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The novel trinuclear platinum complex BBR3464 induces a cellular response different from cisplatin

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Abstract

BBR3464 is a new platinum-based drug non cross-resistant with cisplatin. To characterise the cellular basis of BBR3464 cytotoxicity as opposed to cisplatin, we performed a comparative study of the two drugs in cisplatin-resistant neuroblastoma and astrocytoma cells. In both model systems, BBR3464 proved to be more potent than cisplatin and was able to overcome cisplatin resistance. The higher potency exhibited by BBR3464 correlated with an increased cellular platinum accumulation and DNA–adduct formation. At equitoxic doses, BBR3464 induced apoptosis to a lesser extent than cisplatin and failed to overcome the decreased susceptibility to cisplatin-induced apoptosis in cisplatin-resistant cells. Cell cycle analysis showed a dose-dependent G_2/M arrest by BBR3464. In astrocytoma cells, cisplatin treatment resulted in the upregulation of p53, p21 and bax, while only p21 induction was observed after BBR3464 treatment. In cisplatin-resistant cells, the reduced sensitivity to cisplatin paralleled a resistance to the induction of p53/p21 pathway by cisplatin, while the same doses of BBR3464 induced p21 to a similar extent in the resistant cells as in the parental cells. In conclusion, BBR3464 induces a cellular response that is different from cisplatin, supporting the view that the two drugs act through different mechanisms. Our data indicate that BBR3464 may be a promising agent in the treatment of tumours unresponsive to cisplatin and with a non-functional p53.

Keywords: Cisplatin; BBR3464; Cisplatin resistance; Apoptosis; p53; p21; Cell cycle

1. Introduction

Cisplatin is widely used for the treatment of a number of solid tumours. Although some response is initially observed in previously untreated patients relapse frequently occurs, and subsequent salvage therapy is largely ineffective. Therefore, the identification of new platinum-based drugs with a broader range of antitumour activity has been attempted in order to circumvent intrinsic or acquired cisplatin resistance.

Among the new platinum compounds, the family of polynuclear platinum complexes appears to be one of the most promising. A systematic evaluation of these bifunctional DNA-binding agents indicated BBR3464 (Fig. 1) as a drug non-cross resistant with cisplatin in a panel of human tumour xenografts [1] and *in vitro* selected cisplatin-resistant cell lines, including leukaemia and osteosarcoma cells [2]. The lack of cross-resistance of BBR3464 with cisplatin has been related to different types of drug-induced DNA lesions rather than to BBR3464's capability of overcoming specific cellular alterations [3,4]. A detailed biochemical and biophysical analysis of DNA interaction with BBR3464 has shown that an altered DNA-binding mode of BBR3464 in comparison with that of cisplatin may be responsible for its distinct and unique profile of antitumour activity [5].

An important feature of BBR3464 is its high effectiveness in human tumour xenografts characterised by a mutant p53 [1]. p53 inactivation is found in over 50% of all human cancers, with *TP53* gene mutations being the prevalent mechanisms [6]. Therefore, the potential activity of this new agent in the treatment of cisplatin-resistant or p53-mutated tumours makes BBR3464 a valid candidate for clinical evaluation. Indeed, phase I clinical trials of BBR3464 have finished [7] and the compound has entered phase II studies.

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This study was undertaken to examine the cellular and molecular mechanisms underlying BBR3464 antitumour activity in two paired cell lines with acquired cisplatin resistance, one derived from neuroblastoma, the other from astrocytoma. In both model systems, we evaluated cisplatin and BBR3464 cytotoxicity, cellular platinum accumulation and DNA–adduct formation. In addition, the effects of both drugs on apoptosis induction, cell cycle arrest and p53 protein level was examined. For further insight into the interacting molecular mechanisms, $p21^{Waf1/Cip1}$ and bax expression was also investigated.

2. Materials and methods

2.1. Drugs

Cisplatin, prepared for clinical use, was purchased from Bristol-Myers Squibb (Milan, Italy). BBR3464 was dissolved in 0.9% NaCl before use.

2.2. Cell lines and culture conditions

The U87-MG astrocytoma cell line was purchased from American Type Culture Collection (Rockville, MD), while the BE(2)-M17 neuroblastoma cell line was a gift from Dr. J. Biedler (Memorial Sloan-Kettering Cancer Center, New York). The cisplatin-resistant variants, referred to as U87-MG/Pt and BE(2)-M17/Pt, were generated by continuous exposure of the parental cell lines to stepwise increasing cisplatin concentrations and maintained in a medium always containing 0.5 and 0.8 μM cisplatin, respectively. These cells displayed a slightly increased proliferation rate and a resistance index (RI) of approximately 5–6. All cell lines were grown in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal calf serum (FCS) (Biowhittaker, Walkersville, MD). Experiments were performed on cells grown in a cisplatin-free medium for at least 15 days.

2.3. Growth inhibition assay

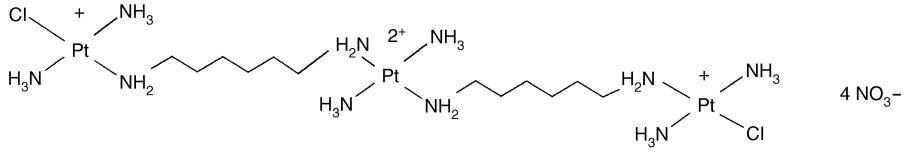
Exponentially growing cells were seeded at the appropriate concentrations to prevent confluence of the cells on the experiment day. After 24 h, the cells were drug treated for 1 h and incubated in a drug-free medium for an additional 72 h. IC_{50} and IC_{80} concentrations were the drug concentrations inhibiting cell growth by 50 and 80%, respectively. Throughout the rest of the paper, IC will refer to the doses of drugs determined by the growth inhibition assay unless otherwise specified.

2.4. Clonogenic survival assay

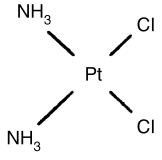
Cells were seeded in T25-cm^2 flasks (neuroblastoma) or 10-cm diameter plates (astrocytoma) at a density of approximately 1000 cells. After 1-h drug treatment, the cells were washed twice with phosphate-buffered saline (PBS) and refed with fresh medium. When colonies had reached a size of approximately 50 cells, the cells were fixed with 70% (v/v) ethanol containing 1% (w/v) methylene blue (Sigma, St Louis, MO, USA). IC_{50} values in the Clonogenic survival assay were the drug concentrations that caused a 50% reduction in the number of colonies formed compared with the untreated cells.

2.5. Platinum drug accumulation

Exponentially growing cells were drug treated for 1 h, washed twice with PBS and harvested by trypsinisation; 2 million cells per sample were pelleted, dried and digested



BBR3464



Cisplatin

Fig. 1. Structures of the platinum complexes BBR3464 and cisplatin.

with 70% (v/v) nitric acid (100 μl) and 30% (w/v) H_2O_2 (30 μl) in a microwave oven. Samples were then added with distilled water (800 μl) and a 30 μl aliquot was injected in a Perkin-Elmer mod 4000 atomic absorption spectrophotometer (AAS). Cell-bound platinum was expressed as nanograms of platinum per million cells.

2.6. Cellular DNA platination

After 1-h drug treatment, cells ($5–10\times10^6$) were harvested and washed twice with PBS. Genomic DNA was extracted according to standard protocols. DNA-bound platinum content was determined by AAS as reported above and expressed as picograms of platinum per micrograms of DNA.

2.7. Cell cycle analysis

Exponentially growing cells were untreated or drug treated for 1 h. Cell cycle analysis was performed 24, 48 and 72 h post treatment by flow cytometry. Briefly, cells were harvested, and nuclei were isolated and stained for at least 24 h using a solution containing 0.1% (w/v) sodium citrate, 0.1% (v/v) Nonidet-P40, 4 mM ethylene diamine tetra acetic acid (EDTA) and 50 μg/ml propidium iodide as a DNA dye. Flow cytometric DNA ploidy analysis was performed by acquiring a minimum of 15,000 nuclei using a Facscan flow cytometer (Becton Dickinson Immunocytometry Systems, San Josè, CA, USA). DNA fluorescence was collected in linear mode and pulse signal processing was used to set a doublet discrimination gate. Cell cycle analysis was performed using the Multicycle software package (Phoenix, San Diego, CA, USA).

2.8. Morphological analysis of apoptosis

BBR3464- and cisplatin-induced apoptosis was measured by the terminal deoxyribonucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP)-biotin *in situ* end labelling (TUNEL) assay using a commercial kit (Apoptosis-I.S., Ylem, Avezzano, Italy), according to the manufacturer's suggestion. Cells were exposed to equitoxic concentrations ($IC_{50–80}$ and multiples) of the drugs for 1 h; after 72 h of culture, a minimum of 500 cells were examined to score apoptotic cells. Count and image analysis was performed using an IAS 2000 System (Delta Sistemi, Rome, Italy).

2.9. Western blot analysis

After 1-h drug treatment, cells were post-incubated in a drug-free medium for 6–48 h. Western blot analysis was performed as previously described [8]. Fifty to 100 μg of total cell protein was electrophoresed on a 10–15% sodium dodecyl sulphate (SDS)-polyacrylamide gel and blotted on nitrocellulose (Amersham, Buckinghamshire, UK). Equal loading was checked by Red Ponceau staining. After destaining, the membranes were probed for 1 h with monoclonal antibodies against mutant and wild type p53 (DO-1; Santa Cruz Biotechnology, Santa Cruz, CA, USA), p21 (Santa Cruz), and polyclonal antibody against bax (Upstate Biotechnology, Lake Placid, NY, USA). All the immunoblots were visualised using the enhanced chemiluminescence (ECL) western blotting Detection System Kit (Amersham) and quantified by densitometry.

3. Results

3.1. Response to cisplatin and BBR3464

The cellular response to cisplatin and BBR3464 in cisplatin-resistant astrocytoma and neuroblastoma cell lines was assessed by two methods — the 3-day growth inhibition assay and the clonogenic survival assay — to allow comparison between short- and long-term effects of the drugs. The two different methods gave comparable values in terms of cisplatin sensitivity (IC_{50}) and fold resistance (Table 1). By contrast, BBR3464 IC_{50}s as determined by the clonogenic survival assay were significantly lower than those obtained in the 3-day growth inhibition assay (P at least <0.05, two-tailed Student's t-test) in all the cell lines but BE(2)-M17, in which the P value was $P<0.08$. With both methods, however, BBR3464 was more potent than cisplatin in both cis-

Table 1
Cytotoxicity of cisplatin and BBR3464 in cisplatin-sensitive and-resistant cell lines

Drug	BE(2)-M17 IC_{50} (μM)	BE(2)-M17/Pt IC_{50} (μM)	RI[a]	U87-MG IC_{50} (μM)	U87-MG /Pt IC_{50} (μM)	RI
Cisplatin[b]	5.10±2.14	28.33±8.04	5.6	5.39±1.47	25.66±7.09	4.8
Cisplatin[c]	5.05±0.64	39.00±15.56	7.7	3.82±1.44	13.60±1.27	3.6
BBR3464[b]	0.39±0.29	2.81±0.53	7.2	0.39±0.12	0.32±0.07	0.8
BBR3464[c]	0.02±0.01	0.02±0.01	1.0	0.08±0.01	0.04±0.01	0.5

[a] RI (resistance index) = cisplatin-resistant/cisplatin-sensitive IC_{50}. Cytotoxicity was assessed by both assays after 1-h drug exposure and expressed as IC_{50} (μM) as described in the Methods. Values are the mean (±standard deviation(S.D.)) of three independent experiments.
[b] 3-day growth inhibition assay.
[c] Clonogenic survival assay.

platin-sensitive (range from ~10 to ~250) and cisplatin-resistant cells (range from ~10 to ~2000). In addition, the resistance index (RI) for BBR3464 in the clonogenic survival assay was 1 for BE(2)-M17/Pt and 0.5 for U87-MG/Pt indicating a complete circumvention of acquired cisplatin resistance.

3.2. Cellular platinum accumulation and DNA platination

Reduced cisplatin accumulation and platinum–DNA adduct formation are common features of cisplatin resistance [9]. To understand the mechanisms by which BBR3464 overcomes cisplatin resistance, we examined the cellular platinum content after cisplatin and BBR3464 exposure. Platinum accumulation was non-saturable and approximately linear with cisplatin concentrations up to 200 μM in both pairs of cell lines (Fig. 2a and data not shown), but the two cisplatin-resistant variants accumulated approximately 2-fold less platinum. Exposure to equimolar BBR3464 concentrations resulted in higher intracellular platinum levels in all the cells. Cisplatin-resistant cells still accumulated less BBR3464 than their wild-type counterparts at high drug concentrations, while similar levels were reached at low concentrations.

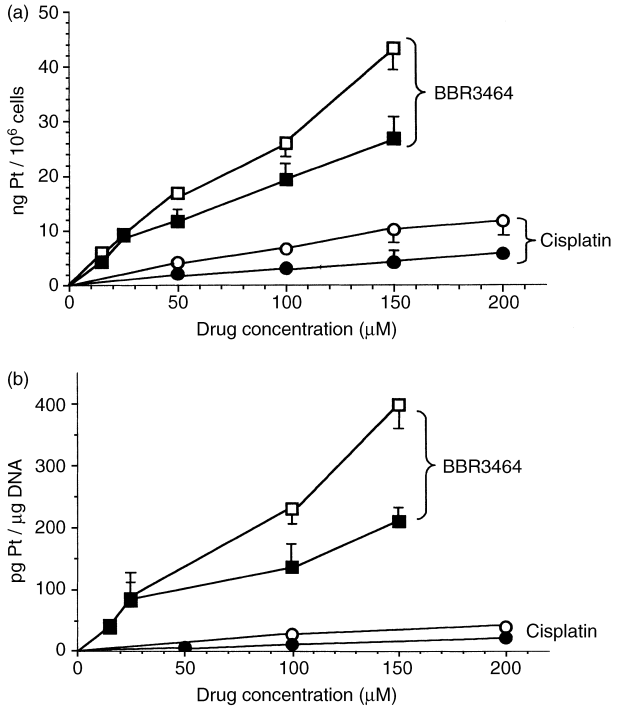


Fig. 2. (a) Cellular platinum accumulation in BE(2)-M17 and BE(2)-M17/Pt after cisplatin or BBR3464 treatmentl (b) DNA platination in BE(2)-M17 and BE(2)-M17/Pt after cisplatin or BBR3464 treatment. In both (a) and (b), open symbols refer to BE(2)-M17, solid symbols refer to BE(2)-M17/Pt. Data points, average values determined from three separate experiments; bars, standard deviation (S.D.). S.D. is not shown when it does not exceed the symbol size.

The pattern of cellular DNA platination at the same drug exposure conditions paralleled intracellular platinum accumulation (Fig. 2b). DNA platination levels were much higher in all the cell lines after BBR3464 treatment. Resistant cells showed reduced DNA platination after both cisplatin and BBR3464 exposure. However, this phenomenon was negligible at low BBR3464 concentrations, when comparable levels of DNA platination were obtained in the two pairs of cell lines (Table 2).

3.3. Induction of apoptosis

One of the mechanisms of cytotoxicity by platinum compounds is the induction of apoptotic cell death [10]. Decreased susceptibility to cisplatin-induced apoptosis is associated with cisplatin resistance [11]. To evaluate whether observed differences in drug sensitivity of our cell lines were related to their proneness to drug-induced apoptosis, we determined the percentage of apoptotic cells after cisplatin and BBR3464 treatment (Fig. 3). In both model systems, apoptosis was only slightly induced by drug concentrations equal to IC_{50} values; however, the phenomenon increased at higher drug concentrations (IC_{80} and multiples), and comparatively more after cisplatin than BBR3464 treatment. Of note, in cisplatin-resistant variants, apoptosis was induced to a lower extent by both cisplatin and BBR3464.

3.4. Cell cycle analysis

In both paired cell lines, cisplatin induced a dose-dependent increase in G_2/M phase (at 24 and 48 h in astrocytoma and neuroblastoma cells, respectively) at the expense of the G_0/G_1 cell population, that was preceded by a substantial increase in S phase at 24 h only in the neuroblastoma model (Fig. 4 and data not shown). Consistent with the RI, cisplatin-resistant cells showed an approximately 5-fold less sensitivity to cisplatin at 24 h and a greater ability to recover from the cytokinetic

Table 2
Cellular DNA platination levels after cisplatin or BBR3464 exposure in cisplatin-sensitive and cisplatin-resistant cell lines[a]

	DNA platination (pg Pt/μg DNA)	
Cell line	Cisplatin	BBR3464
BE(2)-M17	38.7±6.1	37.5±1.1
BE(2)-M17/Pt	22.4±3.6	41.6±13.0
U87-MG	144.3±10.1	128.1±11
U87-MG/Pt	46.3±22.1	137.4±23.5

[a] Cells were exposed to cisplatin and BBR3464 concentrations that yielded equal levels of DNA platination in cisplatin-sensitive cells (for BE(2)-M17 and BE(2)-M17/Pt cells: cisplatin = 200 μM; BBR3464 = 15 μM. For U87-MG and U87-MG/Pt: cisplatin = 150 μM, BBR3464 = 50 μM). Values are the mean (±standard deviation (S.D.)) of three independent experiments.

effects of DNA damage. In both model systems, BBR3464 directly induced a dose-dependent cell cycle arrest in G_2/M 24 h post-treatment with no arrest in S phase. Most remarkably, there were no substantial differences in the cell cycle kinetics of cisplatin-sensitive and cisplatin-resistant cells after the same doses of BBR3464 (Fig. 4).

3.5. p53 expression after cisplatin and BBR3464 treatment

The cellular response to DNA damage may be mediated through p53-dependent as well as p53-independent pathways [12]. p53 has been reported to induce G_1 arrest; however, there is growing evidence that p53 is involved in blocking cells also at the G_2/M checkpoint [13,14].

U87-MG (that are wild-type for *TP53* sequence) [15], and U87-MG/Pt cells were exposed to equitoxic concentrations of cisplatin and BBR3464. Western analysis showed that p53 was induced in a dose-dependent manner up to 11-fold (as determined by densitometric analysis) after cisplatin, while after BBR3464 p53 was only slightly (1.5–2-fold) induced at IC_{50}, with no further increase at very high BBR3464 doses (up to 20 μM) (Fig. 5a; data not shown). With both drugs, p53 expression peaked at 6 h and remained at similar levels up to 48 h (Fig. 5b).

In the neuroblastoma cells, (whose p53 functional status is unknown) [16], no p53 induction was observed in both whole cell lysates and nuclear extracts (data not shown).

3.6. Expression of p21 and bax after cisplatin and BBR3464 treatment

Among the downstream effectors of the p53 signal transduction pathway are the p21 and bax proteins, that control growth arrest and apoptosis, respectively. In addition, p53-independent upregulation of p21 has also been reported in different experimental settings [17–19], including the response to the G_2/M blocker doxorubicin [20].

In both U87-MG and U87-MG/Pt cells, p21 expression correlated with p53 induction after cisplatin exposure; interestingly, p21 was dramatically increased by BBR3464 doses able to only slightly induce p53 expression (Fig. 6a). In fact, a comparison at IC_{50} values between cisplatin and BBR3464 revealed the latter was an even a stronger p21 inducer, mostly in U87-MG/Pt cells. Notably, while p21 was induced by approximately 5-fold more by cisplatin in U87-MG/Pt cells compared with the sensitive parental cells, the same dose of BBR3464 elicited a comparable p21 induction in both parental and cisplatin-resistant cells. Similar results

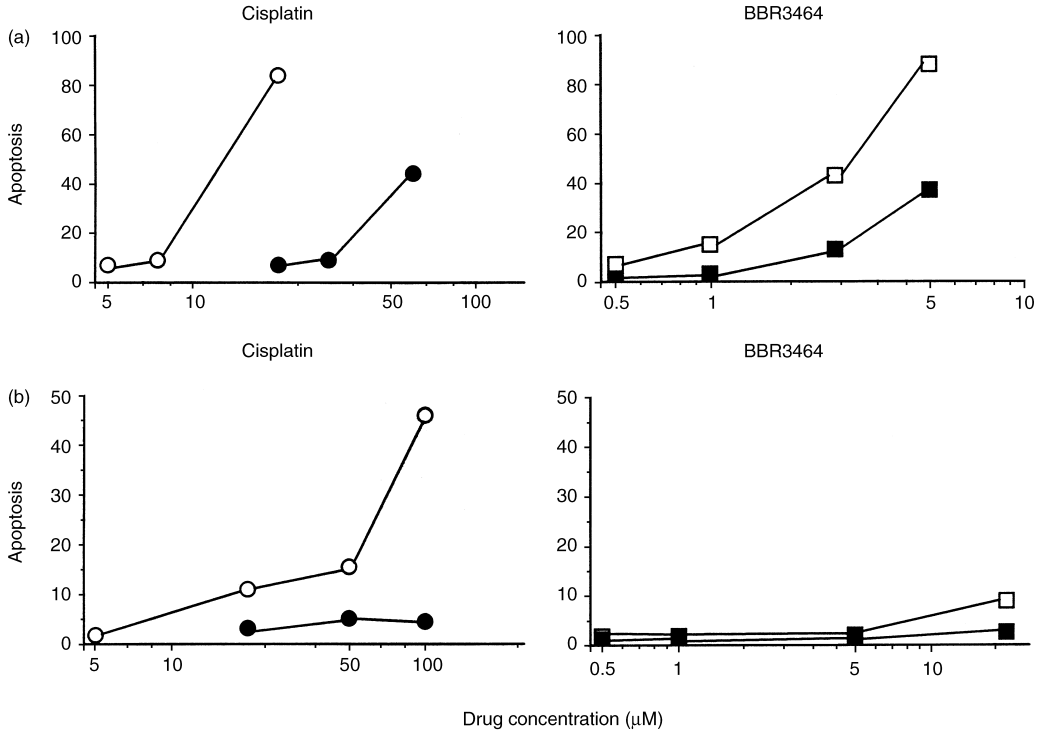


Fig. 3. (a) Cisplatin- and BBR3464-induced apoptosis in neuroblastoma paired cell lines; (b) cisplatin- and BBR3464-induced apoptosis in astrocytoma paired cell lines. In both (a) and (b), open symbols refer to cisplatin-sensitive cells, solid symbols refer to cisplatin-resistant cells. Apoptosis is expressed as the ratio of the apoptosis levels of treated over untreated cells (apoptosis level is defined as the percentage of apoptotic cells in the whole cell populations). One representative experiment out of three is shown.

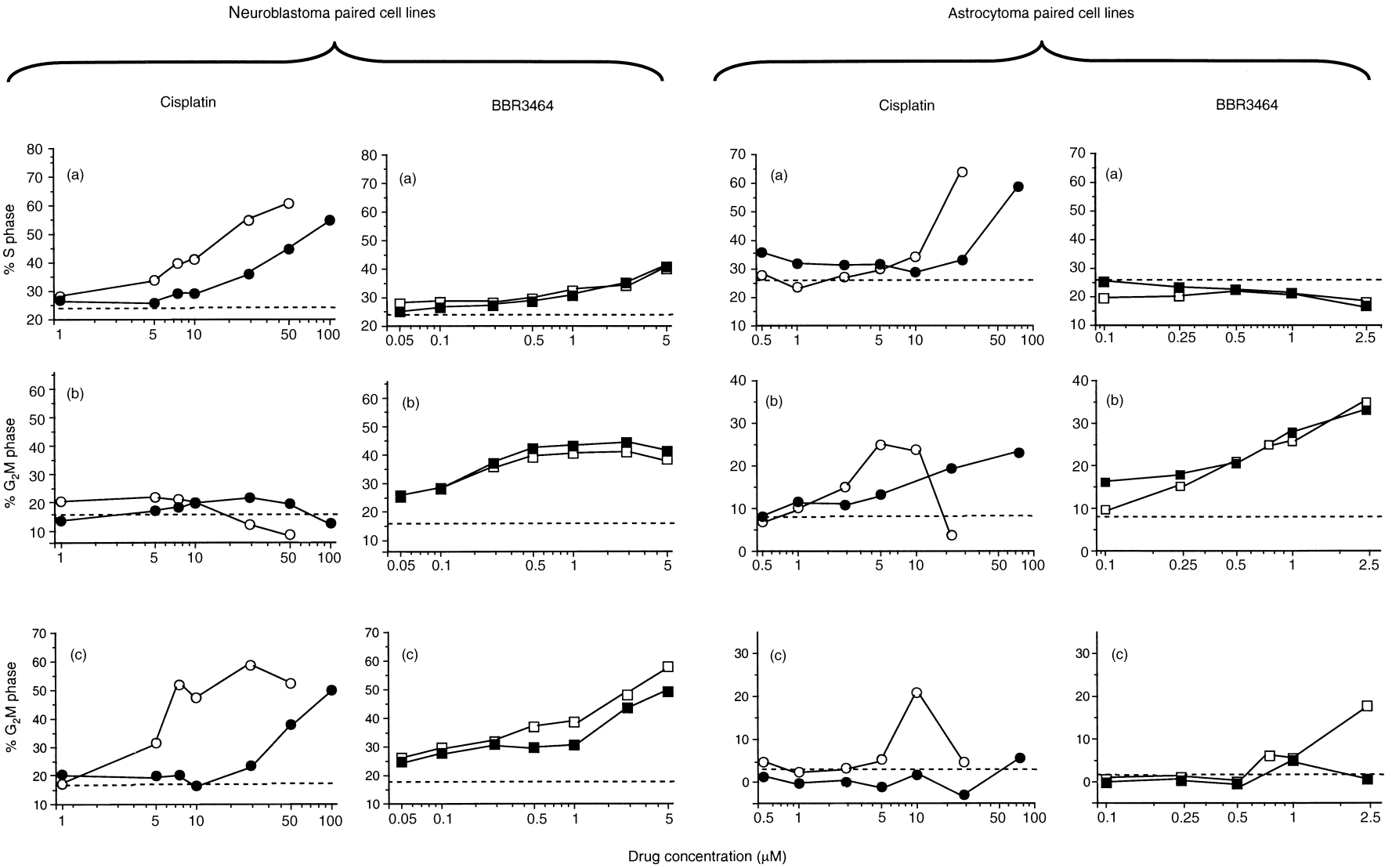


Fig. 4. Cell cycle analysis in neuroblastoma and astrocytoma paired cell lines treated with cisplatin and BBR3464. Cisplatin-sensitive (open symbols) and cisplatin-resistant (solid symbols) cells were treated for 1 h with different doses of cisplatin and BBR3464. DNA analysis was performed after 24 h (a) and (b), 48 h (c) and 72 h (data not shown). Cell percentage in S (a) and G_2/M [(b) and (c)] phases is shown. Dotted lines indicate the control.

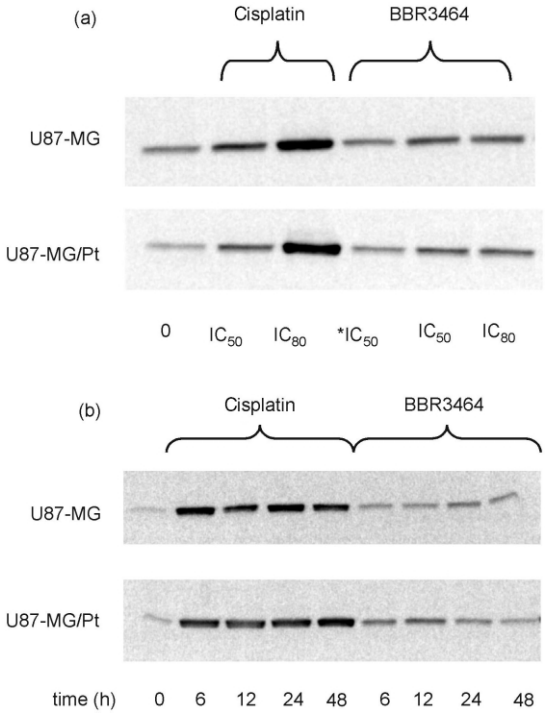


Fig 5. (a) p53 expression in U87-MG and U87-MG/Pt after cisplatin and BBR3464 treatment. Immunoblot analysis was performed 24 h after 1-h exposure to equitoxic drug concentrations: IC_{50} as in Table 1; IC_{80}: 20 and 100 μM cisplatin for U87-MG and U87-MG/Pt, respectively; 2 μM BBR3464; $*IC_{50}$ was as determined by the clonogenic survival assay. (b) p53 Expression versus time after equitoxic (IC_{80}) exposure to cisplatin and BBR3464.

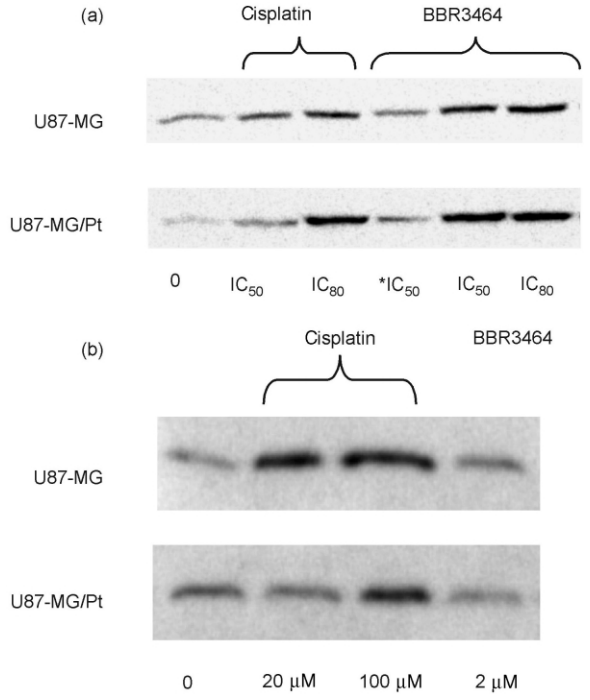


Fig. 6. (a) p21 and (b) bax expression after cisplatin and BBR3464 treatment in U87-MG and U87-MG/Pt cells. Cells were exposed to different concentrations of cisplatin or BBR3464 for 1h. Immunoblot analyses were performed after 24 h (p21) or 48 h (bax).

were obtained after 48 h of culture in a drug-free medium.

We subsequently checked the level of expression of bax protein, which also is under the transcriptional control of p53. Western blot analysis performed in parallel experiments showed that only cisplatin induced a marked increase in bax expression that peaked 48 h after treatment (Fig. 6b and data not shown). In contrast, there was no bax induction after BBR3464 treatment, also at concentrations up to 20 μM (Fig. 6b; data not shown).

In BE(2)-M17 and BE(2)-M17/Pt neither cisplatin, nor BBR3464 induced p21 or bax (data not shown).

4. Discussion

In this paper, we compared the cellular response to BBR3464 and cisplatin in neuroblastoma and astrocytoma cisplatin-resistant cell lines, characterised by reduced cellular cisplatin accumulation and DNA platination. In both model systems, BBR3464 showed a higher potency and the ability to overcome cisplatin resistance. While cisplatin treatment resulted in apoptosis induction and upregulation of p53, p21 and bax proteins, BBR3464 showed less ability to induce apoptosis despite its increased potency and treatment resulted in an upregulation of p21 that was not correlated with the induction of p53.

The different cellular response to cisplatin and BBR3464 provides additional evidence for a different mechanism of action of the two drugs — i.e. the formation of different types of drug–DNA adducts — that could result in a somewhat different mode of cell-death and explains BBR3464 ability to overcome cisplatin resistance. Indeed, in the short- and long-term assays, BBR3464 potency and ability to circumvent cisplatin resistance increased in the long-term assay, while no substantial difference in terms of both sensitivity and fold resistance was found for cisplatin, as has previously been reported [21]. Discrepancy between the short- and long-term assays indicates a different kinetics in growth inhibition and/or a different mode of cell death (reviewed in [22]). Particularly, while short-term assays are markedly affected by the rate at which cells die — which in turn can be dependent on the mode of cell death, long-term assays more reliably assess the overall cell killing after genotoxic agents. Recent investigation has shown that drug-induced cell death can include acute apoptosis, necrosis or a delayed form of cell death, from which cells may undergo apoptosis or necrosis [23,24]. Altogether, our data (i.e. the capacity to induce apoptosis and similar behaviour in the short- and long-term assay) indicate that cisplatin cytotoxicity appears to be mediated by an apoptotic mechanism,

that occurs rapidly after drug exposure [25], while BBR3464 at equitoxic doses exhibits a minor ability to induce apoptosis (short period's observation). Consistently, BBR3464 failed to overcome the decreased susceptibility to cisplatin-induced apoptosis in the cisplatin-resistant variants and to induce bax expression in astrocytoma cells, that responded to cisplatin treatment by upregulating the proapototic gene *bax*. The increased potency of BBR3464 in the long-term assay could be related either to a different mode of cell death or to BBR3464 exerting a longer lasting cytostatic effect [1] as a consequence of cells inability to quickly repair the BBR3464 genotoxic insult. Although this hypothesis has not been tested in our systems, there is recent evidence that the cellular pathways mediating DNA repair after BBR3464 or cisplatin-induced DNA lesions are different. While both nucleotide excision repair (NER) and mismatch repair systems affect the cellular sensitivity to cisplatin, neither of them appear to be involved in modulating the sensitivity to BBR3464 [3,26]. It is also possible that the cellular inability to repair BBR3464-induced DNA damage might be a consequence of the p53-independent mechanism of action of BBR3464, since DNA repair processes, and particularly NER, are facilitated by p53 [14].

The increased potency of BBR3464 in our cells correlates with the increased levels of cell- and DNA-bound platinum observed after exposure to BBR3464. However, the distinct pattern of DNA lesions more than the actual level of platination might be the major determinant of BBR3464 antitumour activity, resulting in the activation of cellular pathways different from those of cisplatin, as suggested in recent studies [3,4,27]. Consistent with this hypothesis, in the astrocytoma model system both cisplatin and BBR3464 exerted a G_2/M arrest. However, and most notably, the checkpoint control of G_2/M phase appeared to be correlated to p53 and p21 upregulation in response to cisplatin, while only p21 was induced in response to BBR3464. In U87-MG/Pt cells, the presence of transactivation-competent p53 protein was confirmed by its ability to activate the *p21/WAF/CIP* and *bax* genes, whose expression is regulated at the transcriptional level by wild-type p53, but not by mutated p53 [28]. Of note, in the U87-MG/Pt cells, the reduced sensitivity to cisplatin treatment paralleled a resistance to the p53/p21 pathway induction by cisplatin; in contrast, the same dose of BBR3464 resulted in a similar p21 induction in both the parental and resistant cells, indicating a complete lack of cross-resistance of BBR3464 with cisplatin at the molecular level.

Further evidence for a likely p53-independent mechanism of action for BBR3464 can be obtained from the data that we obtained in BE(2)-M17 and BE(2)-M17/Pt cells. In these cells, the p53-dependent pathway is very likely not functional, since both cell variants showed constitutive overexpression of p53 and did not respond to cisplatin exposure by upregulating p53 or the p53-related downstream proteins p21 and bax. BBR3464 did not induce either p53 or bax expression, and also failed to induce p21, indicating that there must be other — as yet unidentified — molecular mechanisms responsible for the BBR3464 cytotoxic activity in these cells. The p53-independent mechanism of action shown by BBR3464 in our study is in good agreement with the data reported by Pratesi and colleagues [1], who found a superior BBR3464 antitumour activity against tumours carrying a mutant p53.

In DNA-damaged cells, functional p53 has been implicated as a determinant of chemosensitivity to DNA damaging agents, including cisplatin [29], by regulating both apoptosis and cell cycle arrest [30]. Conversely, a mutant p53 status has been associated with cisplatin resistance [11,31]. p21 Induction in a p53-independent manner after DNA damage might be an alternative pathway for tumour cells with non-functional p53 to arrest [20,32] or to die by chemotherapy-mediated apoptosis [15]. Although in our paper there is no conclusive evidence of a p53-independent p21 upregulation by BBR3464, the experimental data suggests this hypothesis. The protein p21 forms complexes with and inhibits the kinase activity of the cdk-cyclins, which regulate the progression of eucaryotic cells through the cell cycle. Kinase assays will be performed in our laboratory to verify whether kinase activity is changed in response to BBR3464 treatment and to identify the specific target(s) of p21 in this response.

In conclusion, the ability of BBR3464 to bypass the p53-dependent pathway, together with its higher potency and lack of cisplatin cross-resistance, indicate that BBR3464 may elicit improved clinical response in cisplatin-resistant tumours and tumours with a non-functional p53.

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References

1. Pratesi G, Perego P, Polizzi D, *et al*. A novel charged trinuclear platinum complex effective against cisplatin-resistant tumours: hypersensitivity of p53-mutant human tumour xenografts. *Br J Cancer* 1999, **80**, 1912–1919.
2. Giuliani FC, Beggiolin G, Piazzoni L, *et al*. Pattern of cellular sensitivity to BBR3464, a novel trinuclear platinum complex, in

human tumour cell lines: Lack of cross-resistance with cisplatin. In *Proc Am Assoc Cancer Res* San Diego, 12–16 1997 (abstr 2079).
3. Perego P, Caserini C, Gatti L, *et al*. A novel trinuclear platinum complex overcomes cisplatin resistance in an osteosarcoma cell system. *Molec Pharmacol* 1999, **55**, 528–534.
4. Perego P, Gatti L, Caserini C, *et al*. The cellular basis of the efficacy of the trinuclear platinum complex BBR3464 against cisplatin-resistant cells. *J Inorg Biochem* 1999, **77**, 59–64.
5. Brabec V, Kasparkova J, Vrana O, *et al*. DNA modifications by a novel bifunctional trinuclear platinum phase I anticancer agent. *Biochemistry* 1999, **38**, 6781–6790.
6. Harris CC, Hollstein M. Clinical implications of the p53 tumor-suppressor gene. *N Engl J Med* 1993, **329**, 1318–1327.
7. Calvert PM, Highley MS, Hughes AN, *et al*. A phase I study of a novel, trinuclear, platinum analogue, BBR3464, in patients with advanced solid tumours. In *AACR-NCI-EORTC International Conference*, 16–19 November 1999, Washington, USA.
8. Servidei T, Aoki Y, Lewis SE, Symes A, Fink JS, Reeves SA. Coordinate regulation of STAT signaling and c-*fos* expression by the tyrosine phosphatase SHP-2. *J Biol Chem* 1998, **273**, 6233–6241.
9. Chu G. Cellular response to cisplatin. *J Biol Chem* 1994, **269**, 787–790.
10. Sorenson CM, Barry MA, Eastman A. Analysis of events associated with cell cycle arrest at G_2 phase and cell death induced by cisplatin. *J Natl Cancer Inst* 1990, **82**, 749–755.
11. Perego P, Giarola M, Righetti SC, *et al*. Association between cisplatin resistance and mutation of p53 gene and reduced bax expression in ovarian carcinoma cell systems. *Cancer Res* 1996, **56**, 556–562.
12. Clarke AR, Purdie CA, Harrison DJ, *et al*. Thymocyte apoptosis induced by p53-dependent and independent pathways. *Nature* 1993, **362**, 849–852.
13. Agarwal ML, Agarwal A, Taylor WR, Stark GR. p53 controls both the G_2/M and the G_1 cell cycle checkpoints and mediates reversible growth arrest in human fibroblasts. *Proc Natl Acad Sci USA* 1995, **92**, 8493–8497.
14. Gottlieb TM, Oren M. p53 in growth control and neoplasia. *Bioch Biophys Acta* 1996, **1287**, 77–102.
15. Kondo S, Barna BP, Kondo Y, *et al*. WAF1/CIP1 increases the susceptibility of p53 non-functional malignant glioma cells to cisplatin-induced apoptosis. *Oncogene* 1996, **13**, 1279–1285.
16. Darnell G, Richardson DR, *et al*. The potential of iron chelators of the pyridoxal isonicotinoyl hydrazone class as effective antiproliferative agents III: the effect of ligands on molecular targets involved in proliferation. *Blood* 1999, **94**, 781–792.
17. Janson W, Brandner G, Siegel J. Butyrate modulates DNA-damage-induced p53 response by induction of p53-independent differentiation and apoptosis. *Oncogene* 1997, **15**, 1395–1406.
18. Jiang H, Lin J, Su Z, *et al*. Induction of differentiation in human promyelocytic HL-60 leukaemia cells activates p21, WAF1/CIP1, expression in the absence of p53. *Oncogene* 1994, **9**, 3397–3406.
19. Steinman RA, Hoffman B, Iro A, Guillouf C, Liebermann DA, El-Houseini ME. Induction of p21 (WAF-1/CIP1) during differentiation. *Oncogene* 1994, **9**, 3389–3396.
20. Michieli P, Chedid M, Lin D, Pierce JH, Mercer WE, Givol D. Induction of WAF1/CIP1 by a p53-independent pathway. *Cancer Res* 1994, **53**, 3391–3395.
21. Vaisman A, Varchenko M, Said I, Chaney SG. Cell cycle changes associated with formation of Pt-DNA adducts in human ovarian carcinoma cells with different cisplatin sensitivity. *Cytometry* 1997, **27**, 54–64.
22. Brown JM, Wouters BG. Apoptosis, p53, and tumour cell sensitivity to anticancer agents. *Cancer Res* 1999, **59**, 1391–1399.
23. Blagosklonny MN. Cell death beyond apoptosis. *Leukemia* 2000, **14**, 1502–1508.
24. Houghton JA. Apoptosis and drug response. *Curr Opin Oncol* 1999, **11**, 475–481.
25. Eastman A. Activation of programmed cell death by anticancer agents: cisplatin as a model system. *Cancer Cells* 1990, **2**, 275–280.
26. Perego P, Zunino F, Carenini N, Giuliani F, Spinelli S, Howell SB. Sensitivity to cisplatin and platinum-containing compounds of Schizosaccharomyces pombe rad mutants. *Molec Pharmacol* 1998, **54**, 213–219.
27. Manzotti C, Pratesi G, Menta E, *et al*. BBR3464: a novel triplatinum complex, exhibiting a preclinical profile of antitumor efficacy different from cisplatin. *Clin Cancer Res* 2000, **6**, 2626–2634.
28. Harris CC. Structure and function of the p53 tumour suppressor gene: clues for rational cancer therapeutic strategies. *J Natl Cancer Inst* 1996, **88**, 1442–1452.
29. O'Connor PM, Jackman J, Bae I, *et al*. Characterisation of the p53 tumour suppressor pathway in cell lines of the National Cancer Institute anticancer drug screen and correlations with the growth-inhibitory potency of 123 anticancer agents. *Cancer Res* 1997, **57**, 4285–4300.
30. Levine AJ. p53, the cellular gatekeeper for growth and division. *Cell* 1997, **88**, 323–331.
31. Fajac A, Da Silva J, Ahomadegbe JC, *et al*. Cisplatin-induced apoptosis and p53 gene status in a cisplatin-resistant human ovarian carcinoma cell line. *Int J Cancer* 1996, **68**, 67–74.
32. El-Deiry W, Harper JW, O'Connor PM, *et al*. WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. *Cancer Res* 1994, **53**, 1169–1174.