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Cell growth inhibition, G_2M cell cycle arrest and apoptosis induced by the imidazoacridinone C1311 in human tumour cell lines

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Abstract

The cytotoxic activity of the imidazoacridinone C1311 was assessed on two ovarian cancer cell lines (A2780, OAW42) and one osteogenic sarcoma cell line (U2-OS) and their sublines (A2780Cp8, OAW42-MER and U2-OS-R) with experimentally induced resistance to cisplatin. A 1-h exposure to C1311 significantly inhibited the growth of all cell lines, with IC_{50} values ranging from 0.50 $\pm$0.11 to 4.10$\pm$0.36 μM. No or only partial cross-resistance was found between C1311 and cisplatin in the different cell lines. Treatment with equitoxic (IC_{50}) C1311 concentrations consistently induced accumulation of cells in the G_2M phase. The cyclin B1-associated $p34^{cdc2}$ kinase activity in cells arrested in G_2M was superimposable to that of control cells in the OAW42-MER and U2-OS cell lines, whereas a reduction of cdc2 catalytic activity was observed in OAW42 and U2-OS-R cells. Exposure to C1311 (IC_{50}) induced apoptosis in the U2-OS and U2-OS-R cell lines, whereas in the OAW42 and OAW42-MER cell lines there was a negligible percentage of apoptotic cells. In U2-OS, U2-OS-R and OAW42 cells, C1311 induced an increase in p53 expression and an increase in $p21^{waf1}$ protein, whereas p53 failed to transactivate $p21^{waf1}$ in OAW42-MER cells. An almost complete abrogation of bcl-2 was observed in U2-OS-R cells in correspondence with the peak of apoptosis induction. Our results indicate that C1311 is active against human ovarian cancer and osteogenic sarcoma cells and is not cross-resistant with CDDP. Moreover, C1311 blocks cells in the G_2M phase and induces apoptosis in a small percentage of osteogenic sarcoma cells.

Keywords: Imidazoacridinones; C1311; Cisplatin; Ovarian cancer; Osteogenic sarcoma; Cytotoxicity; Cell cycle; Apoptosis

1. Introduction

Imidazoacridinones constitute a new class of antineoplastic agents rationally designed on the basis of structure–activity relationship studies on mitoxantrone [1]. The substituted imidazoacridinone C1311 (Fig. 1), which is the main compound of this group of agents, has demonstrated potent activity against experimental models of murine and human colorectal cancer *in vitro* and in animals [2] and will shortly be entering clinical trials.

The cytotoxicity of C1311 has been initially related to its ability to bind to duplex DNA by intercalation [2] and to inhibit the catalytic activity of topoisomerase II [3]. More recently, a lysosomotropic effect of C1311 has been suggested to be a critical component for the cytotoxic activity of the drug [4]. However, the cellular determinants responsible for the activity of the imidazoacridinone are still largely unknown. It has also been reported that C1311 is able to induce the arrest of tumour cells in the G_2 phase of the cell cycle [5,6], although its interference with proteins that regulate the transition from G_2 to mitosis, i.e. cyclin B1 and cdc2 kinase, has not yet been addressed.

In the present study, we evaluated the cytotoxic activity of C1311 in human ovarian carcinoma and osteogenic sarcoma cell lines. With the aim to better characterise the effects of C1311 on tumour cell proliferation, we assessed the impairment exerted by the imidazoacridinone on the progression of cells throughout the different phases of the cell cycle, as well as the drug's interference with the expression and/or activity of proteins involved in phase-to-phase transition. The ability of C1311 to induce programmed cell death and its interference with proteins involved in the control of the apoptotic process were also investigated.

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Moreover, since C1311 proved to be active in cell lines from human tumour types where cisplatin (CDDP) would be used in the clinical setting, we compared the cytotoxicity of the two drugs in the same cell lines and determined whether C1311 retained its activity in cells with experimentally-induced resistance to cisplatin.

2. Materials and methods

2.1. Cell lines

Three pairs of CDDP-sensitive and CDDP-resistant human tumour cell lines were used in the study. The parental cell lines included the ovarian carcinoma A2780, kindly supplied by Dr R. Ozols, Fox Chase Cancer Center, Philadelphia, USA, the ovarian cystoadenocarcinoma OAW42, kindly supplied by Dr R.A. Britten, University of Liverpool, UK, and the osteogenic sarcoma U2-OS (American Type Culture Collection). The CDDP-resistant counterparts, A2780cp8 and U2-OS-R, were selected after *in vitro* continuous exposure of the parental cell lines to increasing concentrations of CDDP [9]. The OAW42-MER subline was obtained by exposure of the parental cell line to increasing concentrations of melphalan [10], and was cross-resistant with CDDP [11]. All cell lines were maintained in Roswell Park Memorial Institute Medium (RPMI)-1640 containing fetal calf serum, with the exception of U2-OS and U2-OS-R cells, which were grown in McCoy's 5A medium supplemented with 10% fetal calf serum. In the exponential phase of cell growth, the doubling times for the different cell lines were: 23±1 h for A2780, 22±2 h for A2780cp8, 29±3 h for OAW42, 26±2 h for OAW42-MER, 26±2 h for U2-OS and 19±1 h for U2-OS-R.

2.2. Drugs

C1311 (synthesised at the Technical University of Gdansk, Poland, as described elsewhere [1]) and CDDP (Bristol-Myers, Evansville, IL, USA) were initially dissolved in 0.9% sodium chloride. The drugs were diluted with fresh medium immediately before each experiment.

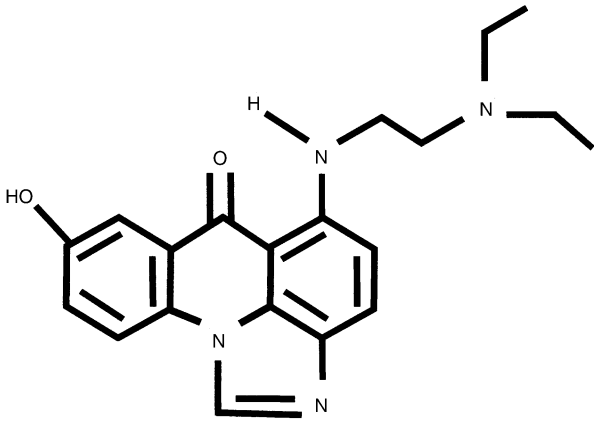

Fig. 1. Structure of the imidazoacridinone C1311.

2.3. Cell growth inhibition assay

To assess the cytotoxic activity of C1311, cells in the logarithmic growth phase were seeded in six-well plates and treated with different concentrations of the drug for 1 h. For comparative purposes, other cells were exposed for 1 h to CDDP at the same concentrations as those used for C1311. At the end of treatment, adherent cells were washed with phosphate-buffered saline (PBS) and incubated at 37 °C in a 5% CO_2 humidified atmosphere for 24, 48 or 72 h in drug-free medium. 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. Viability always exceeded 95%. Each experimental sample was run in triplicate. The results were expressed as the total number of adherent cells in control or drug-treated samples. The *in vitro* activity of the drugs was defined as the concentration able to inhibit cell proliferation by 50% (IC_{50}).

2.4. Cell-cycle distribution analysis

At different intervals (24, 48 and 72 h) after drug treatment, samples (1×10^6) of cells were fixed in 70% ethanol. Immediately before analysis the cells were washed in PBS and stained with a solution containing 50 μg/ml propidium iodide, 50 mg/ml RNAse, and 0.05% Nonidet P-40 (solution A) for 30 min at 4 °C and then analysed with a fluorescent activated cell sorter FACScan flow cytometer (Becton Dickinson, Sunnyvale, CA, USA). The cell cycle distribution was evaluated on DNA plots by CellFit software according to the SOBR model (Becton Dickinson).

2.5. Fluorescence microscopy analysis

Cells were harvested at different intervals (24, 48 and 72 h) following drug treatment; floating and adherent cells were collected separately, washed in PBS and stained with solution A. After staining, the slides were examined using fluorescence microscopy. The percentage of cells with an apoptotic nuclear morphology was determined by scoring at least 500 cells in each sample.

2.6. DNA agarose gel electrophoresis

Floating cells (3×10^6) were lysed in a solution containing 10 mM ethylene diamino tetraacetic acid (EDTA), 5 mM Tris–HCI (pH 8.0) and 0.5% Triton X-100 for 30 min on ice. Samples were centrifuged and the supernatant (low molecular weight DNA) was separated

from the pellet (high molecular weight DNA). Both fractions were digested with RNAse A (500 U/ml) for 1 h at 37 °C and 1% w/v sodium dodecyl sulphate (SDS) detergent containing 0.5 μg/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), precipitated with 2.5 volumes of ethanol and 0.1 volume of sodium acetate (3 M, pH 5.2), and then dissolved in TE buffer (10 mM Tris–HCI (pH 7.5), 1 mM EDTA (pH 8.0)). DNA was electrophoresed in 1.5% agarose gel in 1×TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA). The gel was stained with 10 μg/ml ethidium bromide for 15 min, destained for 20 min in water, and visualised under ultraviolet (UV) light.

2.7. Immunoblotting

Cells were lysed in 1% Nonidet P-40, prepared in PBS containing 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluride (AEBSF), 1 mM Na_3VO_4, 1 mM $NaPPO_4$ and 10 mM NaF. Cells lysates were clarified (15 min, 15 000 rpm), and the resultant supernatants were used for protein analysis. Total cellular lysate (100 μg) was dissolved in 2× sample loading buffer (2% SDS, 5% 2-mercaptoethanol, 20% glycerol, 60 mM Tris, pH 6.8 and 0.0025% bromophenol), separated on 12% SDS-polyacrylamide gel and transferred to nitrocellulose. The filters were blocked in PBS with 5% skim milk and incubated overnight with the primary monoclonal antibodies anti-bax, anti-bcl-2, anti-cyclin B1, anti-cdc2, anti-p53 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-$p21^{waf1}$ (Oncogene Science, Cambridge, MA, USA) or antiphospho-specific cdc2 (Tyr 15) (New England Biolabs, Beverly, MA, USA). All primary antibodies were used at 1 μg per ml. The filters were then incubated with the secondary antibody (anti-mouse or anti-rabbit Ig horseradish peroxidase-linked whole antibody (Amersham, Buckinghamshire, UK)). Bound antibody was detected using the enhanced chemoluminescence western blotting detection system (Amersham). An anti-proliferating cell nuclear antigen (PCNA) monoclonal antibody (Santa Cruz Biotechnology) was used on each blot to ensure equal loading of protein on the gel.

2.8. Immunoprecipitation and assay of cyclin B1-associated cdc2 kinase activity

Cell lysates (0.5 ml) were mixed with a 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 bovine serum albumin (BSA). The cyclin B1 immunoprecipitates were incubated with 3 μg of histone H1 (Boehringer Mannheim, Germany) in 20 μl of kinase buffer containing 20 mM Tris–HCI, pH 7.5, 10 mM $MgCl_2$, 5 μM cold adenosine 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% SDS–polyacrylamide gel. Following autoradiography, the reactions were quantified by densitometry. Kinase activities of control and treated samples were normalised on the number of cells in the G_2M phase and expressed in arbitrary densitometric units.

3. Results

3.1. Cytotoxic activity of C1311

One-hour exposure to 0.5–50 μM C1311 resulted in dose-dependent inhibition of cell proliferation in all experimental models, and the extent of growth inhibition increased as a function of time after drug-washout (Fig. 2). IC_{50} values, calculated from growth inhibition curves obtained 72 h after drug treatment, ranged from 0.5 to 4.10 μM in the ovarian cancer cell lines and from 2.40 to 4.00 μM in the osteogenic sarcoma cell lines (Table 1). Moreover, when the sensitivity of the different cell lines to C1311 was analysed in comparison to that to CDDP, an almost complete lack of cross-resistance between C1311 and CDDP was observed in the OAW42-MER and U2-OS-R cells, whereas only partial cross-resistance was found in A2780cp8 cells (Table 1).

3.2. Effects of C1311 on cell cycle progression

The perturbations induced by C1311 in the distribution of cells in the different phases of the cell cycle were determined by flow cytometry in OAW42, OAW42-MER, U2-OS and U2-OS-R cells at various intervals after a 1-h exposure to equitoxic (IC_{50}) drug concentrations (Table 2). It must be noted that during the time course of the experiment, U2-OS cells were exponentially growing, whereas in the other three cell lines a reduction in the extent of the S-phase cell fraction was observed over time. A drug-induced accumulation of cells in the G_2M phase was observed in all cell lines, although the extent to which cells accumulated in the G_2M phase varied. Specifically, in U2-OS-R cells the G_2M block was significant starting from 24 h after treatment and a continuous increase in the number of cells accumulated in this phase was observed until 72 h. In addition, in the OAW42 and OAW42-MER cells, the drug-induced accumulation of cells in the G_2M compartment was already appreciable at 24 h. However, in these cell lines, the number of cells blocked in this cell cycle compartment did not increase with time. Finally, in the U2-OS cells, the accumulation of cells in the G_2M phase was highest at 24 h and then rapidly decreased.

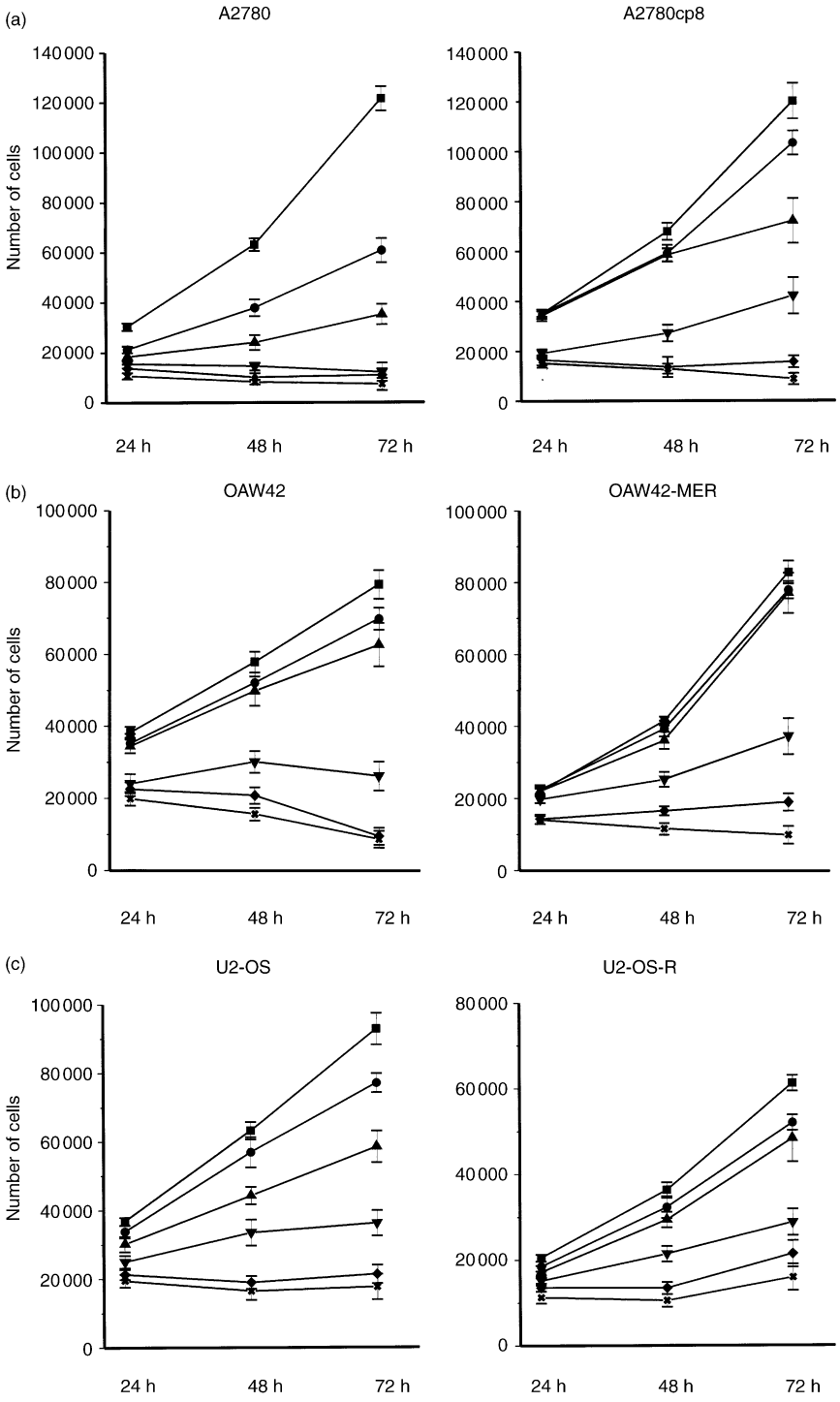

Fig. 2. Growth inhibition curves of ovarian carcinoma (A2780 and A2780cp8 (a); OAW42 and OAW42-MER (b)) and osteogenic sarcoma (U2-OS and U2-OS-R (c)) cell lines treated with C1311. Cells were exposed to the drug for 1 h, washed and incubated in drug-free medium for an additional 24, 48 or 72 h. Data are expressed as the number of cells/ml in control samples (■) and samples exposed to 0.5 μM (●), 1.0 μM (▲), 5.0 μM (▼), 10.0 μM (◆) or 50.0 μM (**X**) of C1311. Values represent mean values (±standard deviation (S.D.)) of three independent experiments.

3.3. Effects of C1311 on proteins involved in G_2 to M transition

Since C1311 consistently induced alterations in cell progression throughout the G_2M phase, the effect of drug treatment on the expression of cdc2 and cyclin B1 proteins, which regulates transition through the G_2 checkpoint [12], was determined next. The results are reported in Fig. 3 and show that exposure to equitoxic (IC_{50}) C1311 concentrations induced a modest increase in the expression of cyclin B1 protein, detectable within 48 h from the end of treatment, in OAW42, U2-OS and

U2-OS-R cells. In the OAW42-MER cells, a slight increase in cyclin B1 protein abundance was only observed at 24 h. As regards cdc2, no appreciable difference with respect to controls was found in the level of protein expression in the drug-treated samples.

The kinase activity of cyclin B1-associated cdc2 was then measured using the substrate histone H1 at different intervals after exposure of the cells to C1311 (Fig. 4a). As shown in Fig. 4b, the overall kinase catalytic activity of treated samples was higher than that of the controls in all cell lines. However, when the results were expressed in terms of the kinase activity value divided by the number of G_2M cells (as detected by flow cytometry), we found that OAW42-MER and U2-OS cells accumulated in G_2M phase after exposure to C1311 showed a cdc2 catalytic activity similar to that of the control cells (Fig. 4c). A slight increase in cdc2 kinase activity was only observed in the OAW42-MER cells 72 h after treatment. Conversely, in the OAW42 and U2-OS-R cells, a cdc2 catalytic activity consistently lower than that of controls was detected (Fig. 4c). Superimposable results were obtained by directly immunoprecipitating cdc2 instead of cdc2 associated with cyclin B1 (data not shown).

To investigate whether differences observed among the four cell lines in the effect exerted by C1311 on cdc2 kinase activity could be ascribed to a different interference of the drug with the pattern of cdc2 phosphorylation, we examined the extent of cdc2 phosphorylation on Tyr15 (Fig. 4d). We found that in the OAW42 and U2-OS-R cells exposed to C1311, cdc2 appeared slightly hyperphosphorylated compared with controls. Conversely, no appreciable difference in the extent of cdc2 phosphorylation was observed between treated and untreated OAW42-MER and U2-OS cells.

Table 1
Sensitivity of human tumour cell lines to C1311 and CDDP

	C1311 IC_{50} (μM)	RI	CDDP IC_{50} (μM)	RI
Ovarian cancer				
A2780	0.50±0.11	–	4.30±0.47	–
A2780cp8	1.95±0.27	3.90	60.00±9.43	13.95
OAW42	2.85±0.32	–	8.67±3.47	–
OAW42-MER	4.10±0.36	1.44	86.70±17.1	10.00
Osteogenic sarcoma				
U2-OS	2.40±0.35	–	8.00±0.30	–
U2-OS-R	4.00±0.56	1.67	60.00±10.00	7.50

RI, resistance index: IC_{50} resistant cell line/IC_{50} parental cell line. The IC_{50} value was determined graphically from the growth inhibition curves (obtained 72 h after exposure of the cells to each drug for 1 h) as the concentration inhibiting cell growth by 50%. Data represent mean values ±standard deviation (S.D.) of three independent experiments.

3.4. Induction of apoptosis by C1311

The ability of C1311 to induce apoptosis in the four cell lines was initially determined by fluorescence microscopy (Fig. 5a) after cell staining with propidium iodide. Fig. 5b shows the percentage of cells with an apoptotic morphology, calculated on the total cell population. In the OAW42 and OAW42-MER cell lines, spontaneous apoptosis was observed in a negligible fraction (<0.1% for OAW42 and <1.5% for OAW42-MER) of the control cells. Exposure to C1311 (IC_{50}) induced a modest increase in the percentage of OAW42 (up to 2.5%) and OAW42-MER (up to 5%) cells with an apoptotic morphology. In U2-OS cells, spontaneous apoptosis was observed in less than 1.5% of the control cells. In C1311-treated U2-OS cells, this percentage increased up to 11% 72 h after drug exposure. The most marked induction of programmed cell death by C1311 was observed in the U2-OS-R cells. In this cell line, the imidazoacridinone was able to cause

Table 2
Cell cycle perturbations induced by C1311[a]

	OAW42		OAW42-MER		U2-OS		U2-OS-R	
	Control	C1311	Control	C1311	Control	C1311	Control	C1311
24 hours								
$G_{0/1}$	49±6	39±12	39±4	31±2	31±2	28±1	29±5	5±1
S	34±4	31±16	41±6	22±10	59±1	18±1	48±2	40±1
G_2M	17±2	30±4	20±1	47±7	10±1	54±5	23±2	55±6
48 hours								
$G_{0/1}$	59±1	53±8	45±2	34±1	39±3	48±4	30±1	16±5
S	24±4	11±7	37±5	28±8	47±5	15±7	47±1	28±1
G_2M	17±4	36±3	18±1	38±9	14±4	37±1	23±2	56±3
72 hours								
$G_{0/1}$	71±9	54±10	56±1	35±1	52±6	51±3	29±1	17±6
S	17±6	17±5	31±1	27±8	38±5	27±2	47±1	15±6
G_2M	12±6	29±7	13±1	38±8	10±1	22±1	24±1	68±7

[a] IC_{50}, 1 h exposure. Data represent mean values ±standard deviation (S.D.) of three independent experiments.

apoptosis in a time-dependent manner, and the rate of apoptotic cells (which was $<5\%$ in control cells) was increased to 21% 72 h after drug exposure. Gel electrophoresis analysis of DNA obtained from C1311-treated U2-OS and U2-OS-R cells showed accumulation of oligonucleosome fragments 72 h after treatment with C1311 (Fig. 6).

Finally, the expression of proteins involved in the control of programmed cell death was assessed in the two cell lines after exposure to C1311 (Fig. 7). In the U2-OS and U2-OS-R cells, an increase in p53 protein expression and transactivation of $p21^{waf1}$ were observed after drug exposure. Similar results were obtained in OAW42 cells. In the OAW42-MER cell line, p53 expression was increased after drug treatment, whereas the $p21^{waf1}$ signal remained undetectable. Drug treatment did not significantly modify bax expression in any of the cell lines. As regards bcl-2, an appreciable decrease in the level of expression of the anti-apoptotic protein was only evident in the U2-OS-R cells 72 h after C1311 exposure, in concomitance with the maximum induction of apoptotic cell death.

4. Discussion

C1311 is the leading compound of a new class of potent anticancer agents, the imidazoacridinones [2]. The compound has already demonstrated significant antitumour activity in experimental models of human and murine colorectal tumours [2] and will soon enter clinical phase I trials. In the present study, we found that C1311 also has significant cytotoxic activity against human ovarian carcinoma and osteogenic sarcoma cell lines, tumour models that have not been tested before for their sensitivity to the drug. Since the cell lines used in the study derive from human tumour types where cisplatin would be used in a clinical setting [13], we compared the cytotoxicity of the two drugs in the same cells. Moreover, in order to ascertain whether or not there was cross-resistance between C1311 and CDDP, we tested the efficacy of the imidazoacridinone against tumour cell variants with experimentally-induced resistance to CDDP. We found an almost complete lack of cross-resistance between the two agents in two tumour cell lines and only partial cross-resistance in the third cell line. This finding, together with previous evidence that C1311 is a weak substrate for P-glycoprotein [8] and, as a consequence, shows only a little cross-resistance to doxorubicin [7], would open the interesting possibility to use C1311 for the clinical treatment of tumours refractory to platinum compounds and anthracyclines. Moreover, C1311 showed an approximately 3- to 30-fold greater potency with respect to CDDP in the different cell lines. This observation is of great interest since it would suggest a relevant antitumour activity of the imidazoacridinone in the clinic. However, even though such a possibility has never been tested in preclinical models, it is possible that C1311 is more toxic than CDDP to normal cells.

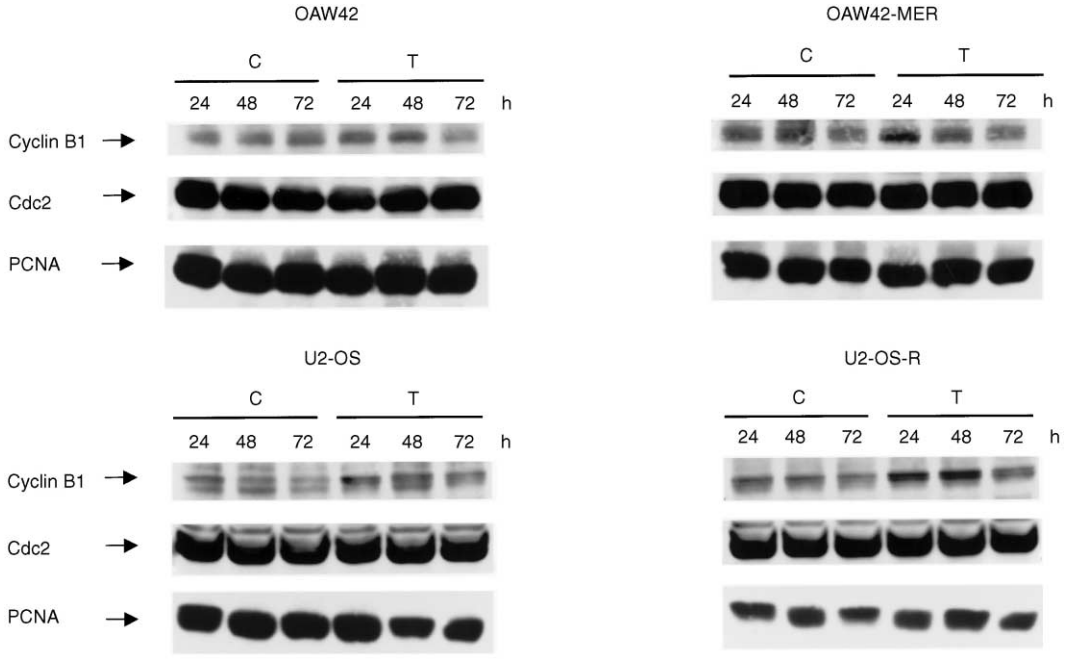

Fig. 3. A representative experiment illustrating the effect of C1311 on the expression of proteins involved in the control of the G_2 checkpoint in ovarian carcinoma and osteogenic sarcoma cell lines. Cells were incubated with solvent (control) or with the IC_{50} concentration of C1311 for 1 h. At the end of treatment, the cells were incubated for an additional 24 h, 48 h and 72 h in drug-free medium. Western blots were probed with monoclonal antibodies for cdc2 and cyclin B1. C, controls, T, treated samples. Proliferating cell nuclear antigen (PCNA) was used as a control for loading.

As regards C1311-induced cell cycle perturbations, treatment of CDDP-sensitive and CDDP-resistant cell lines with equitoxic (IC_{50}) concentrations of the imidazoacridinone induced G_2M accumulation in all cell lines, although to a variable extent. Cell cycle arrest in the G_2 phase after exposure to C1311 had previously been detected in L1210 murine leukaemia [5] and HeLa S3 human cervical carcinoma [6] cell lines. G_2M accumulation was fairly stable over the 72-h observation period in OAW42, OAW42-MER and U2-OS-R cells, whereas in U2-OS cells a progressive decrease in the number of cells arrested in the G_2M phase was observed over time. Such results indicated that the cytotoxic effect of C1311 observed in the different cell lines was not related to the extent or the duration of drug-induced cell cycle perturbations. Moreover, since an appreciable variability in the extent to which the different cell lines accumulated in the G_2M compartment after exposure to equitoxic C1311 concentrations was observed, it is likely that treated cells did not die only in the G_2M compartment, but also in other phases of the cell cycle.

Since the major regulator of G_2 to M transition is the M phase-promoting factor (MPF), a complex constituted by the catalytic subunit cdc2 and the regulatory

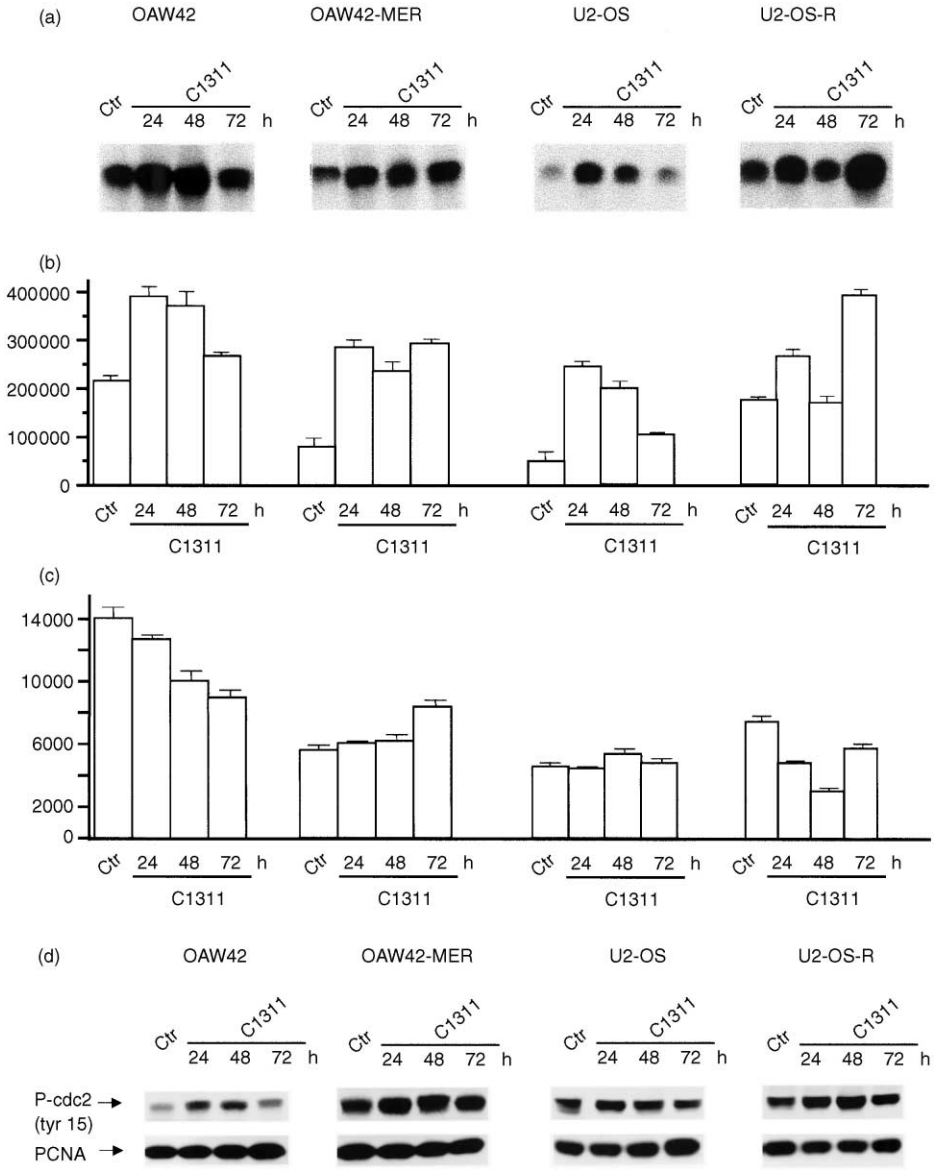

Fig. 4. Effect of C1311 on cdc2 kinase activity in ovarian carcinoma and osteogenic sarcoma cell lines. (a) Cells were incubated with solvent (control; Ctr) or with the IC_{50} concentration of C1311 for 1 h. At the end of treatment, the cells were incubated for an additional 24 h, 48 h and 72 h in drug-free medium. Immunoprecipitation and kinase assays were performed. Following autoradiography, the reactions were quantified by densitometry. For each sample, the kinase activity was expressed in overall arbitrary densitometric units (b) or as arbitrary densitometric units divided by the number of cells in the G_2M phase (c). Data represent mean values (±standard deviation (S.D.)) of three independent experiments. (d) Effect of C1311 on cdc2 phosphorylation. Western blots were probed with monoclonal antibodies for phosphorylated cdc2 (Tyr 15 residue) and proliferating cell nuclear antigen (PCNA).

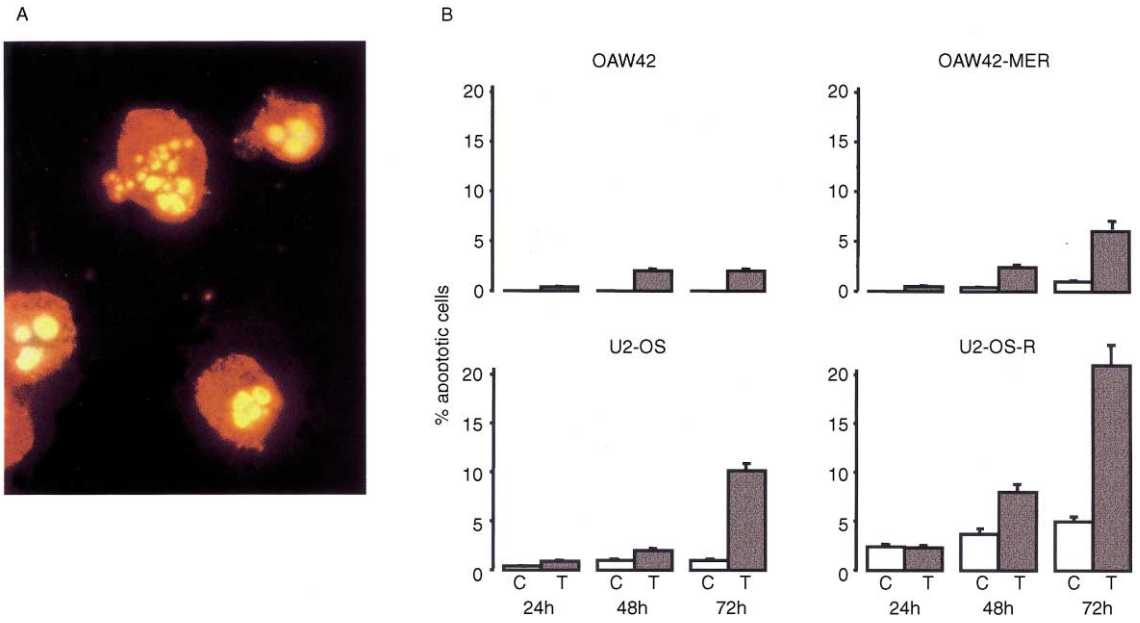

Fig. 5. (a) Propidium iodide staining of U2-OS cells treated with C1311 (IC_{50}). Whole cells were collected, stained with propidium iodide and examined under a fluorescence microscope. (b) Quantification of apoptotic cells in ovarian carcinoma and osteogenic sarcoma cell lines treated with C1311 and collected at different intervals (24 h, 48 h and 72 h) following treatment. Data represent the mean values (±standard deviation (S.D.)) of three independent experiments. C, control; T, treated cells.

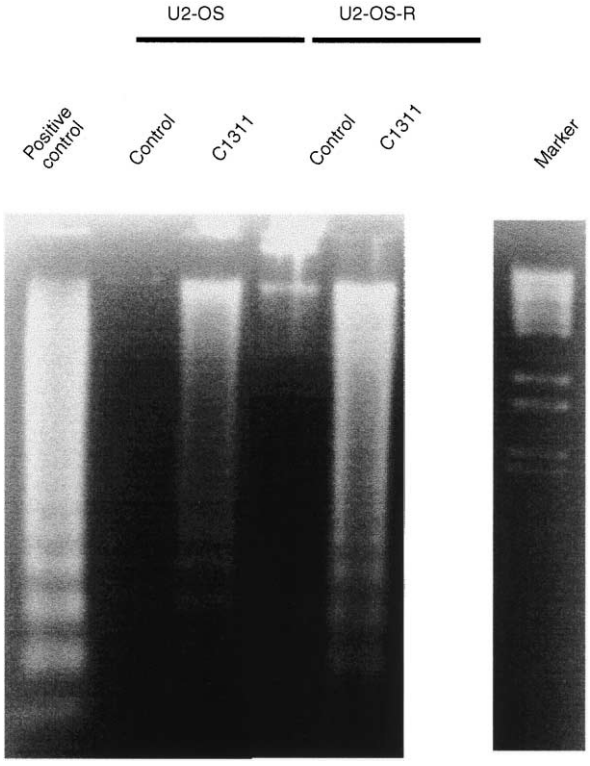

Fig. 6. Electrophoretic pattern of DNA extracted from U2-OS and U2-OS-R cells treated with C1311 (IC_{50}) and collected 72 h after treatment. HL-60 cells treated with 30 μg/ml of VM26 for 8 h were used as positive control for the induction of apoptosis.

subunit cyclin B1 [14,15], we evaluated the effect of C1311 treatment on the cyclin B1-associated cdc2 kinase activity using histone H1 as a substrate. When the 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 and U2-OS-R cells accumulating in the G_2M phase after exposure to C1311 showed a reduced ability to phosphorylate histone H1 with respect to untreated control cells. This observation is in agreement with previous findings obtained after treatment with different DNA damaging agents able to induce G_2M accumulation in other experimental tumour models [16,17]. Since no inhibition of cyclin B1 or cdc2 protein expression was observed after C1311 treatment, the decrease in cdc2 catalytic activity cannot be considered the consequence of a reduced formation of the cyclin B1–cdc2 complex. Results obtained in the two cell lines also showed that C1311 treatment interferes with the pattern of cdc2 phosphorylation, which has been demonstrated to play a role in the control of cdc2 kinase activity [18]. In fact, in treated OAW42 and U2-OS-R cells, cdc2 appeared hyperphosphorylated on Tyr 15 compared with control cells. In OAW42- MER and U2-OS cell lines, cells accumulating in G_2M after exposure to the imidazoacridinone showed a cdc2 kinase activity that was almost superimposable to or slightly higher than that of control cells. The inability of these cells to escape the G_2 block

could be tentatively explained by assuming that the active cyclin B1–cdc2 complexes are confined to the cytoplasmic compartment. In fact, it has previously been demonstrated that exclusion of cdc2 kinase activity from the nucleus may contribute to the cell cycle delay occurring after irradiation in HeLa-S1 cells [19].

Since apoptosis is a major mode of cell death induced by several DNA damaging agents [20], we evaluated the induction of apoptosis after exposure of our tumour cell models to C1311. Fluorescence microscopy analysis indicated the presence of a negligible percentage of cells with an apoptotic nuclear morphology in the OAW42 and OAW42-MER cell lines. A slightly greater extent of apoptosis induction was observed in U2-OS and U2-OS-R cells after exposure to C1311. In these cell lines, the occurrence of apoptosis was also confirmed by the presence of oligonucleosomal DNA fragmentation. However, the onset of apoptosis induced by the imidazoacridinone in these cell lines was delayed since the presence of a significant percentage of cells with an apoptotic morphology was appreciable only from 48 h after drug treatment. As previously suggested by Burger and colleagues [4], this finding would indicate that the process of programmed cell death is not directly activated by C1311, but might be triggered by cell autolysis. In fact, the same authors demonstrated that in HT29 human colon cancer cells the imidazoacridinone induced lysosomal rupture and a significant increase in acid phosphatase activity at earlier time points with respect to those in which apoptosis was observed [4]. As a consequence, the very low level of apoptosis after C1311 treatment in the OAW42 and OAW42-MER cell lines could be explained on the basis of a reduced susceptibility to the lysosomotropic effect of C1311 or, alternatively, to the presence of intrinsic cytoprotective mechanisms that specifically counteract the efficacy of the imidazoacridinone. However, all these data would suggest that the induction of apoptosis is not the major mechanism of cell kill induced by the imidazoacridinone.

At the molecular level, we also investigated whether the different susceptibility of our tumour cell models to undergo C1311-induced apoptosis might be sustained by a different expression of proteins involved in the

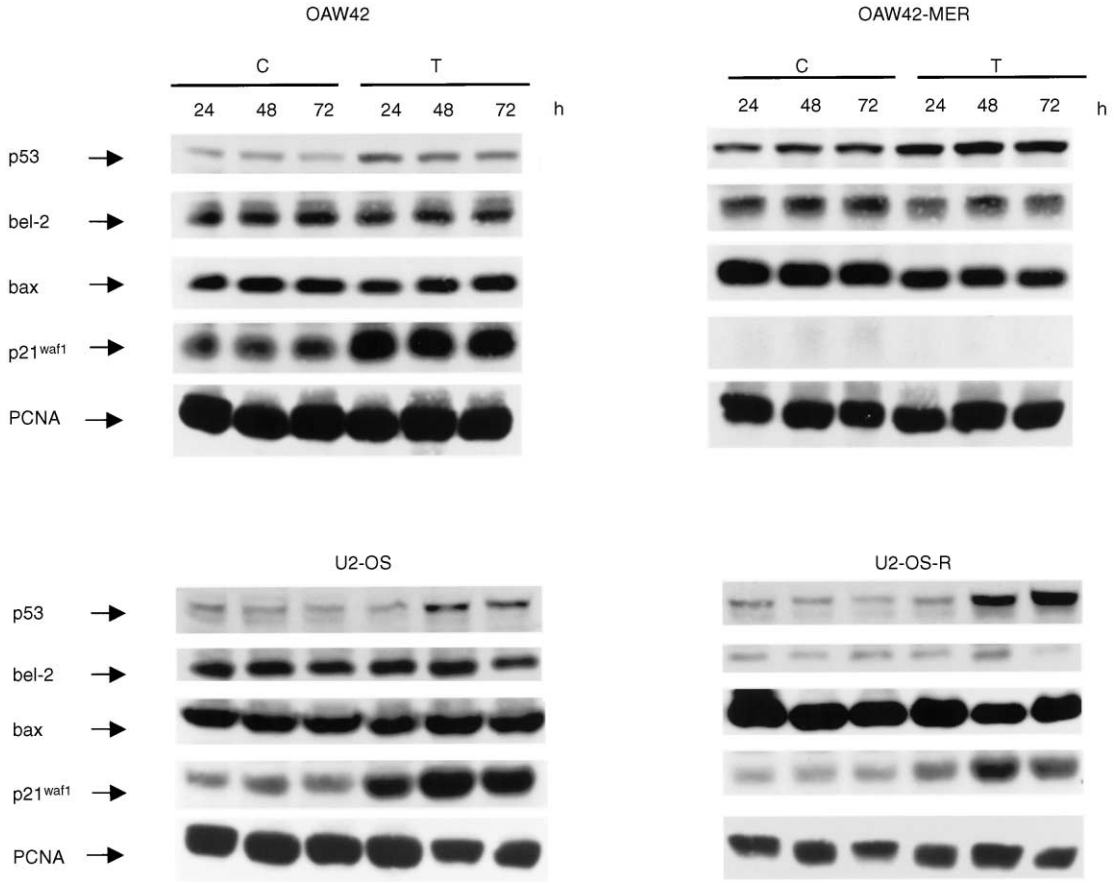

Fig. 7. A representative experiment illustrating the effect of C1311 treatment on the expression of proteins involved in the control of apoptosis in ovarian carcinoma and osteogenic sarcoma cell lines. Cells were incubated with solvent (control) or with the IC_{50} of C1311 for 1 h. At the end of treatment, the cells were incubated for an additional 24 h, 48 h and 72 in drug-free medium. A 100-μg aliquot of whole cell extract was separated and electrophoretically blotted. Western blots were probed with antibodies for p53, bcl-2, bax and $p21^{waf1}$. Proliferating cell nuclear antigen (PCNA) was used as a control for loading.

control of apoptosis such as p53 [21] and some of the major downstream genes controlled by this transcription factor. No appreciable variation in the levels of expression of the pro-apoptotic protein bax was observed after drug treatment in the different cell lines, whereas an almost complete abrogation of the expression of the anti-apoptotic protein bcl-2 was detected in U2-OS-R cells, in concomitance with the peak of apoptosis. However, it must be stressed that the loss of bcl-2 protein may simply reflect the cleavage/degradation of the protein by caspases. In fact, it has been demonstrated that the loop domain of bcl-2 is cleaved by caspase 3 [22]. As regards $p21^{waf1}$, an increased expression of the cyclin-dependent kinase inhibitor was observed in the U2-OS, U2-OS-R and OAW42 cell lines after exposure to C1311. Conversely, $p21^{waf1}$ was always undetectable in the OAW42-MER cells. This last finding would suggest that the presence of an apparently non-functional p53 protein (as indicated by the lack of $p21^{waf1}$ induction in OAW42-MER cells), which is detrimental for the susceptibility of cells to other DNA damaging agents such as CDDP [23,24], is not a determinant of tumour cell resistance to C1311. Moreover, results obtained in this cell line suggest that cell cycle perturbation induced by the drug (i.e. G_2M cell accumulation) is not the consequence of a p53-mediated induction of p21 protein expression.

Overall, our results indicate that C1311 is an active compound against human ovarian cancer and osteogenic sarcoma cell lines, and that it retains its cytotoxic effect and the ability to promote apoptosis also in tumour cells that are refractory to CDDP, probably because different molecular targets are involved in the pathways of cellular response to the two drugs. Such findings suggest that further studies should be undertaken to identify possible preferential targets of C1311 in human tumours with a view to the clinical use of the compound.

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