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Ewa Augustin · Denys N Wheatley · Justin Lamb
Jerzy Konopa

Imidazoacridinones arrest cell-cycle progression in the G2 phase of L1210 cells

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Abstract Imidazoacridinones are a new class of highly potent antineoplastic agents synthesised at the Technical University of Gdansk. The pharmacophoric alkyldiamine group, which is also present in anthracenediones (e.g. ametantrone, mitoxantrone), has been shown to be responsible for their antineoplastic activity. In view of their chemical similarity to anthracenediones, we anticipated that the imidazoacridinones would have a mechanism of action similar to that of these agents and that this would be reflected by a similar influence on cell-cycle progression. Flow cytometry was used to monitor the effect of three derivatives of imidazoacridinone (C-1263, C-1310 and C-1311) on L1210 cell cycle traverse at concentrations ranging from 0.01 to 0.9 μg/ml, corresponding to their 50% and 90% effective concentrations (EC_{50} and EC_{90} values), over times of drug treatment ranging from 1 to 48 h. The results demonstrate that all of the compounds produced a similar effect, inducing preferential and complete arrest (accumulation) of cells in the G2 phase of the cell cycle (i.e. G2 block). The kinetics of the induction of G2 arrest were dependent on both the dose and the duration of treatment. Cell-cycle arrest was reversible for up to about 3 h of treatment, being quite irreversible at longer incubation times. Microscopic inspection of cells performed in parallel with flow cytometry confirmed that imidazoacridinones induced a G2, not a G2/M, block.

Key words Imidazoacridinones · Cell cycle · *G*2 arrest Flow cytometry

E. Augustin · J. Konopa (✉)
Department of Pharmaceutical Technology and Biochemistry, Technical University of Gdansk, Narutowicza Street 11/12, 80-952 Gdansk, Poland

D.N. Wheatley · J. Lamb
Cell Pathology Laboratory, Department of Pathology, University Medical School, Foresterhill, Aberdeen AB9 2ZD, Scotland, UK

Introduction

Imidazoacridinones are a new group of potent antitumour compounds whose development was based on the results of our studies on the mechanism of action of mitoxantrone [10, 11]. Diaminoalkyl groups in the side chains of mitoxantrone undergo metabolic activation [11] and subsequently play a key role in the formation of covalent interstrand DNA cross-links. Indeed, the presence of these moieties is a prerequisite for the biological activity of this compound (see [4] for review). The polycyclic anthraquinone nucleus seems to be needed both to effect appropriate steric orientation and docking of the pharmacophoric dialkylamino grouping within DNA and to modulate its metabolism.

On the basis of these findings it was proposed that attachment of the diaminoalkyl group to polycyclic moieties with DNA-intercalative properties would yield a new series of antineoplastic agents. This hypothesis was corroborated by our successful development of two classes of highly active antitumour derivatives, viz., the imidazoacridinones [5, 7] (see Fig. 1) and the triazoloacridinones [6]. Besides the diaminoalkyl residue, the hydroxyl group in position 8 of the imidazoacridinone core is significant for the biological activity of these compounds; i.e. imidazoacridinones and triazoloacridinones possessing 8-hydroxy or 8-methoxy groups were much more active than their unsubstituted analogues [5–7].

The biological activity of the imidazoacridinones has now been extensively investigated. In addition to in vitro studies against murine leukaemia cells [5, 7], imidazoacridinones have displayed significant differential cytotoxicity in vitro against the 64 human tumour lines of the National Cancer Institute (NCI) screen. Several of the derivatives that performed well in this primary screen have been further evaluated on human tumour xenografts in nude mice (unpublished data). Other in vivo studies have shown that

imidazoacridinones possess strong antitumour activity against leukaemia P388 [5, 7], melanoma B16, ascites colon 26 adenocarcinoma, and colon 38 adenocarcinoma in mice [12].

The biochemical mechanism of action of the most active imidazoacridinones is currently under investigation. Although imidazoacridinones intercalate DNA, there has been no obvious direct or positive correlation between this ability and their cytotoxic properties (unpublished data). Inhibition of topoisomerase II is an activity exhibited by several antineoplastic agents, including mitoxantrone [17], and is an activity shared with imidazoacridinones. The interaction of imidazoacridinones with the enzyme caused the inhibition of catalytic activity, stabilisation of cleavable complexes of DNA topoisomerase II, as well as influencing the decatenation properties of topoisomerase II (unpublished data).

The structural similarity of the imidazoacridinones to the anthracenediones, mitoxantrone and ametantrone (particularly the presence of the same diaminoalkyl groups in these compounds), together with their common molecular activities, indicate that several other steps involved in their mode of action might also be comparable. Given that the first biological effect observed in tumour cells exposed to low concentrations of mitoxantrone is preferential accumulation of cells in the G2 phase of the cell-cycle [8, 18], we decided to investigate whether the imidazoacridinones share these cell cycle effects and to determine the relative efficacies of several of the more powerful agents in this series in this regard.

Materials and methods

Reagents and chemicals

Imidazoacridinones were synthesised in the laboratories of the Technical University of Gdansk. Solutions of the compounds in distilled water, sterilised by filtration, were prepared immediately before use. Nonidet P40 was obtained from BDH Chemicals Ltd. (UK), and spermine tetrahydrochloride, trypsin, trypsin inhibitor, ribonuclease A, propidium iodide and tris-(hydroxymethyl)-aminomethane were supplied by Sigma Chemical Company (UK). All other reagents were of analytical grade and were obtained from local sources. Cell-culture supplies, including RPMI 1640 medium and foetal calf serum, were acquired from Life Technologies (UK). Antibiotics were obtained from Serva (Heidelberg, Germany). Chicken red blood cells (CRBC) were kindly provided by Craibstone Poultry Unit (Bucksburn, Aberdeen, Scotland, UK).

Cell culture

L1210 murine leukaemia cells (purchased from Gibco Europe Ltd.) were maintained in exponential growth at 37°C in a humidified 5% CO_2 atmosphere in RPMI 1640 medium supplemented with 10% foetal calf serum and antibiotics (100 μg/ml streptomycin, 100 U/ml penicillin). Under these growth conditions the cell-doubling time was 12–13 h.

Cytotoxicity assay

The cytotoxic activity of the imidazoacridinones against L1210 cells was determined after 48 h of continuous treatment with the drugs. Exponentially growing cultures were exposed to drug concentrations ranging from 0.005 to 0.5 μg/ml. After 48 h of incubation, cellular protein was assayed by the method of Lowry et al. [14] as modified by Schacterle and Pollack [15]. Cytotoxic activity was expressed as an EC_{50} value—the concentration required to inhibit the increment of cellular protein by 50%—determined as such from dose-response curves.

Flow-cytometry analysis

L1210 cultures were exposed in the logarithmic phase of growth to drugs at concentrations ranging from 0.01 to 0.9 μg/ml for between 1 and 48 h. Cells were harvested by centrifugation, washed twice with ice-cold phosphate-buffered saline (PBS), resuspended in 50 μl PBS and mixed with the same volume of a CRBC suspension. The nuclei were isolated and stained with propidium iodide by the method of Vindeløv et al. [19]. Nuclear fluorescence was recorded for each population, with doublet discrimination, using an EPICS Profile-II flow cytometer (Coulter Electronics Inc., Hialeah, Fla., USA). In all 10,000 nuclei were processed per sample. The resulting histograms of DNA-content frequency were decomposed using the CYTOLOGIC software package (Coulter Electronics Inc.).

For studies on the reversibility of the cell-cycle effects induced by imidazoacridinones, exponentially growing cells were exposed to the compounds at the indicated concentrations for periods ranging from 1 to 24 h. The medium was withdrawn and the cells were washed twice with PBS, resuspended in fresh medium and incubated for a further period of up to 48 h. Cell-cycle analysis was conducted as described above.

Mitotic index determination

After incubation with drug for the appropriate times, cells were centrifuged for 5 min at 800 *g* and washed twice with PBS. The supernatants were withdrawn and the cells were resuspended in 0.5 ml of fixing solution (3 parts methanol to 1 part acetic acid). The cells were respun, the fixative was entirely removed and the pelleted cells were resuspended in three drops of crystal violet solution (0.02% in 1% acetic acid). The mitotic index of each sample was determined microscopically by scoring at least 1,000 cells for mitotic frequency as a percentage.

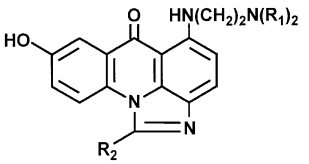
Results

Cytotoxic activity

Growth-inhibition curves obtained for L1210 cells after 48 h of continuous exposure to the chosen imidazoacridinones demonstrated a concentration-dependent effect (data not shown). The EC_{50} and EC_{90} values for each of the compounds studied are shown in Fig. 1.

Flow cytometry

The influence of the three imidazoacridinone derivatives (C-1263, C-1310 and C-1311) on the cell-cycle



compound	R_1	R_2	EC_{50} (μg/ml)	EC_{90} (μg/ml)
C-1263	CH_3	H	0.02	0.3
C-1310	CH_2CH_3	CH_3	0.1	0.9
C-1311	CH_2CH_3	H	0.01	0.3

Fig. 1 Chemical structure of chosen imidazoacridinones and their EC_{50} and EC_{90} values

progression of L1210 cells was studied over concentrations ranging from 0.01 to 0.9 μg/ml (including those levels corresponding to their EC_{50} and EC_{90} values) and over continuous exposure times varying from 1 to 48 h. Histograms representing the distribution of cells through the cycle following continuous treatment with the aforementioned compounds at one representative concentration (0.05 μg/ml) are shown in Fig. 2, whereas the cycle-phase distributions over the complete treatment matrix are shown in Fig. 3.

In the cultures treated with C-1263 (Fig. 3a) at a concentration of 0.01 μg/ml, cells accumulated in the S phase by 6 h. This effect was transient, however, and by 12 h there was an increase in the number of cells in the G2 phase, with a corresponding decrease in the G1- and S-phase fractions. At between 24 and 48 h after the treatment there was a trend towards a return to control-phase distribution. After treatment with C-1263 at a concentration of 0.05 μg/ml (corresponding to the EC_{50} level), there was a more dramatic change in cell-cycle distribution. Transient S-phase accumulation was again observed, with cells preferentially accumulating in the G2 phase by 24 h of treatment, giving a G2-phase fraction of around 88%. An apparent increase in the G1- and S-phase populations, with a concomitant decrease in the G2 fraction, was observed after 36 and 48 h. This effect was presumably the result of a loss of G2 cells from the culture, possibly due to phase-selective cell disintegration. At the highest concentration examined (0.3 μg/ml, EC_{90} value), the effects were qualitatively similar to those observed for treatment at 0.05 μg/ml. All S-phase fractions were higher at this concentration, indicating greater impediment to S-phase transit, but preferential G2 accumulation was again evident, the G2 fraction approximating 70% by 36 h.

Fig. 3b illustrates the cell-cycle distribution of L1210 cells exposed to C-1310. Changes in cell-cycle distribution were evident after 6 h at all concentrations studied, and the effects were similar to those observed for C-1263. Treatment at a concentration of 0.1 μg/ml (corresponding to the EC_{50} value) led to G2-phase accumulation, which reached a maximal level of around 75% by 24 h. Treatment at a concentration of 0.9 μg/ml (EC_{90} value) resulted in the G2-phase fraction increasing with exposure time from 57% by 12 h, through 79% by 24 h, to 86% by 36 h, the latter being maintained to 48 h.

Cell-cycle effects exhibited by C-1311 are shown in Fig. 3c. The effects were again similar to those observed for C-1263. At a concentration of 0.05 μg/ml (EC_{50} value), we observed an accumulation of cells in the G2 phase, with the fraction increasing with exposure time (72% by 12 h, 90% by 24 h, 94% by 36 h). At a concentration of 0.3 μg/ml (EC_{90} value), the cell-cycle distribution pattern was virtually identical to that observed at the comparable level of C-1263. G2-phase accumulation was evident, being maximal at 66% after 24 h.

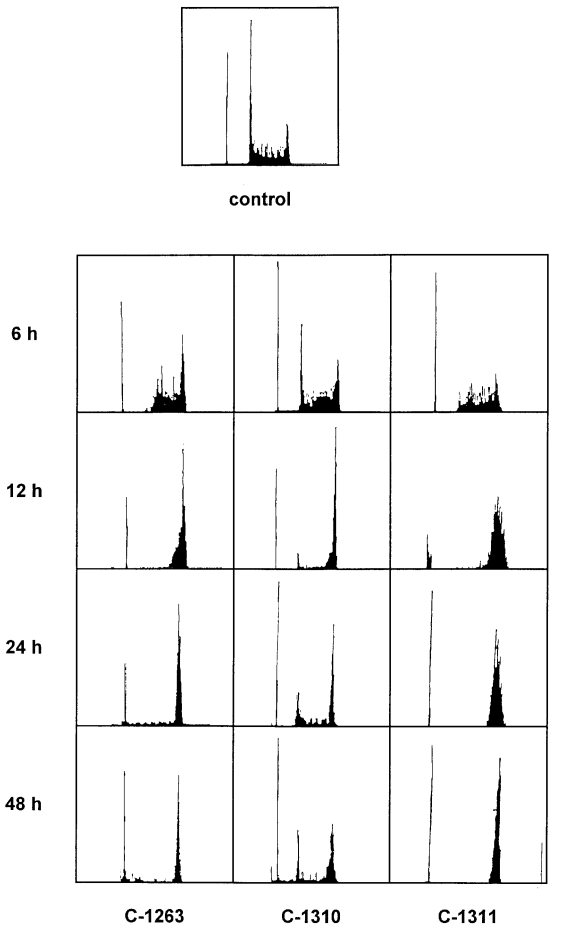
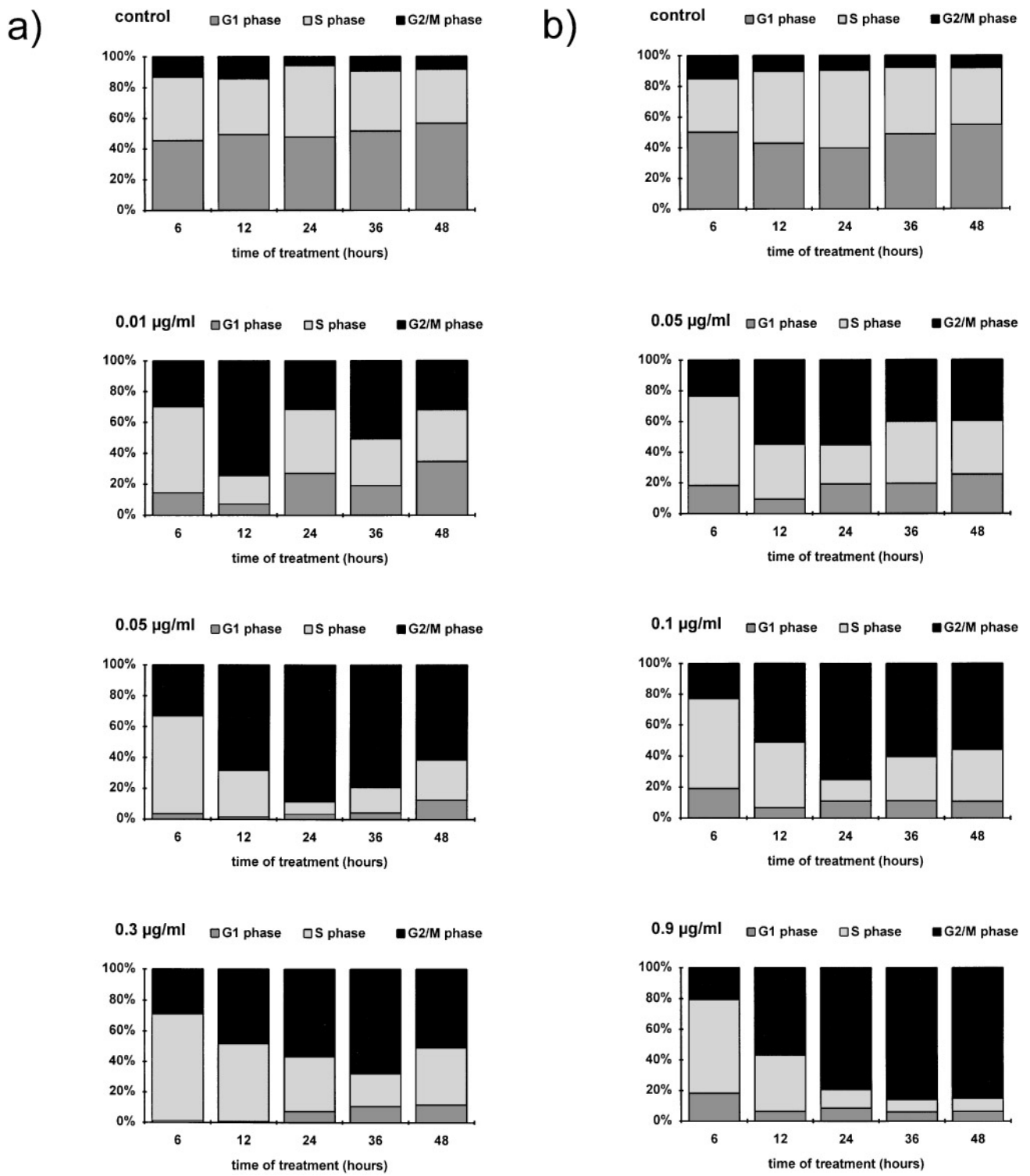


Fig. 2 Histograms representing the distribution of L1210 cells through the cell cycle following treatment with each imidazoacridinone at a concentration of 0.05 μg/ml for different times. Each histogram is a plot of nuclear DNA content against frequency. *The left-most peak* in each histogram is an internal reference standard

Our data show that all three imidazoacridinones induced preferential accumulation of L1210 cells in the G2 phase at concentrations that inhibited cell proliferation. The kinetics of the induction of G2 arrest by the imidazoacridinones depended on both the dose and the time of incubation.

Results obtained in our study on the reversibility of the drug-induced cell-cycle effects were similar for all three compounds; therefore, data are presented for C-1311 only (Fig. 4). This compound induced a temporary inhibition of cell-cycle traverse with a G2-phase accumulation after treatment for up to 3 h. This cycle



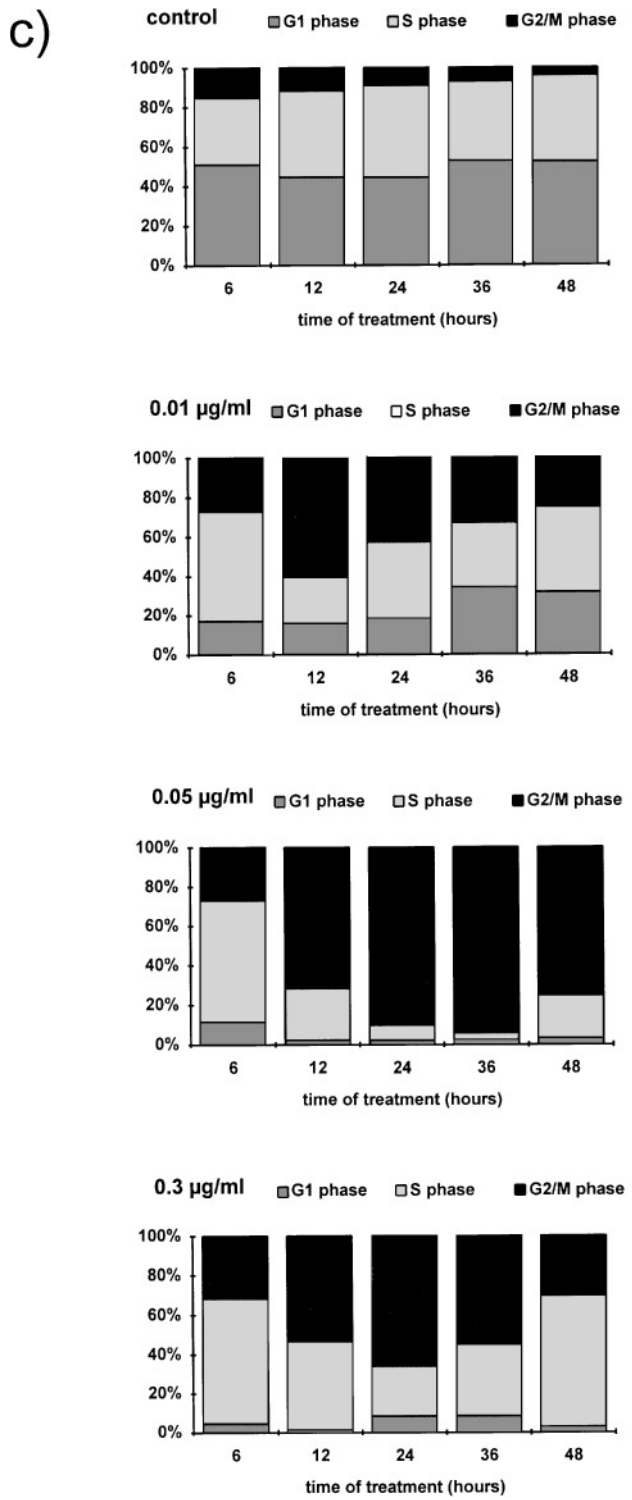


Fig. 3a–c Distribution of L1210 cells following treatment with **a** C-1263, **b** C-1310 and **c** C-1311 for the times indicated

perturbation was overcome, and a control-cycle distribution was restored by 48 h post-exposure (Fig. 4A). At longer exposure times, the G2 accumulation produced by C-1311 became increasingly persistent and was accompanied by evidence of cell disintegration (Fig. 4B).

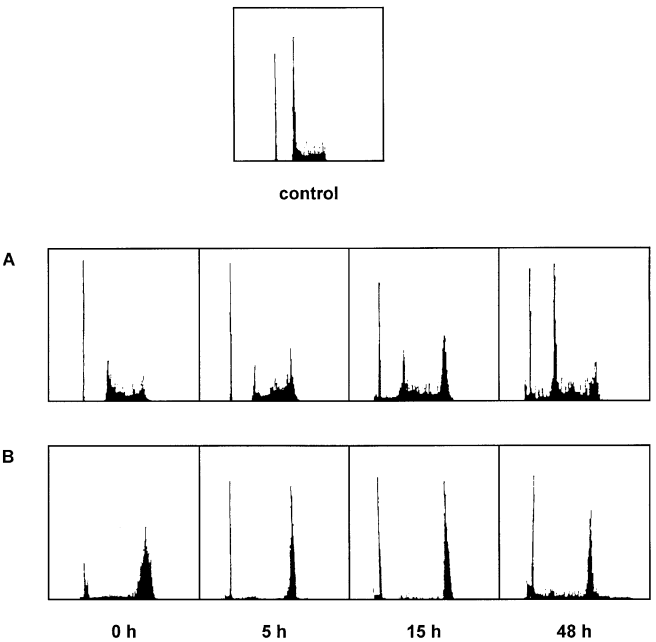


Fig. 4A, B Reversibility of the G2 block following incubation with C-1311 at 0.05 µg/ml for **A** 3 h and **B** 12 h and at different post-incubation times for (details, see Fig. 2)

Mitotic index determination

All the imidazoacridinones studied induced a G2 block, not a G2/M block, as judged by the absence of mitotic figures (data not shown). Indeed, mitotic figures disappeared within 1 h from cultures exposed to doses of imidazoacridinones that caused complete G2 arrest. The G2/M phase has a duration of 2–3 h in the L1210 cell line.

Discussion

Inhibition of the (tumour) cell cycle in the G2 phase by imidazoacridinones leads to an immediate arrest of proliferation. Together with the fact that G2 arrest is the first easily visible biological effect induced by imidazoacridinones, it contradicts generally accepted opinion that the basic phenomenon underlying the activity of antitumour compounds interacting with DNA, imidazoacridinones included, is inhibition of DNA replication, in other words, induction of an S-phase block [9].

The ability of imidazoacridinones to induce G2-phase accumulation confirms a resemblance in mode of action to that of the structurally similar anthracenediones, especially their major representative, mitoxantrone [8, 18]. The irreversibility of the G2 block induced by imidazoacridinones resembles that of other groups of potent antitumour compounds, e.g. cisplatin [3] and doxorubicin (Adriamycin) [1].

The origin of the G2 arrest induced by the imidazoacridinones may reside in an ability to cause interstrand DNA cross-links. Mitoxantrone, which shares structural similarities with the imidazoacridinones, exhibits this activity [10] and all DNA-cross-linking agents are known to induce G2 block as the first biological effect [9]. Recently, the imidazoacridinones have been identified as topoisomerase II inhibitors (unpublished data), and this activity, also being associated with induction of G2-phase accumulation, may provide a mechanistic basis for the observed cell-cycle perturbations. The ability of the imidazoacridinones to arrest the cell cycle in the G2 phase could originate from both of these effects: interstrand DNA cross-linking and inhibition of topoisomerase II.

Demonstration that the irreversible arrest of cell-cycle progression in the G2 phase represents the first biological effect induced by imidazoacridinones in vitro allows one to expect a similar effect under in vivo conditions. If this is the case, as has been discussed elsewhere [9], therapeutic doses used in clinical practice should be as low as required to induce exclusive G2 block. At higher doses, arrest in other phases of the cell cycle (i.e. S and G1) can be expected, and undesirable toxic effects will be observed [9].

The question as to what happens to tumour cells irreversibly blocked in the G2 phase as a result of treatment with imidazoacridinones arises. By analogy to cisplatin [2] and anthracyclines [13, 16], which also induce this block as the first biological effect, cells might spontaneously undergo programmed cell death (apoptosis). The cytostatic action of the imidazoacridinones may therefore be spontaneously transformed into a cytotoxic effect, and this phenomenon is now being investigated in greater depth.

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