Comparative evaluation of C1311 cytotoxic activity and interference with cell cycle progression in a panel of human solid tumour and leukaemia cell lines

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Abstract. The cytotoxic activity of the imidazoacridinone C1311 was related to its effect on cell cycle progression in 16 human cell lines from different solid tumour types and leukaemias. A 72-h exposure to C1311 induced a wide range of growth inhibition (IC_{50} values ranging from 0.0094 to 0.8 μM; median, 0.279 μM), with the highest activity in the 2 gastric cancer cell lines (IC_{50}, 0.0094 and 0.0098 μM). No significant association was found between *in vitro* sensitivtity to C1311 and doxorubicin or taxol. Moreover, the activity of C1311 was independent of the p53 gene status of the cell line. Twenty-four-hour exposure to C1311 led to a marked increase in the number of cells in the G_2M phase in the majority of cell lines, although the extent of such accumulation was independent of the level of drug cytotoxic activity. C1311 did not generally affect the expression level of cyclin B1 or Cdk1 (p34cdc2) proteins. Conversely, when normalised on the basis of the number of cells arrested in the G_2M compartment, Cdk1 kinase activity appeared lower than that of untreated cells in the 4 cell lines showing the most pronounced accumulation in G_2M. Overall, such data show that C1311 is active against a variety of human tumour cell lines and strongly support further evaluation of the drug in clinical trials.

Introduction

Imidazoacridinones are a group of antineoplastic agents rationally designed on the basis of structure-activity relationship studies on mitoxantrone (1). The most promising analogue, C1311 (Symadex™; Fig. 1), which contains the hydroxyl group in position 8 of the imidazoacridinone core, is currently in phase II clinical trial. The compound, which is rapidly transported to tumour cells and accumulates in the nucleus (2,3), has shown potent activity against experimental models of murine and human colorectal cancer *in vitro* and in animals (4), and has shown limited mutagenic potential (2) and poor capacity to generate oxygen radicals (5), which suggests a lack of cardiotoxic properties.

The drug, which undergoes enzymatic oxidative activation (6,7), has been shown to intercalate into DNA (8) and to trap topoisomerase II cleavable complexes (9). A lysosomotropic effect of C1311 has also been observed in colon cancer cells following continuous drug exposure (10) but not pulse treatment (11). As far as cell kinetic effects are concerned, it has been reported that C1311 is able to induce the arrest of tumour cells in the G_2M phase of the cell cycle (12,13). However, the cellular determinants responsible for the activity of the imidazoacridinone have not yet been characterised.

In the present study we evaluated the cytotoxic activity of C1311 in a large panel of cell lines derived from human solid tumours and leukaemias, and compared it with the cytotoxic activity of doxorucin and taxol, two drugs that are currently used in the clinical treatment of a wide spectrum of malignancies. Taxol was included since it also causes G_2M accumulation (14), and doxorubicin was included as a prototype of a DNA-damaging agent. To obtain insight into the molecular mode of action of C1311, the cytotoxic activity of the compound was also correlated with the p53 gene status of the tumour cell models. Moreover, in order to better characterise the effects of C1311 on tumour cell proliferation and to define their relevance in the overall tumour cell response to the drug, we assessed the impairment induced by the imidazoacridinone in cell cycle progression as well as the drug's interference with the expression and/or activity of cyclin B1 and Cdk1 (p34cdc2) proteins, which regulate G_2 to M phase transition (15).

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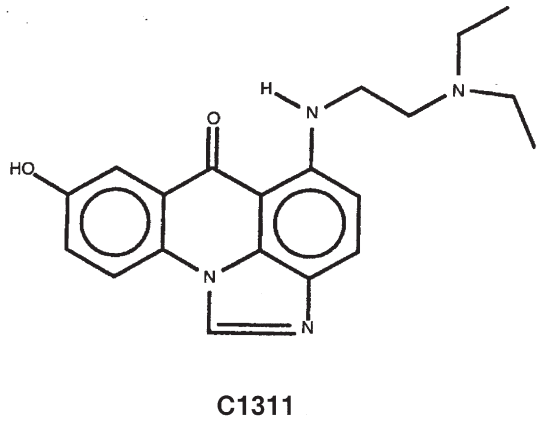

C1311

Figure 1. Structure of the imidazoacridinone C1311.

Materials and methods

Cell lines. Sixteen human cell lines from different tumour types including colon (LoVo, HT29, WiDr, SW948, Colo320), lung (H460, H322, GLC4), ovarian (OAW42, OAW42mer), and gastric cancers (GK2, KKP), melanoma (JR8, SK-MEL-5) and leukaemias (CEM, Jurkat) were used in the study. The cell lines were maintained in specific culture medium containing fetal calf serum. Eight cell lines were characterised by a wt p53 gene (LoVo, GLC4, H460, OAW42, OAW42mer, SK-Mel-5, GK2, KKP), 7 carried a mutant p53 gene (WiDr, SW948, Colo320, HT29, H322, CEM, JR8) and one was p53-null (Jurkat).

Drugs. C1311 [synthesised at the Technical University of Gdansk, Poland, as described elsewhere (1)] and doxorubicin (Pharmacia-Upjohn, Uppsala, Sweden) were initially dissolved in 0.9% sodium chloride, whereas taxol (Sigma Chemicals) was dissolved in dimethylsulphoxide. The drugs were then diluted with fresh medium immediately before each experiment.

SRB cytotoxicity assay. Cytotoxicity was determined by the sulforhodamine B (SRB) assay using 96-well microtitre plates (16). Each cell line was plated at an appropriate density in 100 μl complete culture medium/well, and 24 h later 100 μl drug-containing medium was added. Different concentrations ranging from 0.001 to 10 μM for C1311, from 0.001 to 10 μM for doxorubicin, and from 0.0001 to 1 μM for taxol were tested. After 72 h of incubation, cells were fixed with cold 50% trichloroacetic acid solution for 1 h, and 0.4% SRB was added to each well. After a 30-min incubation, the plates were washed and bound dye was dissolved by 10 mM Tris base (pH 10.5). Optical density (OD) was read at 550 nm on a microplate reader. The results were expressed as the OD values in treated samples compared with control samples. Dose-response curves were created and IC_{50} values (i.e., concentrations able to inhibit cell growth by 50%) were determined graphically from the plots for each drug.

Cell-cycle distribution analysis. Twenty-four hours after exposure of cells to C1311, doxorubicin and taxol (IC_{50} and 10 x IC_{50}), samples of $1x10^6$ cells were fixed in 70% ethanol.

Table I. Cytotoxic activity of C1311, doxorubicin and taxol in the different cell lines

	IC_{50} (μM)		
	C1311 (mean ± SD)[a]	Doxorubicin (mean ± SD)[a]	Taxol (mean ± SD)[a]
Colon cancer			
WiDr	0.740±0.297	0.138±0.064	0.022±0.010
SW948	0.478±0.297	0.013±0.008	0.027±0.007
HT29	0.231±0.040	0.027±0.010	0.017±0.007
Colo320	0.141±0.038	0.064±0.026	0.650±0.149
LoVo	0.039±0.015	0.005±0.003	0.042±0.020
Lung cancer			
H322	0.800±0.284	0.083±0.011	0.049±0.018
GLC4	0.357±0.050	0.094±0.005	0.143±0.062
H460	0.032±0.015	0.007±0.003	0.034±0.010
Ovarian cancer			
OAW42mer	0.743±0.314	0.097±0.026	0.0004±0.0001
OAW42	0.507±0.163	0.090±0.017	0.0010±0.0002
Melanoma			
SK-MEL-5	0.440±0.126	0.045±0.012	0.0003±0.0001
JR8	0.327±0.096	0.056±0.022	0.0003±0.0001
Gastric cancer			
GK2	0.0098±0.0002	0.015±0.0004	0.937±0.186
KKP	0.0094±0.0006	0.010±0.0003	0.013±0.002
Leukaemia			
CEM	0.138±0.027	0.133±0.058	0.139±0.038
JURKAT	0.200±0.059	0.146±0.055	0.106±0.055

[a]Data represent mean values ± standard deviations of at least 3 independent experiments.

Immediately before analysis the cells were washed in phosphate-buffered saline (PBS) and stained with a solution containing 50 μg/ml propidium iodide, 50 mg/ml ribonuclease, and 0.05% Nonidet P-40 (solution A) for 30 min at 4°C and then analysed with a 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).

Immunoblotting. Cells exposed to C1311 (IC_{50} and 10 x IC_{50}) for 24 h 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 fluoride, 1 mM Na_3VO_4, 1 mM $NaPPO_4$, and 10 mM NaF. Cell 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 2X 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

Table II. Two-way drug sensitivity comparisons for C1311, doxorubicin and taxol.

	S/S	R/R	S/R	R/S	Agreement rate (%)	Cosensitivity (%)	Coresistance (%)
C1311 versus doxorubicin	5	5	3	3	62.5	45.5	45.5
C1311 versus taxol	2	3	5	6	31.3	15.4	21.4

Cell lines were arbitrarily defined as sensitive (S) or resistant (R) to individual drugs on the basis of the specific median IC_{50} value of each compound: 0.279 μM for C1311, 0.064 μM for doxorubicin and 0.027 μM for taxol.

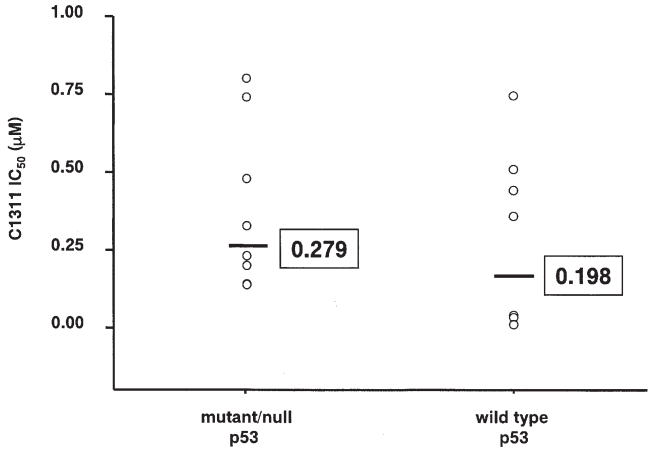

Figure 2. Cytotoxic activity of C1311 (in terms of IC_{50}) as a function of p53 gene status in individual cell lines. The median IC_{50} values of the 2 groups of cell lines are indicated.

blocked in PBS with 5% skim milk and incubated overnight with the primary monoclonal antibodies anti-cyclin B1 and anti-Cdk1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). 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 chemiluminescence Western blotting detection system (Amersham). An anti-PCNA monoclonal antibody (Santa Cruz Biotechnology) was used on each blot to ensure equal loading of protein on the gel.

Immunoprecipitation and assay of cyclin B1-associated Cdk1 kinase activity. Cell lysates (0.5 ml) were mixed with a mouse monoclonal anti-cyclin B1 (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 washed twice with lysis buffer for the kinase assay and then twice in the same buffer minus bovine serum albumin. 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 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 2X 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. The kinase activity of control and treated samples was expressed in arbitrary densitometric units and normalised on the basis of the number of cells in the G_2M phase.

Results

Seventy-two-hour exposure to different concentrations (from 0.001 to 10 μM) of C1311 resulted in dose-dependent inhibition of cell proliferation in all cell lines, and the IC_{50} values, calculated from growth inhibition curves, ranged from 0.0094 to 0.8 μM (Table I). A variable degree of sensitivity to C1311 was also observed within individual tumour types. Among the different cell lines, the highest sensitivity to imidazoacridinone was found in the 2 gastric cancer cell lines KKP and GK2 (IC_{50}, 0.0094 and 0.0098 μM, respectively), which were also more sensitive to C1311 than to doxorubicin (IC_{50}, 0.01 and 0.015 μM) and taxol (IC_{50}, 0.013 and 0.937 μM) (Table I).

When the different cell lines were arbitrarily defined as sensitive or resistant to individual drugs on the basis of the specific median IC_{50} value of each compound (0.279 μM for C1311, 0.064 μM for doxorubicin and 0.027 μM for taxol), no significant association between the *in vitro* responses to C1311 and doxorubicin (concordance rate 10/16 cell lines; 62.5%) or to C1311 and taxol (concordance rate 5/16 cell lines; 31.2%) was found (Table II).

The *in vitro* activity of C1311 was independent of the p53 gene status of the cell lines. Specifically, among the 8 cell lines with mutant or null p53, the IC_{50} of C1311 ranged from 0.138 to 0.8 μM (median 0.279 μM), whereas in the 8 cell lines carrying a wt p53 the IC_{50} values varied from 0.0094 to 0.749 μM (median 0.198 μM) (Fig. 2).

The perturbations induced by C1311 and the 2 reference drugs, taxol and doxorubicin, in the distribution of cells in the different phases of the cell cycle were determined by flow cytometry in 11 cell lines after a 24-h exposure to equitoxic (IC_{50} and 10 x IC_{50}) drug concentrations (Table III). In agreement with what was observed in previous studies (12,13), a C1311-induced increase in the number of cells in the G_2M phase (Fig. 3A) was observed in 9 cell lines after exposure to at least one drug concentration. The extent of such accumulation in the G_2M phase varied among cell lines, ranging from +3% to +50% compared to untreated control cells (Fig. 3B), and it was similar to or greater than that observed with equitoxic taxol concentrations in 5 cell lines (CEM, Jurkat, OAW42, OAW42mer and JR8) and lower than that induced by the taxane in the remaining 4 cell lines (Table III). Moreover, C1311-induced accumulation of cells

Table III. Cell cycle perturbations induced by C1311, doxorubicin and taxol in the different cell lines.

		C1311		Doxorubicin		Taxol	
	Control	IC_{50}	10 x IC_{50}	IC_{50}	10 x IC_{50}	IC_{50}	10 x IC_{50}
WiDr							
$G_{0/1}$	58±1	50±4	28±2	47±4	24±3	16±2	24±9
S	18±3	18±3	25±4	18±3	26±6	12±1	15±4
G_2M	24±1	32±3	47±5	35±5	50±8	72±7	61±10
LoVo							
$G_{0/1}$	54±4	53±5	42±2	51±5	43±6	31±2	27±6
S	18±2	15±2	16±3	19±2	22±4.6	19±2	28±3
G_2M	28±4	31±4	42±8	30±4	35±8	50±7	45±11
GLC4							
$G_{0/1}$	45±1	31±6	34±2	29±6	46±2	27±3	17±6
S	24±4	31±6	37±7	24±3	31±3	19±1	17±1
G_2M	31±1	38±3	28±3	47±8	23±4	54±6	66±8
H460							
$G_{0/1}$	56±9	60±12	39±10	53±9	56±9	39±6	35±9
S	18±1	20±9	14±2	20±6	15±5	21±3	20±5
G_2M	26±3	20±1	47±8	27±1	29±2	40±2	45±4
OAW42mer							
$G_{0/1}$	40±3	21±5	34±5	39±3	36±4	28±5	25±6
S	40±2	19±4	25±4	41±2	33±3	32±3	27±4
G_2M	20±4	60±3	41±3	20±5	31±3	40±4	48±4
OAW42							
$G_{0/1}$	48±5	30±4	42±3	40±3	41±5	29±4	27±4
S	42±4	30±4	40±4	45±3	36±2	36±5	34±6
G_2M	10±4	40±3	18±2	15±4	23±3	35±3	39±4
JR8							
$G_{0/1}$	46±5	28±4	34±5	42±3	40±5	36±2	31±5
S	41±2	22±3	26±4	38±5	26±7	30±4	29±4
G_2M	13±1	50±4	40±3	20±3	34±6	34±3	40±3
GK2							
$G_{0/1}$	45±3	44±5	38±3	43±4	41±3	36±3	32±1
S	28±7	30±2	35±4	29±3	27±2	29±2	31±3
G_2M	27±4	26±4	27±2	28±2	32±4	35±4	37±5
KKP							
$G_{0/1}$	40±2	42±1	43±4	35±2	32±4	36±7	34±4
S	31±4	30±6	31±4	31±4	33±1	30±5	28±6
G_2M	29±3	28±4	26±5	34±3	35±4	34±2	38±1
CEM							
$G_{0/1}$	40±5	12±3	34±3	21±8	36±5	37±4	20±4
S	38±4	46±8	52±8	53±5	54±6	47±8	52±4
G_2M	22±2	42±4	14±4	26±9	10±5	16±21	28±4
Jurkat							
$G_{0/1}$	60±3	14±3	20±2	19±5	47±2	44±14	16±4
S	19±1	15±4	46±3	15±1	44±6	21±1	22±5
G_2M	21±3	71±2	34±4	66±10	9±2	35±9	62±15

Data represent mean values ± standard deviations of at least 3 independent experiments.

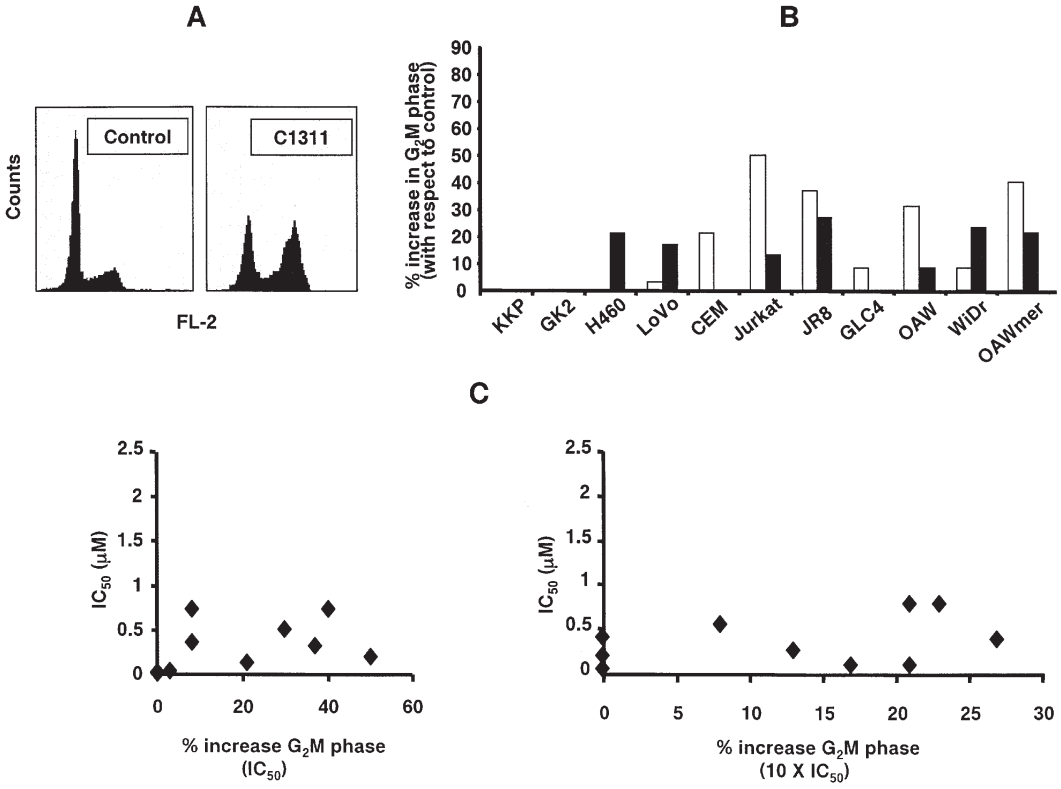

Figure 3. (A) Results of flow cytometric analysis of OAW42 cell cycle distribution after 24-h exposure to C1311 (IC_{50}). (B) Quantitation of the percentage increase in G_2M phase of different cell lines after 24-h exposure to C1311 (empty column, IC_{50}; black column, 10 x IC_{50}). (C) Correlation between C1311 cytotoxic activity (in terms of IC_{50}) and percentage increase in G_2M phase after exposure to C1311 (IC_{50} and 10 x IC_{50}).

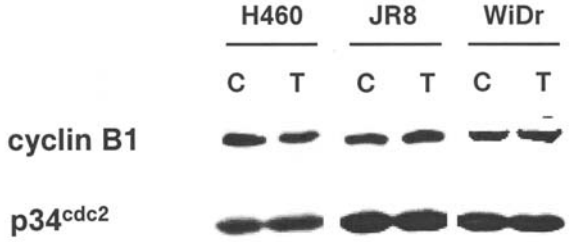

Figure 4. A representative experiment illustrating the effect of C1311 on the expression of proteins involved in the G_2 checkpoint in 3 human tumour cell lines. Cells were incubated with solvent (control) or with the IC_{50} concentration of C1311 for 24 h. Western blots, obtained as described in Materials and methods, were probed with monoclonal antibodies for Cdk1 (p34cdc2) and cyclin B1. C, controls; T, treated samples.

in the G_2M compartment was independent of the level of C1311 cytotoxic activity in individual cell lines (Fig. 3C).

The effect of a 24-h exposure to C1311 (IC_{50}) on the expression of Cdk1 and cyclin B1 proteins, which regulate transition through the G_2 checkpoint, was next determined in the 7 cell lines in which an increase in the G_2M cell fraction >20% with respect to controls was observed. C1311 treatment generally did not affect the expression of cyclin B1 and Cdk1 (Fig. 4). The kinase activity of cyclin B1-associated Cdk1 was then measured on the substrate histone H1. As shown in Fig. 5A, the overall kinase catalytic activity of C1311-treated samples was generally higher than that of controls in the different cell lines. However, when the results were expressed in terms of the kinase activity value divided by the number of G_2M cells [i.e., the cells that together with late S-phase cells mainly account for this specific kinase activity (17)], we found a Cdk1 catalytic activity lower than that of untreated cells in the 4 cell lines showing the most pronounced G_2M accumulation (Jurkat, JR8, OAW42 and OAW42mer) or similar to that of control cells in the remaining 3 cell lines (CEM, H460 and WiDr) (Fig. 5B). We obtained superimposable results by directly immunoprecipitating Cdk1 instead of Cdk1 associated with cyclin B1 (data not shown).

Discussion

C1311 is an investigational compound which has already been proved to have significant antitumour activity in experimental models of human and murine colorectal tumours (4) as well as in human ovarian carcinoma and osteogenic sarcoma cell lines (18). In this study we demonstrated appreciable cytotoxic activity of this compound in a large panel of cell lines derived from human leukaemias and solid tumours, some of which (such as lung and gastric cancers and melanoma) had not been tested before for their sensitivity to the drug. We compared the cytotoxicity of C1311 with that of doxorubicin and taxol as reference drugs in the same cells to ascertain whether or not there was cross-resistance between the compounds. No significant cross-resistance between these agents was observed in our cell lines. This finding, together with previous evidence indicating that C1311 is a weak substrate for P-glycoprotein (19) in contrast to doxorubicin and taxol, would suggest the

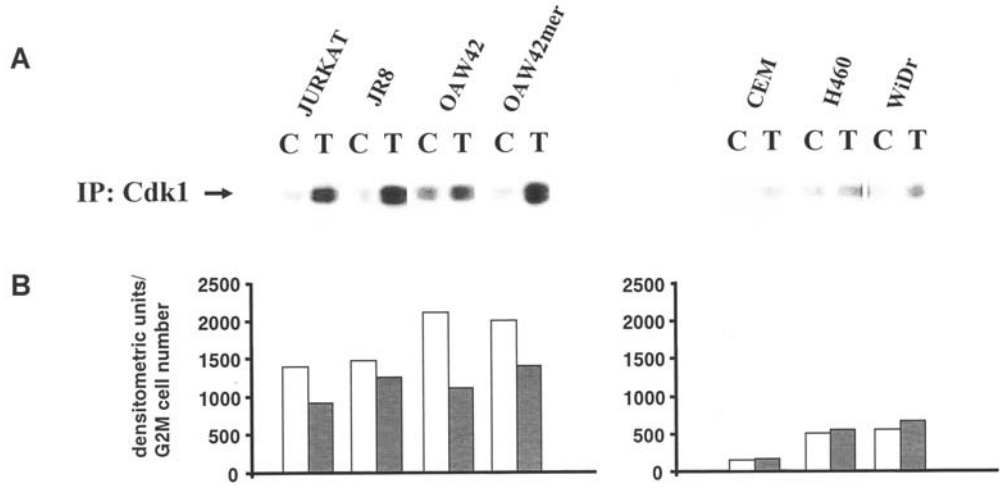

Figure 5. Effect of C1311 on Cdk1 kinase activity in human tumour cell lines. (A) Cells were incubated with solvent (control) or with the IC_{50} concentration of C1311 for 24 h. Immunoprecipitation and kinase assay were performed as described in Materials and methods. (B) Following autoradiography, the reactions were quantified by densitometry. For each sample the kinase activity was expressed as arbitrary densitometric units divided by the number of cells in the G_2M phase.

interesting possibility that C1311 could be used for the clinical treatment of tumours refractory to anthracyclines and taxanes. Moreover, although the analysis was carried out in a panel of cell lines with different genetic backgrounds, our results would suggest that p53 gene status is not a major determinant of tumour cell sensitivity to C1311.

Interestingly, the highest sensitivity to C1311 was observed in the 2 gastric cancer cell lines, which were also more sensitive to the imidazoacridinone than to doxorubicin and taxol. Although several drugs belonging to different classes have proven clinical activity in gastric cancer, approximately half of all patients are resistant to chemotherapy (20). Experimental studies have related such chemoresistance to the inducible activity of gene products, such as the transcription factor NF-κB (21) and others (22), which counteract apoptosis. Based on these findings, new and more effective drugs and treatment strategies for gastric cancer are needed, and C1311 could be a good candidate for phase II clinical trials in this malignancy.

When we assessed the interference of C1311 with cell cycle progression, we observed significant C1311-induced G_2M accumulation in 7 of 11 cell lines tested in this study, in accordance with previous findings obtained in L1210 murine leukaemia cells (12), and in human cell lines from cervical (13) and ovarian carcinoma, and osteosarcoma (18). Moreover, in line with our previous observations (18), we found that the cytotoxic effect of C1311 observed in the different cell lines was not related to the extent of G_2M accumulation. Since there was considerable variability in the extent to which the different cell lines showed accumulation in the G_2M phase after exposure to equitoxic C1311 concentrations, 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 complex constituted by the catalytic subunit Cdk1 and the regulatory subunit cyclin B1 (17), we evaluated the effect of C1311 treatment on the cyclin B1-associated Cdk1 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 the 4 cell lines with the greatest extent of G_2M accumulation, Jurkat, JR8, OAW42 and OAW42mer, 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 (23). Since no significant decrease in cyclin B1 or Cdk1 protein expression was observed after C1311 treatment, the fall in Cdk1 catalytic activity cannot be considered the consequence of a reduced formation of the cyclin B1-Cdk1 complex. The inability of these cells to escape the G_2 block could be tentatively explained by assuming that the active cyclin B1-Cdk1 complexes are confined to the cytoplasmic compartment. 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 (25).

Flow cytometric analysis carried out after 24-h exposure to C1311 failed to indicate the presence of the apoptotic pre-G_1 cell peak in all cell lines but the Jurkat leukaemia cell line, in which a modest fraction of cells with an apoptotic DNA content was observed after exposure to the highest drug concentration (data not shown). This finding would suggest that the apoptosis programme is not directly activated in C1311-treated cell lines, in accordance with our previous observation indicating that the onset of apoptosis induced by the imidazoacridinone in human osteosarcoma cells was delayed until 48 h after drug treatment (18). Burger *et al* (10) suggested that the process of programmed cell death in C1311-treated cells could be the result of cellular autolysis. In fact, these authors demonstrated that in HT29 human colon cancer cells continuous exposure to C1311 induced lysosomal rupture and a significant increase in acid phosphatase activity at earlier time points with respect to those at which apoptosis was observed (10). More recently, Hyzy *et al* (11) showed that a

pulse (3 h) exposure of the same cell line to C1311 failed to induce apoptosis but caused delayed cell death in a process resembling abortive mitosis or mitotic catastrophe. All these data seem to indicate that the induction of apoptosis is not the main cell killing mechanism induced by the imidazoacridinone and may explain, at least in part, the negligible effect of p53 gene status on the tumour cell response to this agent.

Overall, our results suggest that C1311 is active against a large panel of cell lines derived from human solid tumours and leukaemias. The lack of cross-resistance of this compound with doxorubicin and taxol suggests that different molecular targets are involved in the pathways of cellular response to the drugs. Such findings indicate that it would be interesting to carry out further studies for the identification of possible preferential targets of C1311 in human tumours with a view to evaluating the potential clinical use of the compound.

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