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Effects of a novel trinuclear platinum complex in cisplatin-sensitive and cisplatin-resistant human ovarian cancer cell lines: interference with cell cycle progression and induction of apoptosis

L. Orlandi [a], G. Colella [a], A. Bearzatto [a], G. Abolafio [a], C. Manzotti [b], M.G. Daidone [a], N. Zaffaroni [a,*]

[a] *Dipartimento di Oncologia Sperimentale, Unita' Operativa # 10, Istituto Nazionale per lo Studio e la Cura dei Tumori, 20133 Milan, Italy*
[b] *Novuspharma S.p.A., 20052, Monza, Milan, Italy*

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Abstract

We evaluated the effects of the trinuclear platinum complex, BBR 3464, in a human ovarian carcinoma cell line (OAW42) and in its cisplatin (CDDP)-resistant counterpart (OAW42MER). A 14-fold increased sensitivity to a 1-h BBR 3464 exposure was found in OAW42MER cells compared with their parental cell line. Flow cytometric experiments showed that BBR 3464 was able to induce a persistent block of OAW42 and OAW42MER cells in the G_2M phase, whereas CDDP caused an initial accumulation of cells in the S phase followed by an increase in the G_2M cell fraction in both cell lines. Exposure to equitoxic (IC_{50}) drug concentrations induced programmed cell death in both cell lines. However, the percentage of cells with an apoptotic nuclear morphology was slightly higher after CDDP than BBR 3464 treatment in OAW42 cells, whereas the opposite pattern was observed in OAW42MER cells. Degradation of the nuclear lamin B was detected in OAW42 cells after exposure to each drug. Conversely, in OAW42MER cells lamin B cleavage was only appreciable after BBR 3464 exposure. In OAW42 cells, CDDP and BBR 3464 did not appreciably affect the mitochondrial membrane potential ($\Delta\psi_{mt}$), whereas in the OAW42MER cell line a marked $\Delta\psi_{mt}$ reduction was observed after exposure to BBR 3464, but not to CDDP. The results of the study would suggest that the sensitivity to BBR 3464 observed in the CDDP-resistant OAW42MER cell line might be attributable to the ability of the trinuclear platinum complex to modify DNA in a way which is different from that of CDDP and, as a consequence, to induce different cellular responses to DNA damage such as the triggering of specific apoptotic pathways.

Keywords: Cisplatin; BBR 3464; Apoptosis; Cell cycle perturbation; Kinase activity; Ovarian cancer; Mitochondrial membrane potential

1. Introduction

Cisplatin (CDDP) is an effective drug used for the treatment of a wide spectrum of human solid tumours including ovarian, testicular, head and neck and non-small cell lung cancers [1,2]. However, its clinical therapeutic effect is often limited by intrinsic or acquired tumour cell resistance, which also affects the response to other mononuclear platinum analogues such as carboplatin as a consequence of the similarity in the structure and mechanisms of action of these compounds.

Multinuclear platinum complexes represent a new class of anticancer drugs that contain two reactive platinum centres stably linked by a variable length alkanediamine chain [3,4] and are characterised by a different DNA binding profile with respect to that of their mononuclear counterparts [5]. BBR 3464 (Fig. 1) has been identified as the most active member of this class of compounds. It is more potent than CDDP, and retains significant activity in human tumour cells lines [6] and xenografts [7] refractory or poorly responsive to CDDP.

The molecular mechanisms by which BBR 3464 is able to overcome CDDP resistance are largely unknown. However, its ability to induce peculiar platinum–DNA adducts (such as 'long-distance' intra- and inter-strand crosslinks [3,4]) which are not produced by conventional mononuclear platinum compounds suggests that BBR 3464 may escape, at least in part, the classical mechanisms of CDDP resistance related to DNA damage recognition and repair [8,9]. Moreover, due to its ability to modify DNA in a way which is different from that of CDDP, BBR 3464 could differently

* Corresponding author. Tel.: +39-2-2390-700; fax: +39-2-2364-366.

E-mail address: zaffaroni@istitutotumori.mi.it (N. Zaffaroni).

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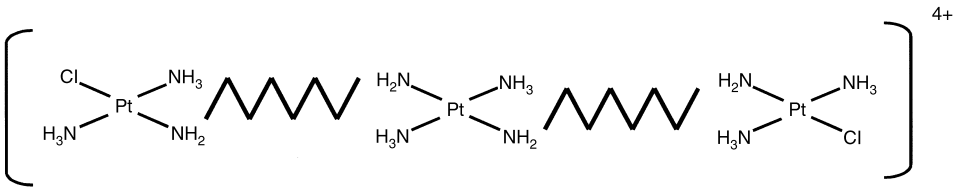

Fig. 1. Chemical structure of the multinuclear platinum complex BBR 3464.

evoke pathways of cellular response to DNA damage such as triggering of the apoptotic pathway, as a function of the genetic background of the tumour model. In fact, it has recently been demonstrated that unlike CDDP, which is generally less active against tumour models carrying a mutated *TP53* gene [10], BBR 3464 displays high activity in human tumour cell lines and xenografts characterised by mutant *TP53* [7], probably as a consequence of its ability to induce p53-independent programmed cell death.

In the present study, we evaluated the cytotoxic activity of BBR 3464 in two human ovarian carcinoma cell lines, one sensitive (OAW42) and one with experimentally induced resistance to melphalan and cross-resistance to CDDP (OAW42MER). Since the OAW42MER cell line proved to be more sensitive (14-fold) to BBR 3464 than the parental OAW42 cell line, experiments were carried out to verify whether the peculiar drug sensitivity profiles observed in our cellular models were attributable to a different effect exerted by CDDP and BBR 3464 on cell cycle progression or to their different ability to activate the apoptotic pathways.

2. Materials and methods

2.1. Cell lines

OAW42 is a cell line derived from the ascites of a patient with a papillary serous cystadenocarcinoma of the ovary. The melphalan-resistant OAW42MER subline was obtained by exposure of the OAW42 line to stepwise increasing concentrations of melphalan [11] and has been demonstrated to be cross-resistant to CDDP [12]. Both cell lines were kindly supplied by R.A. Britten (Department of Radiation Oncology, University of Liverpool, UK). The cell lines were maintained as monolayers at 37°C in a 5% CO_2 humidified atmosphere in air, using Dulbecco's Modified Eagle's Medium (DMEM)/F12 medium supplemented with 10% (v/v) fetal calf serum, 0.25 U/ml insulin (Sigma) and 0.1% (v/v) gentamycin. OAW42MER cells were maintained in medium containing 1 μg/ml melphalan and passaged for 2 weeks in melphalan-free medium before any experiment was carried out. Both cell lines are characterised by a wild-type *TP53* gene, as detected by single-strand conformation polymorphism (SSCP) and direct DNA sequencing analysis (data not shown).

2.2. Drugs

Cisplatin (CDDP) (Platamine, Pharmacia & Upjohn, Uppsala, Sweden) was dissolved in a 0.9% (w/v) NaCl solution. BBR 3464 was obtained from Novuspharma S.p.A. (Monza, Italy) as a NO_3^- salt and dissolved in saline before use.

2.3. Cell proliferation assay

After harvesting in the logarithmic growth phase, cells were seeded in six-well plates and treated with varying doses of BBR 3464 and CDDP for 1 h. The medium was then removed and the cells were rinsed in phosphate-buffered saline (PBS) and incubated in fresh medium for an additional 72 h at 37°C in a 5% CO_2 humidified atmosphere. The cells were then trypsinised and counted in a particle counter (Coulter Counter, Coulter Electronics, Luton, UK). The percentages of adherent viable cells were determined by the trypan blue dye exclusion test. The viability always exceeded 95%. Each experimental sample was run in triplicate. The results were expressed as the total number of adherent cells in treated samples compared with control samples. *In vitro* drug activity was expressed in terms of the concentration able to inhibit cell proliferation by 50% [IC_{50}].

2.4. Cell cycle analysis

At different intervals after drug treatment, samples of 1×10^6 of OAW42 and OAW42MER cells were fixed in 70% (v/v) ethanol. Before analysis, the cells were washed in PBS and stained with solution A containing 50 μg/ml propidium iodide, 50 mg/ml RNAse, and 0.05% (v/v) Nonidet P40 (NP40) for 30 min at 4°C and then analysed with a flourescence activated cell sorter (FACScan) can flow cytometer (Becton Dickinson, Sunnyvale, CA). For each sample, 30 000 events were collected and stored in listmode (Lysis II software, Becton Dickinson). A peak width versus area cytogram was used to discriminate and gate out doublets and debris from the analysis (Doublet Discrimination Mode, Becton Dickinson). The percentages of cells in the $G_{0/1}$, S and G_2M phases were evaluated on DNA plots by CellFit software according to the sum of broadened rectangles (SOBR) model (Becton Dickinson).

2.5. Mitochondrial membrane potential ($\Delta\psi_{mt}$)

Alterations in the $\Delta\psi_{mt}$ were studied by flow cytometry using the $\Delta\psi_{mt}$-sensitive dye JC-1 (5,5′ 6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (Molecular Probes, Inc., Eugene, OR, USA) [13]. In brief, 48 h after a 1 h exposure to CDDP or BBR 3464, OAW42 and OAW42MER cells were harvested, washed once with PBS, resuspended in complete medium and incubated with 10 μg/ml JC-1 at 37° C for 15 min in the dark. Stained cells were then washed once in PBS and analysed by flow cytometry. A FACScan flow cytometer (Becton Dickinson) was used to analyse a minimum of 30 000 cells per sample. Data were acquired in list mode and evaluated using Lysis II software. Forward and side scatter were used to gate the viable population of cells. JC-1 monomers emit at 527 nm (FL-1 channel), and J-aggregates emit at 590 nm (FL-2 channel). Duplicate samples of control cells were used for compensation (FL-1–FL-2), and flow cytometric profiles from these cells defined the 590 nm cut-off for drug-treated samples.

2.6. Cell lysis and immunoblotting

Cells were lysed as previously described [14]. In brief, total cellular lysate (75 μg) was separated on a 10–12% (w/v) sodium dodecyl sulphate (SDS)-polyacrylamide gel and transferred to nitrocellulose. The filters were blocked in PBS with 5% (w/v) skim milk and then incubated overnight with the primary antibody anti-p53, anti-Bax, anti-Bcl-2, anti-cyclin B_1, anti-cyclin dependent kinase 1 (cdk1), anti-lamin-B (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-$p21^{waf1}$ (Oncogene Science, Cambridge, MA). The filters were then incubated with the secondary antimouse or anti-rabbit Ig horseradish peroxidase-linked whole antibody (Amersham Pharmacia Biotech, Uppsala, Sweden). Bound antibody was detected using the enhanced chemoluminescence western blotting detection system (Amersham). An antitubulin α monoclonal antibody (NeoMarkers, Fremont, CA, USA) was used on each blot to ensure equal loading of protein on the gel. All the primary antibodies were used at a binal concentration of 1 μg/ml and the second antibodies were diluted 1:2500 in PBS with 5% w/v of bovine serum albumin (BSA).

2.7. Immunoprecipitation and assay of cyclin B_1-associated cdk1 kinase activity

Cells (1×10^6) were washed once with ice-cold PBS and lysed on ice in lysis buffer for kinase assay (1% (v/v) NP40 prepared in PBS containing 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) and 1% (w/v) BSA). Cell lysates were clarified (30 min, 20 600*g*) and 0.5 ml of lysate from 1×10^6 cells was mixed with 5 μg mouse monoclonal anti-cyclin B_1 (Santa Cruz Biotechnology) in the presence of 100 μl of a 20% (v/v) protein A-sepharose slurry (Amersham) followed by rotation for 4 h at 4°C. The immune complexes were then washed twice with lysis buffer for kinase assay and then twice in the same buffer minus BSA. The cyclin B1 immunoprecipitates were incubated with 3 μg of histone H1 (Boehringer Mannheim, Mannheim, Germany) in 20 μl of kinase buffer containing 20 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 5 μM cold adenosine 5′-triphosphate (ATP) and 10 μCi of [γ^{32}P]ATP for 20 min at 30°C. The reaction was terminated by adding an equal volume of 2×SDS sample loading buffer. The mixture was then boiled for 5 min before loading onto a 12% (w/v) SDS-polyacrylamide gel. Following autoradiography, reactions were quantified by densitometry. Kinase activities of control and treated samples were normalised on the number of cells in G_2M and expressed in arbitrary densitometric units.

2.8. Evaluation of apoptotic morphology by fluorescence microscopy

Cells were harvested at different intervals after drug treatment; floating and adherent cells were collected separately, washed in PBS and stained with solution A. After staining, the slides were observed by fluorescence microscopy. The percentage of apoptotic cells was determined by scoring at least 200 cells in each sample.

2.9. DNA agarose gel electrophoresis

Adherent and floating cells (3×10^6) were lysed as previously described [15]. Briefly, the supernatant (low molecular weight DNA) was separated from the pellet (high molecular weight DNA) and was digested with RNAse A (500 U/ml) for 1 h at 37°C and with 1% w/v SDS detergent containing 0.5 mg/ml proteinase K for 3 h at 50°C. The samples were extracted once with phenol and once with phenol chloroform isoamyl alcohol (25:24:1, v/v/v), and precipitated with 2.5 volumes of ethanol and 0.1 volume of sodium acetate (3 M, pH 5.2). The DNA was then electrophoresed in 1.5% (w/v) agarose gel containing ethidium bromide and visualised under ultraviolet (UV) light.

2.10. Statistics

The Student *t*-test was used to compare the percentages of cells present in the different cycle phases in drug-treated and control samples. A P value of <0.05 was considered significant.

Table 1
Cell cycle perturbation induced by CDDP and BBR 3464[a]

	Time[b]								
	24 h			48 h			72 h		
	$G_{0/1}$	S	G_2M	$G_{0/1}$	S	G_2M	$G_{0/1}$	S	G_2M
OAW42 cells									
Control	63±5	24±5	13±1	62±5	25±	13±1	66±7	21±7	13±1
BBR 346[c]	37±6	18±7	45±5[d]	44±8	11±1	45±8[d]	51±8	10±1	38±9[d]
CDDP[d]	33±2	52±8[d]	15±9	21±3	22±7	57±6[d]	45±3	9±2	46±6[d]
OAW42MER cells									
Control	50±7	33±5	17±2	53±5	34±4	13±1	58±5	29±4	13±1
BBR 3464[c]	29±3	29±3	42±5[d]	40±3	22±2	38±1[d]	40±1	34±3	26±2
CDDP[c]	17±3	56±6[d]	27±5	35±4	29±1	36±3[d]	48±1	35±1	17±1

[a] Data represent mean values±standard deviation (S.D.) of three independent experiments.
[b] Calculated from the end of a 1 h treatment.
[c] Specific IC_{50} concentration.
[d] $P < 0.05$, Student's *t*-test, compared with controls.

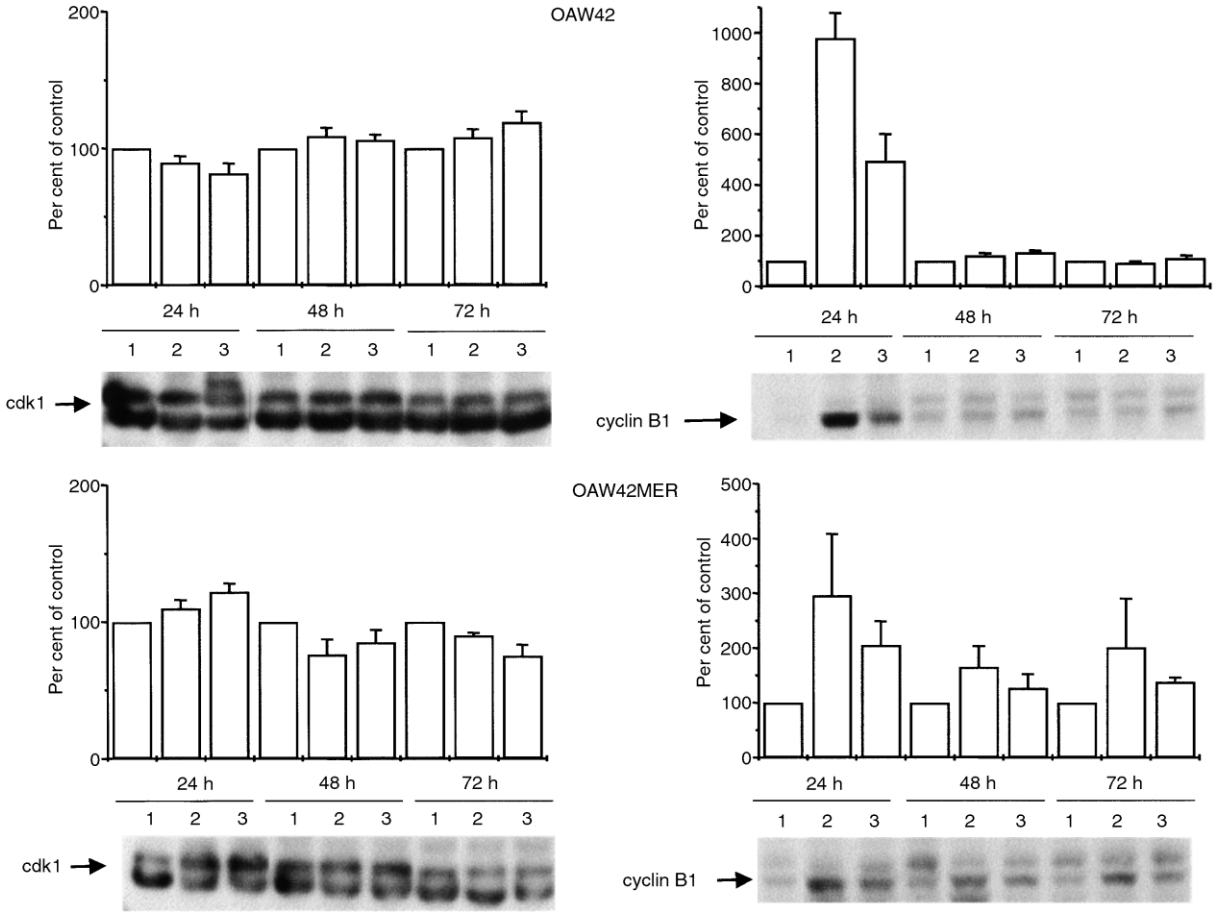

Fig. 2. Effect of BBR 3464 and CDDP on the expression of proteins involved in the control of G_2 checkpoint in OAW42 and OAW42MER cells. Cells were incubated with solvent (control, lane 1) or with the IC_{50} concentration of BBR 3464 (lane 2) or CDDP (lane 3) for 1 h. At the end of treatment, the cells were incubated for an additional 24, 48 and 72 h in drug-free medium. Western blots were probed with antibodies for cyclin dependent kinase 1 (cdk1) and cyclin B1. The densitometric values of band intensities are indicated above the corresponding blots and represent the mean values±standard deviation (S.D.) of three independent experiments.

3. Results

The sensitivity to a 1 h exposure to CDDP or BBR 3464 was markedly different for the OAW42 and OAW42MER cells. In fact, whereas the OAW42MER cell line was 10-fold more resistant to CDDP than the parental OAW42 cell line (IC_{50} values, 25 ± 5.6 versus 2.5 ± 0.84 μg/ml), it showed a remarkable sensitivity to BBR 3464 (IC_{50} values, 0.45 ± 0.18 versus 6.5 ± 1.6 μg/ml).

DNA flow cytometric analysis was performed to determine whether cell cycle perturbations could be responsible for the different drug sensitivity pattern observed in the two cell lines (Table 1). In OAW42 cells, CDDP induced a transient accumulation of cells in the S phase, perceptible 24 h after the end of treatment, which was followed by a stable increase in the G_2M cell fraction, still present after 72 h. After exposure to the trinuclear platinum complex BBR 3464, OAW42 cells were stably blocked in the G_2M compartment until 72 h. In OAW42MER cells, exposure to CDDP induced an accumulation of cells in the S phase at 24 h followed by an increase in the G_2M cell fraction, which was almost completely resolved at 72 h. In this cell line, BBR 3464 caused a persistent accumulation of cells in the G_2M phase, although less pronounced than that observed in OAW42 cells.

Since CDDP and BBR 3464 mainly induced alterations in cell progression throughout the G_2M phase, the effect of drug treatment on the expression of cdk1 and cyclin B1 proteins, key regulators of the G_2 checkpoint, was determined (Fig. 2). Only differences in band intensities greater than 25% with respect to control were considered as significant.

Based on such criteria, in OAW42 and OAW42MER cells, both drugs induced a marked increase in cyclin B1 protein expression that was highest 24 h after treatment. The accumulation of cyclin B1 protein was still present, although at lower levels, 48 and 72 h after treatment in the OAW42MER cells, whereas in the OAW42 cells, the level of protein expression in the treated cells was similar to that of controls at such time points. As regards cdk1, when the overall quantification of the faster and slower migration bands was performed, no appreciable difference with respect to controls was found in treated samples of either cell line. When the ratio of the upper band (representing the phosphorylated/inactive form of cdk1) to the lower band (representing the dephosphorylated/active form of cdk1) was considered, no major differences in the treated samples compared with controls were evidenced in the OAW42 cells. In contrast in OAW42MER cells, an increase in the upper inactive form of cdk1 was evident 24 h after treatment with BBR 3464, and 24 and 72 h after exposure to CDDP.

The kinase activity of cyclin B1-associated cdk1 on the substrate histone H1 was also measured after drug treatment (Fig. 3). When the results were expressed in terms of the value for the kinase activity divided by the number of G_2M cells (as detected by flow cytometry), we found that OAW42 cells accumulating in G_2M phase after CDDP exposure showed a cdk1 catalytic activity consistently lower than that of control cells at all time points considered. In OAW42 cells exposed to BBR 3464, an unexpected and marked increase in cdk1 kinase activity was observed 24 h after treatment, then the enzyme catalytic activity dropped to values lower than those observed in control cells. As regards the OAW42-MER cell line, CDDP and BBR 3464-treated cells were characterised by levels of cdk1 kinase activity generally comparable to those of controls until 48 h after treatment, whereas a marked increase in enzyme catalytic

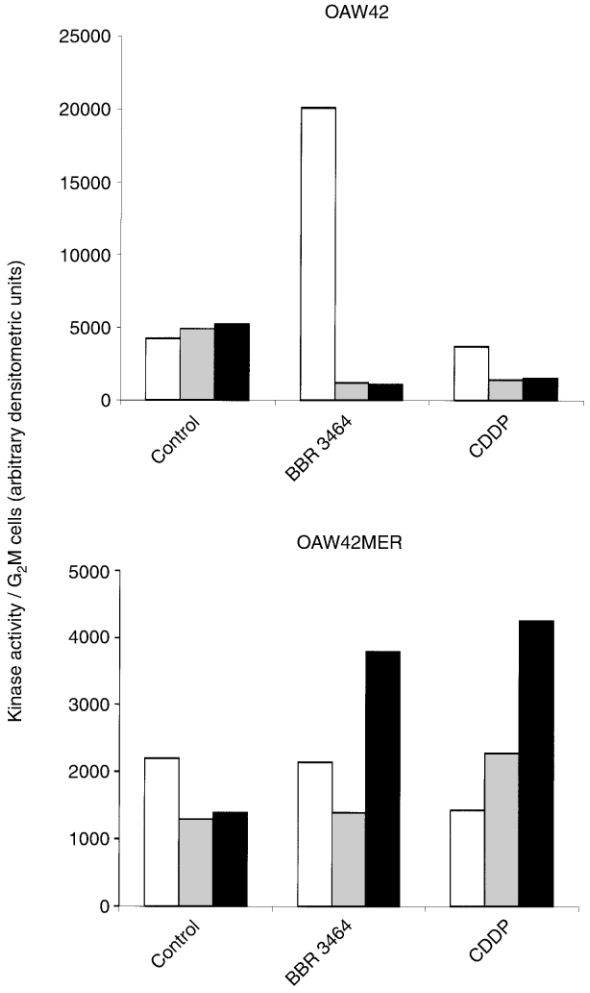

Fig. 3. Effect of BBR 3464 and CDDP on cyclin B1/cdk1 kinase activity in OAW42 and OAW42MER cells. Cells were incubated with solvent or with the IC_{50} concentration of BBR 3464 or CDDP for 1 h. At the end of the treatment, cells were incubated for an additional 24, 48 and 72 h in drug-free medium. Immunoprecipitation and kinase assays were performed. Following autoradiography, reactions were quantified by densitometry; for each sample, the results obtained at 24 h (□), 48 h (■) and 72 h (■) were expressed in terms of kinase activity (arbitrary densitometric units) divided by the number of cells in the G_2M phase as assessed by flow cytometry.

activity was observed at 72 h in cells exposed to either drug (Fig. 3).

The ability of CDDP and BBR 3464 to induce apoptosis in the two cell lines was determined by considering different endpoints. Fig. 4 shows the percentage of cells with an apoptotic morphology, determined by fluorescence microscopy after staining of cells with propidium iodide and calculated on the total cell population. Spontaneous apoptosis was observed in a negligible fraction ($<0.1\%$) of OAW42 control cells. Drug treatment induced a time-dependent increase in the percentage of OAW42 cells with an apoptotic morphology. This effect was slightly more pronounced for CDDP (14–18%) than for BBR 3464 (3–12%). In untreated OAW42MER cells spontaneous apoptosis was seen in approximately 1% of the overall cell population. This percentage increased after exposure of cells to CDDP (7–9%) or BBR 3464 (8–14%). Gel electrophoresis analysis of DNA obtained from OAW42MER cells showed the accumulation of oligonucleosome fragments 48 and 72 h after treatment with CDDP or BBR 3464 (Fig. 5). Similar results were obtained in OAW42 cells (data not shown).

Degradation of nuclear lamin B, as demonstrated by the appearance of a 45 kD cleavage product and a concomitant decrease in the 66 kD full-length protein level, was detected in the OAW42 cells after exposure to CDDP or BBR 3464 (Fig. 6). Conversely, in the OAW42MER cells, the 45 kD cleavage product was only detectable after BBR 3464 treatment (Fig. 6).

Since the disruption of mitochondrial membrane potential ($\Delta\psi_{mt}$) has been linked to the induction of

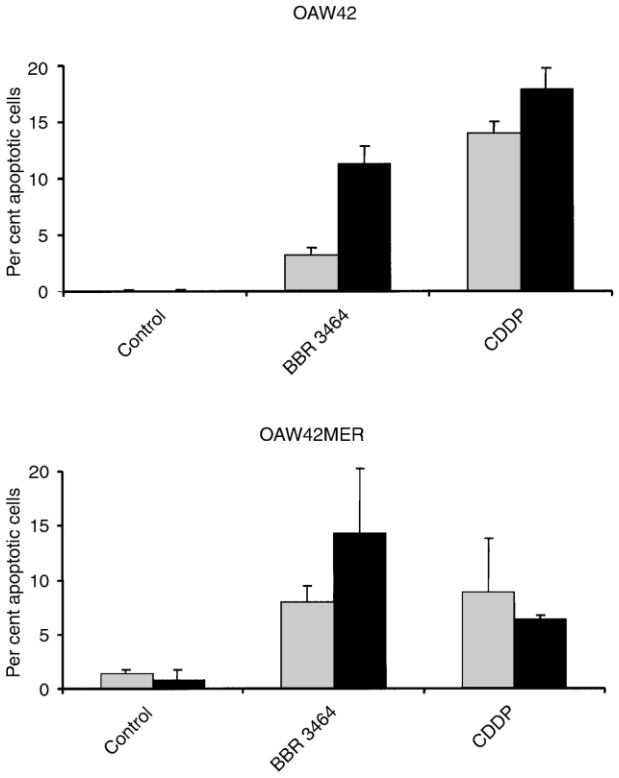

Fig. 4. Induction of apoptosis by BBR 3464 and CDDP in OAW42 and OAW42MER cells. After a 1 h treatment with solvent (control) or with the IC_{50} concentration of BBR 3464 or CDDP, samples were incubated in drug-free medium and harvested after 48 h (▒) and 72 h (■). The cells were then stained with propidium iodide and the slides were examined by fluorescence microscopy. The percentage of apoptotic cells with respect to the total cell number was determined by scoring at least 200 cells in each sample. Data are means±standard deviation (S.D.) of three independent experiments.

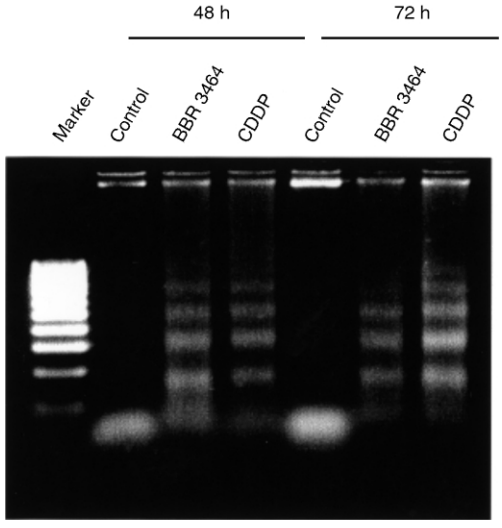

Fig. 5. Electrophoretic pattern of DNA extracted from OAW42MER floating cells. After a 1 h treatment with solvent (control) or with the IC_{50} concentration of BBR 3464 or CDDP, cells were incubated in drug-free medium and harvested after 48 and 72 h.

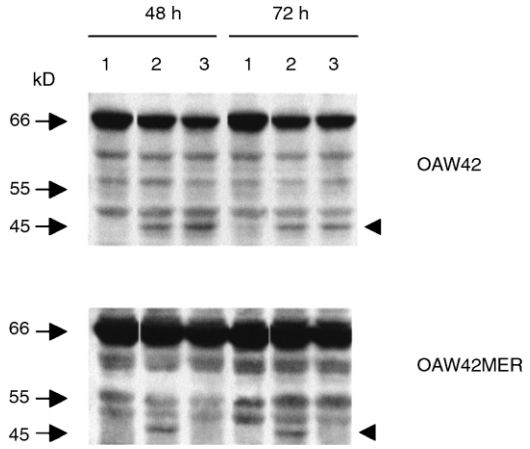

Fig. 6. Degradation of lamin B induced by BBR 3464 or CDDP in OAW42 and OAW42MER cells. After a 1 h treatment with the IC_{50} concentration of BBR 3464 or CDDP, cells were incubated in drug-free medium and harvested after 48 and 72 h and western blotting was performed. The numbers on the left correspond to the migration position of the protein markers. Lamin B-specific cleavage product bands are indicated on the right. Lanes are as described in Fig. 2. The blot of OAW42MER cells has been overexposed to evidence the cleavage product.

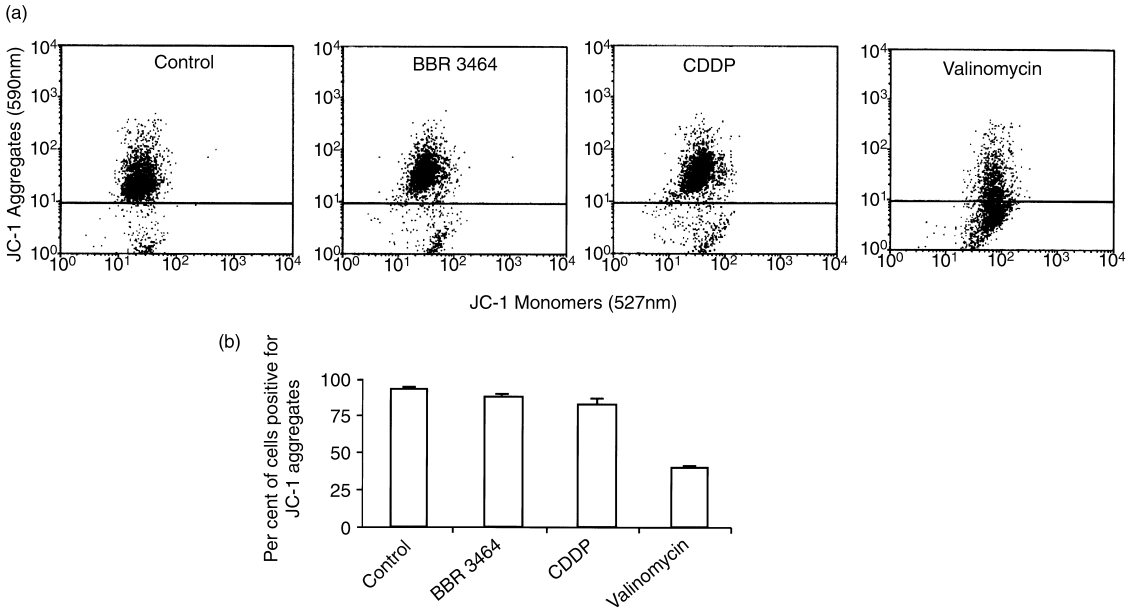

Fig. 7. Effect of BBR 3464 and CDDP on the $\Delta\psi_{mt}$ in OAW42 cells. After a 1 h treatment with IC_{50} concentration of BBR 3464 or CDDP, cells were incubated in drug-free medium, stained with JC-1 and analysed by flow cytometry. Cells treated for 30 min at 37°C prior to JC-1 addition with 5 μM valinomycin were used as positive control for $\Delta\psi_{mt}$ disruption. (a) Representative examples of the fluorescence pattern of cells incubated with the solvent (control) or with BBR 3464 or CDDP; (b) percentage of cells staining positive for J-aggregate formation (emitting at 590 nm) in untreated (control) and in BBR 3464- or CDDP-treated cell populations. Data are means ± standard deviation (S.D.) of three independent experiments.

apoptosis by different stimuli, we investigated whether the $\Delta\psi_{mt}$ was altered by CDDP or BBR 3464 treatment in the two cell lines. For this purpose, control and drug-treated cells were stained with the $\Delta\psi_{mt}$-sensitive dye JC-1. At relatively high $\Delta\psi_{mt}$, the dye forms J-aggregates, which emit at 590 nm in the orange range of visible light. Conversely, in the absence of or at low $\Delta\psi_{mt}$, JC-1 exists as a monomer, remaining in the cell, but emitting at 527 nm in the green range. Representative flow cytometric data, in which fluorescence at 590 nm is plotted against fluorescence at 527 nm, are shown in Figs. 7 and 8. In untreated cells, JC-1 exists predominantly in a highly aggregated form indicated by intense fluorescence emission at 590 nm. Conversely, in

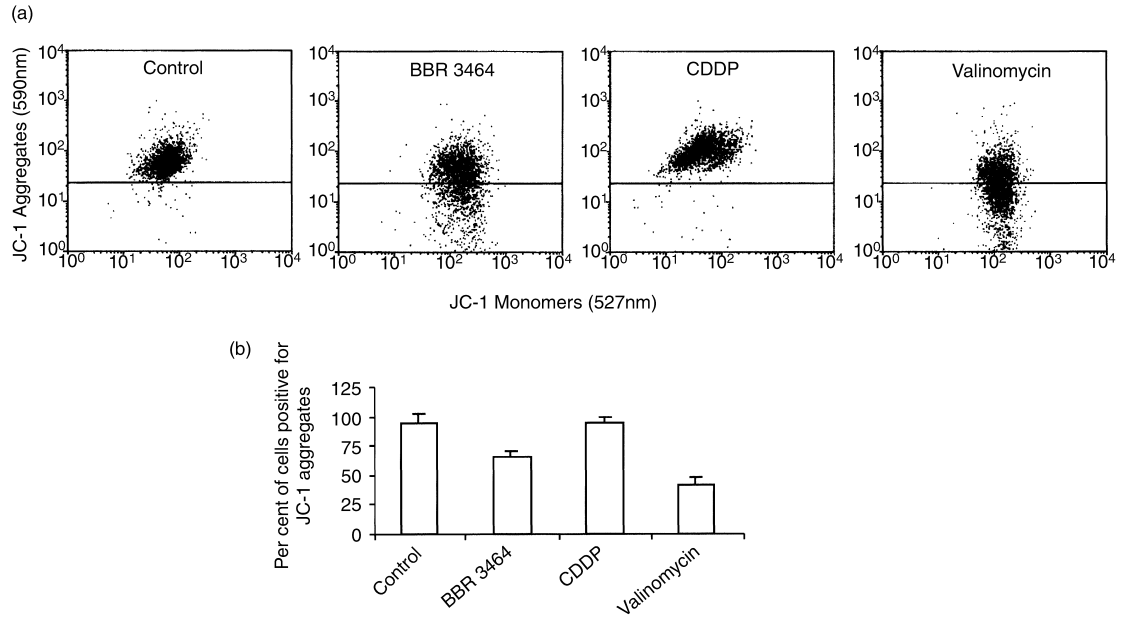

Fig. 8. Effect of BBR 3464 and CDDP on the $\Delta\psi_{mt}$ in OAW42MER cells. (a) Representative examples of the fluorescence pattern of cells incubated with the solvent (control) or with BBR 3464 or CDDP; (b) percentage of cells staining positive for J-aggregate formation (emitting at 590 nm) in untreated (control) and BBR 3464 or CDDP-treated cell populations. Data are means ± standard deviation (S.D.) of three independent experiments.

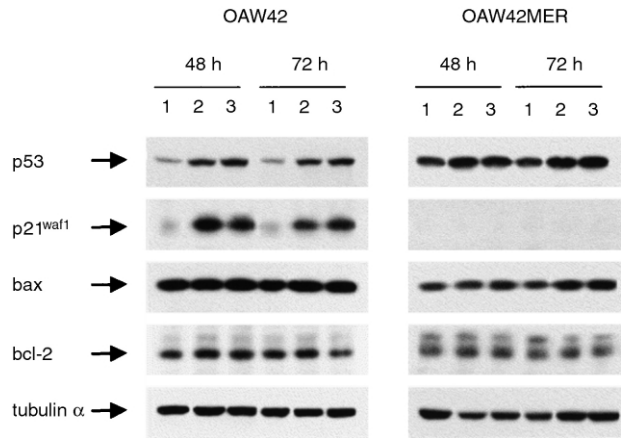

Fig. 9. Effect of BBR 3464 and CDDP on the expression of proteins involved in the control of apoptosis in OAW42 and OAW42MER cells. Cells were incubated with solvent (control) or with the IC_{50} concentration of CDDP or BBR 3464 for 1 h. At the end of the treatment, the cells were incubated for an additional 48 and 72 h in drug-free medium. Western blots were probed with antibodies for p53, bcl-2, bax, and $p21^{waf1}$. Antitubulin α monoclonal antibody was used to ensure equal loading of protein on the gel. Lanes are as described in Fig. 2.

cells exposed to valinomycin, a K+ ionophore that uncouples oxidative phosphorylation, JC-1 was largely present in its monomeric state, thus indicating a low $\Delta\psi_{mt}$. Following drug treatment, CDDP and BBR 3464 induced negligible decreases in $\Delta\psi_{mt}$ of OAW42 cells (Fig. 7b). Conversely, in OAW42MER cells, CDDP had almost no effect on the $\Delta\psi_{mt}$, whereas after treatment of cells with BBR 3464 the $\Delta\psi_{mt}$ was markedly diminished, as demonstrated by the 29% reduction of cells positive for J-aggregates compared with controls (Fig. 8b).

The expression of proteins involved in the control of programmed cell death was then assessed after drug exposure in the two cell lines (Fig. 9). In the OAW42 cells, an increase in p53 protein expression and a consequent transactivation of $p21^{waf1}$ were observed after exposure to CDDP or BBR 3464. In the OAW42-MER cell line p53 expression was also slightly increased after drug treatment, whereas the $p21^{waf1}$ signal remained undetectable. Moreover, drug treatment did not significantly modify bax expression in the two cell lines. A slight reduction in bcl-2 protein levels was perceptible 72 h after CDDP exposure in the OAW42 cell line.

4. Discussion

BBR 3464 is the leading compound of a new class of anticancer agents that was rationally designed to bind to DNA in a different way from that of its mononuclear counterparts [3,4]. In the present study we evaluated the cytotoxic activity of BBR 3464 in two human ovarian carcinoma cell lines (one sensitive and one with experimentally induced resistance to melphalan and cross-resistance to CDDP [12]) and showed that the resistant OAW42MER cell line displayed a marked sensitivity to BBR 3464 (i.e. it was approximately 14 times more sensitive to the drug than the parental OAW42 cell line). This finding is in agreement with results recently obtained by Pratesi and colleagues [7], who documented an important antitumour activity of BBR 3464 in human tumour xenografts refractory or poorly responsive to CDDP.

In OAW42MER cells, resistance to CDDP appears to be related to defects in the recognition of DNA damage, as suggested by the absence of the mismatch repair proteins hMLH1 and hPMS2 [35], as well as to an enhanced nucleotide excision repair pathway, as indicated by the markedly higher *ERCC1* and *ERCC2* mRNA levels present in this cell line compared with those observed in the parental CDDP-sensitive OAW42 cells [12]. Since alterations in DNA repair systems do not negatively affect the sensitivity of OAW42MER cells to BBR 3464, it may be hypothesised that DNA lesions induced by the trinuclear platinum complex are differently recognised and repaired by the cells with respect to those caused by CDDP. As a consequence, the two drugs could induce different cellular responses to DNA damage in the same tumour model. In this context, we decided to comparatively evaluate in our ovarian cancer cell lines the effects of CDDP and BBR 3464 in terms of interference with cell cycle progression and the induction of apoptosis.

As regards the drug effects on OAW42, cell cycle progression, a 1 h treatment with the IC_{50} CDDP concentration induced an early increase in the S-phase fraction that was followed by accumulation of cells in the G_2M compartment, whereas BBR 3464 [IC_{50}] caused a persistent arrest of cells in the G_2M phase. Similar, although less pronounced perturbations, were observed in OAW42MER cells after drug treatment.

Since the cyclin B1-cdk1 complex is the key regulator of the G_2 to M transition [16], we next measured the effect of drug treatment on the cyclin B1-associated cdk1 kinase activity using histone H1 as a substrate. When kinase activity was expressed as a function of the number of cells in the G_2M compartment (i.e. the cells that, together with late S-phase cells, mainly account for this specific kinase activity), we found that OAW42 cells accumulating in the G_2M phase after exposure to CDDP or BBR 3464 generally showed a reduced ability to phosphorylate histone H1 with respect to untreated control cells. This observation is in agreement with previous findings obtained after CDDP treatment in other experimental tumour models [17] . The only exception was a marked increase in cyclin B1-associated kinase activity found in cells accumulated in the G_2M phase 24 h after treatment with BBR 3464. This increase could

be sustained by the high level of cyclin B1 induced by treatment at this time point, which could allow increased formation of the cyclin B1-cdk1 active complex.

In OAW42MER cells accumulating in G_2M after exposure to CDDP or BBR 3464, the cyclin B1-associated kinase activities were similar to those of the control cells until 48 h after treatment. The inability of the treated cells to escape the G_2 block could be explained by assuming that the active cyclin B1-cdk 1 complexes are confined to the cytoplasmic compartment. In fact, it has previously been demonstrated that exclusion of cdk1 kinase activity from the nucleus may contribute to the cell cycle delay occurring after irradiation in HeLa-S1 cells [18]. An increased kinase activity was observed at later timepoints (72 h) after CDDP or BBR 3464 treatment in correspondence with the resolution of the G_2M blocks.

Since apoptosis is a major mode of cell death induced by several DNA damaging agents [19], we evaluated the induction of apoptosis after exposure of OAW42 and OAW42MER cells to CDDP or BBR 3464. Fluorescence microscopy analysis indicated the presence of cells with an apoptotic nuclear morphology in both cell lines after treatment with either drug. The occurrence of apoptosis was also confirmed by the presence of DNA fragmentation in both cell lines. Although the percentage of apoptotic cells was generally modest, there was a trend towards a correlation between cell sensitivity to a specific drug and apoptotic response. Specifically, the parental OAW42 cells (which are sensitive to CDDP) showed a slightly higher percentage of apoptotic cells after treatment with CDDP than BBR 3464, whereas in OAW42MER cells (which are sensitive to BBR 3464) an inverse pattern was observed.

At the molecular level, we investigated the effects of drug treatment on the expression of proteins involved in the control of apoptosis such as p53 [20] and some of the major downstream genes controlled by p53 including *p21*^{waf1}, *bax* and *bcl-2* [21]. In the OAW42 cell line, exposure to CDDP or BBR 3464 caused a marked increase in p53 expression and a consequent transactivation of p21^{waf1}. Moreover, CDDP-induced down-regulation of bcl-2 was found in concomitance with the peak of apoptosis induction. Conversely, in the OAW42MER cells, CDDP and BBR 3464 induced a very slight increase in p53 protein expression, whereas p21^{waf1} was undetectable. Moreover, no variation in the levels of bax and bcl-2 expression was observed after drug treatment.

Our findings indicate that the presence of a wild-type and functional p53 protein that allows cells to undergo p53-dependent apoptosis is an important determinant of tumour cell sensitivity to CDDP, as already demonstrated in experimental and clinical studies [22–24]. Conversely, the presence of an apparently non-functional p53 protein, as indicated by the lack of p21^{waf1} induction in OAW42MER cells, is not detrimental for the susceptibility of cells to BBR 3464. This observation is in agreement with previous findings of Pratesi and colleagues [7], who demonstrated superior activity of the trinuclear platinum complex against *TP53*-mutant human tumour xenografts compared with those carrying the wild-type gene. These authors also showed that the transfer of functional p53 in a *TP53*-null human osteosarcoma cell line resulted in a marked reduction of cellular sensitivity to BBR 3464. The efficacy of the trinuclear platinum complex in tumour cells with mutant or non-functional p53 protein suggests that the drug is able to induce a p53-independent response through the induction of specific DNA lesions. However, since in OAW42MER cells the apoptotic response to BBR 3464 is quite modest, the efficacy of the trinuclear platinum complex could also be due to the induction of a persistent cytostatic effect resulting from the inability of cells to recognise and repair drug-induced DNA lesions.

There is increasing evidence for a major role of the mitochondria in the apoptotic process. In fact, it has been demonstrated that apoptosis-inducing stimuli can trigger uncoupling of electron transport from ATP production, leading to a decrease in the $\Delta\psi_{mt}$ and a corresponding production of reactive oxygen species that are responsible for the oxidative degradation of mitochondrial components [25]. The occurrence of mitochondrial changes during the apoptotic process induced by CDDP in sensitive HeLa cells has recently been reported [26]. Moreover, a more general role of mitochondrial damage in determining the cytotoxic activity of this compound has previously been proposed by Zhang and colleagues [27], who reported that the toxic activity of CDDP in renal cortical slices was related to a rapid loss of mitochondrial protein-SH followed by a substantial decrease in Ca2+ uptake and a decline in mitochondrial membrane potential. We found that in the OAW42 cells, CDDP and BBR 3464 did not appreciably affect the $\Delta\psi_{mt}$. Conversely, in the OAW42MER cells, a marked decrease in $\Delta\psi_{mt}$ was evident after exposure to BBR 3464 only, which indicates the impairment of mitochondrial membrane function as a possible determinant of the chemosensitivity to the trinuclear platinum complex observed in this cell line.

Disruption of mitochondrial membrane function also leads to the release of protease activators [28]. In particular, the release of cytochrome C from the mitochondria into the cytoplasm may play a central role in the activation of the executioner phase of apoptosis through its participation in the proteolytic activation of caspases [29]. To investigate whether different executioners of apoptosis are involved in the pathways of programmed cell death induced by the two drugs in the same cellular models, we assessed the expression of lamin B, a well-known caspase substrate, in drug-treated cells. In fact,

proteolysis of lamin A and B has been reported to occur during apoptosis induced by different stimuli [30–32] in various cell lines. Cleavage of lamin B was clearly detected in the OAW42 cells after treatment with BBR 3464 or CDDP. Conversely, in the OAW42MER cells it was only observed after exposure to the trinuclear platinum complex. Since nuclear lamin disassembly and proteolysis involves hyperphosphorylation of lamins by lamin kinases and cleavage by caspases [33,34], it may be hypothesised that in the different cellular settings the two drugs may differently activate the lamin pathway leading to apoptosis.

In conclusion, the results of our study would suggest BBR 3464 to be a promising drug for the treatment of human tumours resistant to CDDP as a consequence of alterations in the DNA repair systems or the inactivation of p53 function. Moreover, the evidence that BBR 3464 is able to induce apoptotic pathways which are different from those caused by CDDP in the same tumour models suggests that multinuclear platinum complexes represent an entirely new class of DNA binding agents rather than simple CDDP analogues.

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