulation of PKCα expression. In particular, to define the possible involvement of PKCα in regulating cellular sensitivity to platinum drugs, we performed a growth-inhibition assay after 1 h drug exposure. Under our experimental conditions, A2780/BBR-PKCα cells displayed an increased resistance to cisplatin as compared to empty vector-transfected cells ($P<0.05$, ANOVA; Table 3), whereas cell sensitivity to BBR3464 was unchanged. Using a colony-forming assay, to detect long-term survival of treated cells, a slight but not significant increase of BBR3464 cytotoxicity in A2780/BBR-PKCα cells was observed (IC_{50} was 1.3±0.3 μg/ml in PKCα transfected cells and 1.68±0.25 μg/ml in empty vector-transfected cells).

As exposure to platinum drugs is known to induce cell cycle perturbations which play a protective role in response to DNA damage, we examined cell cycle distribution in empty vector- and A2780/BBR-PKCα cells following 1 h exposure to cisplatin or BBR3464 and 24 h incubation in drug-free medium. BBR3464 produced G1 arrest in both cell lines at all tested concentrations (Table 4). At 30 μg/ml BBR3464, a partial G2 arrest was also evident in PKCα overexpressing cells. Cisplatin exposure resulted in G1 arrest at low concentrations in both cell lines, whereas a S phase and G2 accumulation of cells was found at higher drug concentrations.

Based on the observed cell cycle perturbations, we explored drug-induced modulation of proteins involved in checkpoint control. Western blot analysis of $p21^{WAF1}$ revealed that the protein was induced after cisplatin and BBR3464 treatment, the extent being lower in A2780/BBR-PKCα cells as compared to empty vector-transfected cells (Fig. 3A). The up-regulation of p53 after DNA damage was evident in both cell lines and was more marked after cisplatin exposure (Fig. 3A).

To investigate whether the differential cell sensitivity to platinum drugs of PKCα overexpressing cells was associated with changes in apoptotic response, we examined cleavage of caspase 3 following exposure to 30 μg/ml BBR3464 or cisplatin. No marked difference in caspase 3 cleavage was observed between A2780/BBR-PKCα and A2780/BBR-e cells after BBR3464 exposure, whereas reduced levels of cleaved caspase were found after cisplatin exposure (Fig. 3B). The reduction of cleaved caspase 3 was dose-dependent (Fig. 3C). The observation is consistent with the reduced antiproliferative effect observed following cisplatin exposure in A2780/BBR-PKCα cells.

3.6. Combination of PKC modulators and platinum compounds

To investigate whether the PKCα inhibitor Gö6976 was capable of modulating cell sensitivity to cisplatin and BBR3464, we performed growth-inhibition assays after 1 h cisplatin or BBR3464 exposure followed by 72 h exposure to Gö6976 in A2780 cells. The modulation of drug effect by Gö6976 was assessed using *R* index, whose values are greater than 1 when synergistic interaction occurs. Under our experimental conditions, Gö6976 produced a marginal sensitization to cisplatin, but not to BBR3464, at cytotoxic concentrations (i.e., >1 μM; Fig. 4).

We examined the effect of short- and long-term exposures to the PKC activator PDBu on cisplatin sensitivity. PDBu was chosen because it is less hydrophobic then TPA and therefore more appropriate for in vitro experiments. Cells were treated for 15 min (short-term exposure) or 24 h (long-term exposure) with 1 μM PDBu, and then exposed to cisplatin for 1 h. The results,

Table 4
Cell cycle perturbations induced by cisplatin or BBR3464[a]

	A2780/BBR-e			A2780/BBR-PKCα		
	G1	S	G2/M	G1	S	G2/M
Control	59.9	27.2	12.9	52.6	31.3	16.1
1 μg/ml BBR3464	68.5	19.8	11.7	67.7	20.1	12.2
3 μg/ml BBR3464	77.3	13.4	9.3	73	13.9	13
10 μg/ml BBR3464	83.5	7.3	9.2	75.6	10.7	13.8
30 μg/ml BBR3464	84.2	7.9	7.9	67	11.5	21.5
1 μg/ml Cisplatin	63.8	21.4	14.8	57.7	28.4	14
3 μg/ml Cisplatin	73.2	16.7	10.1	65.3	20.3	14.4
10 μg/ml Cisplatin	61.1	9.7	29.2	59.7	17.2	23.2
30 μg/ml Cisplatin	23.5	41.5	35.0	28.6	51.1	20.3

[a] Cells were exposed to drug for 1 h and harvested 24 h after drug removal. Values represent percent of cells in different cell cycle phases. A2780/BBR-e and A2780/BBR-PKCα cells were derived by transfection of A2780/BBR3464 cells with empty or PKCα vector.

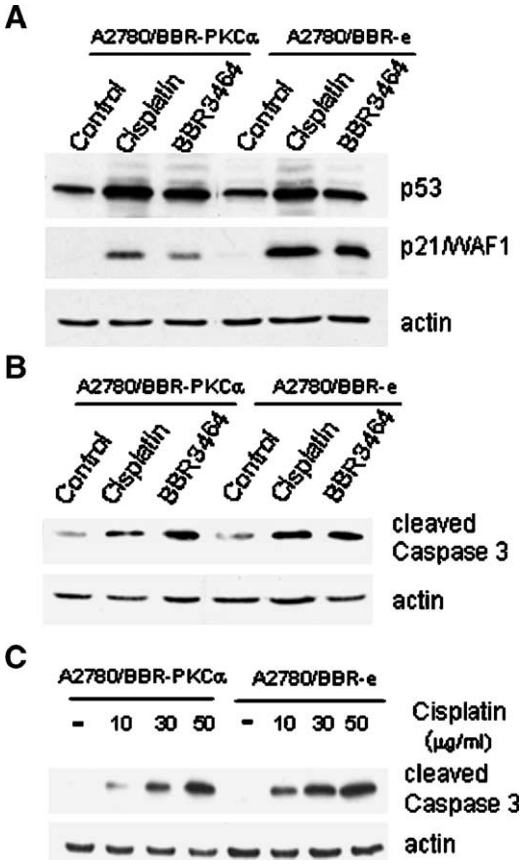

Fig. 3. Modulation of selected proteins by drug treatment in PKCα-transfected cells. (A) Western blot analysis of p53 and $p21^{WAF1}$. Cells were exposed for 1 h to 30 μg/ml cisplatin or BBR3464 and harvested 24 h later. Control loading is shown by actin. (B) Western blot analysis of cleaved caspase 3. Cells were exposed for 1 h to 30 μg/ml cisplatin or BBR3464 and harvested 24 h later. Control loading is shown by actin. (C) Western blot analysis of cleaved caspase 3. Cells were treated for 1 h with different cisplatin concentrations and harvested 24 h later. Control loading is shown by actin. A2780/BBR-e and A2780/BBR-PKCα cells were derived by transfection of empty or PKCα-containing vector in A2780/BBR cells.

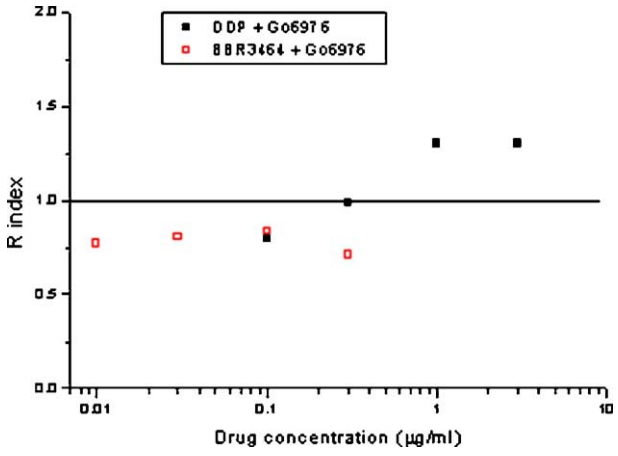

Fig. 4. Combination of platinum drugs and Gö6976 in A2780 cells. Cells were exposed for 1 h to cisplatin or to BBR3464 and then incubated for 72 h with 0.03 μM Gö6976. Cell sensitivity to treatment was assessed by growth-inhibition assay and the effect of the combination was evaluated using the *R* index.

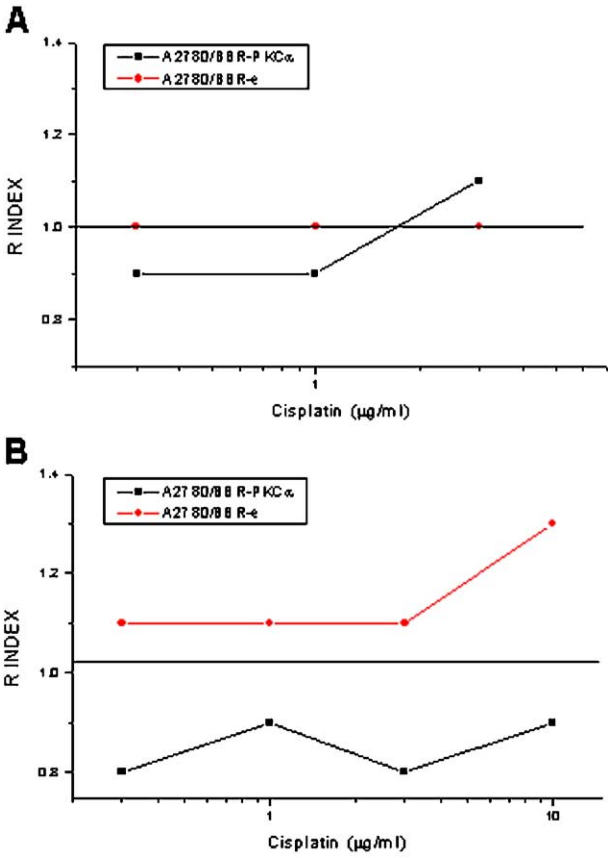

Fig. 5. Combination of cisplatin and PDBu in PKCα overexpressing cells. Cells were exposed to cisplatin for 1 h after 15 min (A) or 24 h (B) pre-incubation with 1 μM PDBu. Cell sensitivity to treatment was assessed by growth-inhibition assay. The results are reported in terms of *R* index. A2780/BBR-e, empty vector transfected cells; A2780/BBR-PKCα, A2780/BBR cells overexpressing PKCα.

expressed as *R* index, indicated no significant modulation of cisplatin sensitivity after short-term exposure (Fig. 5). Conversely, long-term treatment with PDBu (that results in PKC down-regulation) [30] produced a marginal sensitization to cisplatin only in A2780BBR-e cells at highly cytotoxic concentrations (10 μM), therefore suggesting that in PKCα-overexpressing cells a complete down-regulation of PKCα could not be achieved by treatment.

4. Discussion

The present study shows modulation of several transcripts, including MARCKS, NF-L, CAS, beta-glucoronidase, S19 ribosomal protein, and acetoacetyl CoA thiolase in cells resistant to platinum drugs. Among the modulated transcripts, we focused on the myristoylated alanine-rich C kinase substrate (MARCKS, Fig. 1A, B), because it is the major and ubiquitous substrate of PKC, and the PKC pathway is known to be implicated in modulation of several critical events including cell growth, cell cycle progression and apoptosis [27]. Transfection of a MARCKS-containing plasmid into A2780 cells did not result in a significant change in sensitivity to platinum drugs. This observation does not support a role of MARCKS itself in modulating drug sensitivity. On the basis of the substantial up-

regulation of the MARCKS protein in the drug-resistant subline, the down-regulation of PKCα was an unexpected finding (Fig. 1C). In contrast, we did not observe modulation of PKCδ (Fig. 1C), which has been associated with modulation of sensitivity to cisplatin [31–34].

PKCs are a family of isoenzymes differentially implicated in the transduction of signals for proliferation, differentiation and apoptosis, but the role of specific PKC isoforms in drug resistance remains to be defined [27,35]. PKC signal transduction pathway has been reported to play a role in modulating cell death mediated by cisplatin [34,36]. Some PKC members are substrates for caspase 3 activated during drug-induced apoptosis and proteolytic activation of PKC-δ has been implicated in cellular response to cisplatin [31–34]. In contrast, PKCα has been described as an antiapoptotic protein [28]. However, the development of resistance to BBR3464 and cross-resistance to cisplatin in A2780/BBR cells, characterized by down-regulation of PKCα, suggest that the PKCα pathway was not implicated in the resistance mechanisms. In addition, our results do not support a general protective function of PKCα, because transfection of A2780/BBR cells with PKCα resulted in an increased resistance only to cisplatin, as shown by increase in IC_{50} value as well as by decreased cleavage of caspase 3 (Table 3, Fig. 3B, C). In keeping with this finding is the observation that the PKCα inhibitor Gö6976 potentiated the effect of cytotoxic concentrations of cisplatin but not of BBR3464 in A2780 cells.

As already reported for other PKC isoforms [34], the function of PKCα could be dependent on the nature of cellular response which may be related to the extent and type of DNA damage. The controversial data reported on the role of PKCα could reflect the complexity of multiple functions. PKCα has been implicated as a prosurvival factor in response to cytotoxic injuries and therefore it is recognized as a potential determinant of drug resistance [35]. Since cisplatin and BBR3464 are known to induce different patterns of cellular response [37], it is possible that PKCα may have a different role in the various pathways activated by specific types of injury. An additional protective function of PKCα could be ascribed to functional activation of enzymes involved in mechanisms of cellular protections through xenobiotic metabolism. In particular, the activation of glutathione-*S*-transferase P1 (GST-P1) by PKC-dependent phosphorylation could contribute to cell protection resulting in tumor cell resistance to agents metabolized by GST (e.g., electrophilic compounds) [38]. A tentative explanation for the differential response of PKCα-transfected cells to cisplatin and to the multinuclear platinum complex could be a different GSH interaction of the two complexes, resulting in an increased inactivation of cisplatin. GST-P1 itself plays a role in stress response and signaling [39,40].

In conclusion, the development of resistance in the A2780/BBR subline and the cross-resistance to cisplatin, in spite of down-regulation of PKCα, support that the resistance mechanism is independent of PKCα function. The available results do not support a general protective role of PKCα in response to genotoxic stress. Its antiapoptotic function or its regulatory function on cellular response pathways likely depends on particular circumstances related to the nature of cytotoxic stress or to the biological context.

Acknowledgments

This work was partially supported by the Associazione Italiana Ricerca sul Cancro, Milan, by the Ministero della Salute, Rome and by the Ministero Istruzione, Università e Ricerca (FIRB Project), Rome, Italy. We are grateful to Dr. P.J. Blackshear and Dr. D.J. Stumpo (Research Triangle Park, NC, USA) and to Dr. P.J. Parker (London, UK) for providing the plasmids.

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