G2 cell cycle arrest and apoptosis are induced in Burkitt's lymphoma cells by the anticancer agent oracin

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Abstract The cytotoxic effect of the new potential intercalating anticancer drug oracin was studied on Burkitt's lymphoma cell line that overexpressed bcl-2 (BL bcl-2) and a control transfectant without the bcl-2 gene (BL SV2). Oracin showed a marked cytostatic effect on both BL SV2 and BL bcl-2 cells. IC_{50}, as measured by the MTT assay, was approx. 5-times greater for BL bcl-2 cells (5.0 μmol/l) than for BL SV2 cells (1.0 μmol/l). There was no significant increase in apoptosis after 24 h of treatment with oracin (1.0 μmol/l) in both cell lines. However, after 48 h from the removal of oracin in BL SV2 culture the levels of apoptotic and secondary necrotic cells increased to 20 and 37%, respectively. In contrast, BL bcl-2 cells treated in a similar manner showed only basal levels of apoptotic and secondary necrotic cells. Analysis of the cell cycle profiles showed a significant increase of S and G2/M phases of the cell cycle in both cell lines after 6 h of drug treatment (1.0 μmol/l). The cells were arrested in G2/M phase of the cell cycle after 24 h, with no significant changes in cell viability. After 72 h, the viable BL SV2 cells were still in G2/M, however, the viability of this culture had fallen to approx. 5%. Flow cytometry analysis of the DNA content revealed the presence of a 'sub-G2' region, which represented the apoptotic cells. The BL SV2 cells died after 72 h while they were in the G2/M phase. Although the treated BL bcl-2 cells were similarly arrested in the G2/M phase, they nevertheless remained with a relatively high viability (68%).

Key words: Apoptosis; Cell cycle; Oracin; G2 arrest; bcl-2; Anticancer agent

1. Introduction

Oracin is a novel anticancer agent of the idenoisoquinoline chemical group and it is both structurally and pharmacologically related to the clinically used anti-cancer agent mitoxantron (Novatron). The pharmacological, biochemical and toxicological characterisation of oracin has been reported by Melka [1]. The report indicated that oracin has a significant cytotoxicity against a wide spectrum of tumor and leukaemia cells both in vitro and in vivo. The planar fundamental structure of the compound is typical for intercalating antitumor agents (anthracyclines, e.g. adriamycin-like drugs and/or anthracenediones, e.g. mitoxantrone-like agents). The main advantage of oracin is the lack of cardiotoxicity which is a dose-limiting adverse effect of the doxorubicin-like drugs. There is also a possibility of its oral administration.

Treatment of tumor cells with cytotoxic agents or with ionising radiation usually results in the breakdown of the cell cycle machinery. The subsequent decision of the treated cell 'to die or not to die' is the outcome of the balance of promoting and protecting factors in which the capacity of DNA repair plays a crucial role [2]. Diverse anti-cancer agents may induce a mode of death which has characteristics of 'programmed' cell death or apoptosis [3]. Apoptosis is a process whereby intrinsic or extrinsic stimuli activate a molecular programme to implement a specific series of events that culminate in cell death. The entry of a cell into apoptosis can be restricted to a particular phase of the cell cycle [4,5], although this cell cycle dependence does not appear to be a general process [6]. Moreover, circumstantial evidence of the induction of apoptosis due to damage to the cell cycle or to DNA integrity and the presence of many oncogenes that promote cell cycle progression also inducing apoptosis has implied the existence of a direct linkage between the two antagonistic processes [7].

It is known that the bcl-2 gene plays an important role in the regulation of apoptosis [8]. The bcl-2 gene product is a membrane-associated GTP-binding protein that has been shown to inhibit apoptosis triggered by a variety of stress stimuli, including serum deprivation, radiation and cytotoxic agents [9–14]. It is well documented that bcl-2 becomes deregulated in tumor cells [4] which may constitute a barrier to the success of cancer therapy. Therefore, we studied in vitro the protective effect of bcl-2 on cell cycle arrest induced cell death in Burkitt's lymphoma cells by the cytotoxic agent oracin.

2. Materials and methods

All reagents, except fetal calf serum (Gibco Ltd), were from Sigma Ltd. Oracin was provided by the Research Institute for Pharmacy and Biochemistry, Prague (Czech Republic).

The cell lines, Burkitt's lymphoma transfected with control (BL SV2) and bcl-2 (BL bcl-2) plasmids, were kindly provided by Dr. C.D. Gregory (Department of Immunology, University of Birmingham, UK). Details of the transfection protocols have been described by Milner et. al. [15]. Cells were cultured in RPMI 1640 containing 10% fetal calf serum, at 37°C in a humid atmosphere of 5% CO_2/air.

2.1. Cytotoxicity assay

Different concentrations of oracin were added to the cells at a density of 6×10^5 cells/ml (concentration of DMSO was maximally 1% v/v). After 24 h incubation in 5% CO_2, incubator samples were analysed by standard MTT assay as described by Al-Rubeai [16]. The IC_{50} values of oracin were then calculated for both BL SV2 and BL bcl-2 cell lines.

2.2. Apoptotic response

Cells suspension were mixed with an equal volume of staining solution containing 10 μg/ml acridine orange and 10 μg/ml propidium iodide as described by Simpson et. al. [14]. The percentages of viable, apoptotic and necrotic cells were determined in an improved Neubauer rhodium-coated haemocytometer under a fluorescence microscope. Results were expressed as a proportion of the total cell number (%), established by nucleus morphology and propidium iodide exclusion.

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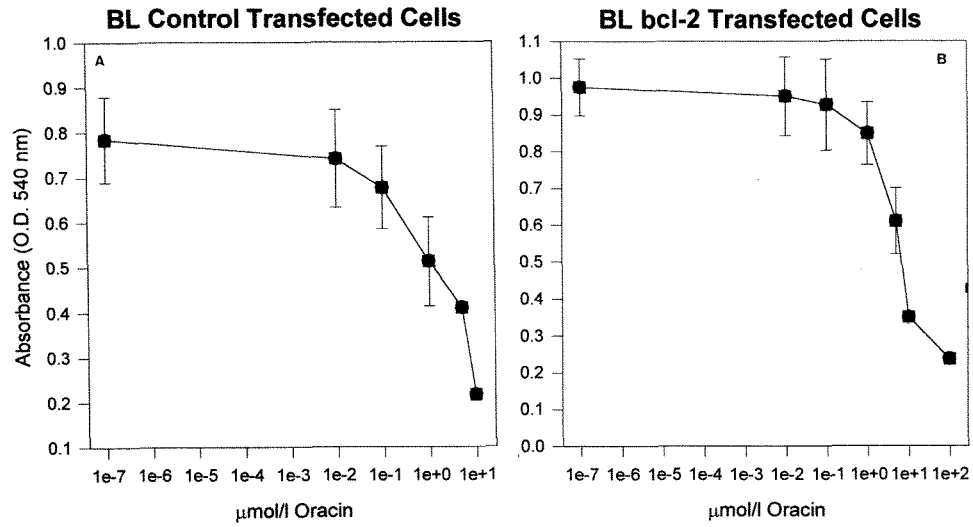

Fig. 1. (A,B) Cytotoxic effect of oracin on Burkitt's lymphoma (BL) cells. Cells were treated with different concentrations of the drug for 24 h and analysed by the MTT assay.

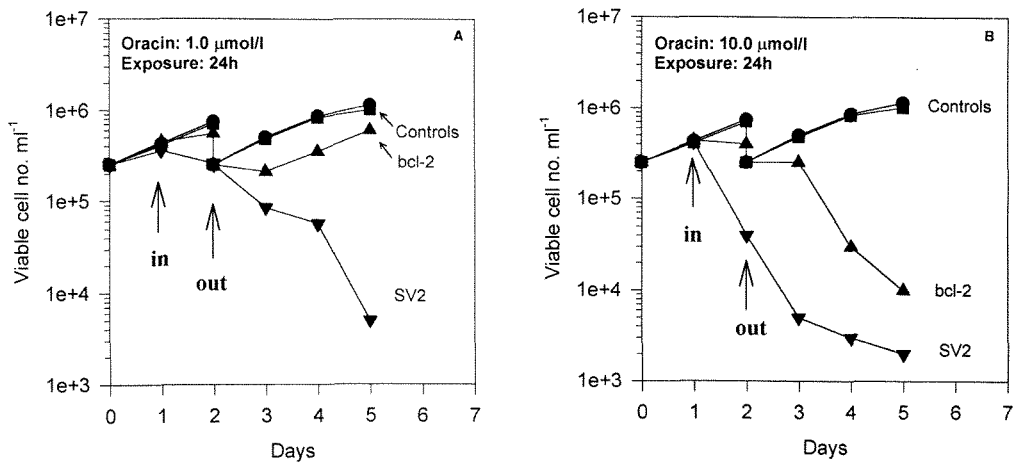

Fig. 2. (A,B) Effect of 0, 1, and 10 µmol/l oracin on growth of BL cells. Cells were treated with oracin for 24 h at day 1. On day 2 the cells were washed and incubated in fresh medium. bcl-2, BL transfected with bcl-2; SV2, BL transfected control.

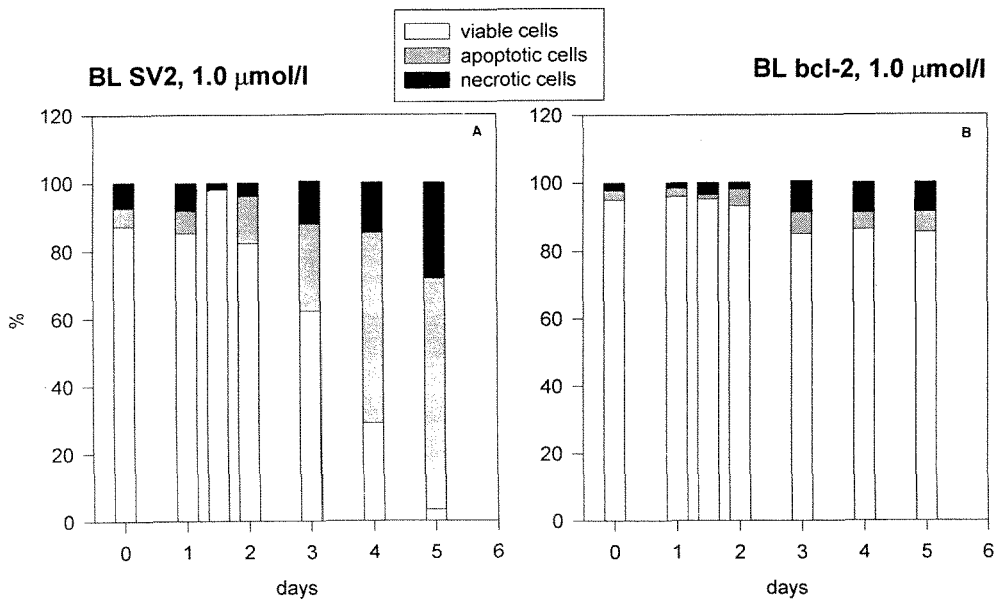

Fig. 3. (A,B) Percentage of viable, apoptotic and necrotic BL cells. Non-fixed cells were stained with acridine orange and propidium iodide.

2.3. Cell cycle and cell size analysis

BL cells were inoculated into 20-ml T-flasks (with viable cell density 3×10^5 cells/ml). After 24 h incubation in CO_2 incubator different concentrations of oracin were added. Samples of cell density approx. 1×10^6 cells/ml were removed at intervals, spun down (1000 rpm, 5 min), washed once with PBS and fixed with cold 70% ethanol and kept at 4°C. After 24 h the samples were centrifuged as before, washed once with PBS and incubated with 100 μl of 0.5 mg/ml RNase for 30 min. Cells were washed once with PBS and resuspended in 0.5 ml of PBS and 40 μl of 0.5 mg/ml propidium iodide. The samples were analysed on a Coulter Epics Elite flow cytometer.

3. Results and discussion

MTT assay was used to determine the cytotoxic effect (IC_{50}) of oracin on BL SV2 cells and BL bcl-2 cells (Fig. 1A,B). Drug treatment for 24 h led to IC_{50} values of 1 ± 0.1 and 5 ± 0.2 μmol/l for BL SV2 and BL bcl-2 cells, respectively. An approx. 5-times higher value of IC_{50} for BL cells with bcl-2 over-expression supports the hypothesis that expression of bcl-2 provides protection against cytotoxic drugs. Treatment with oracin (1.0 μmol/l) significantly decreased the viability of control SV2 transfectants (Fig. 2A). This loss of viability was

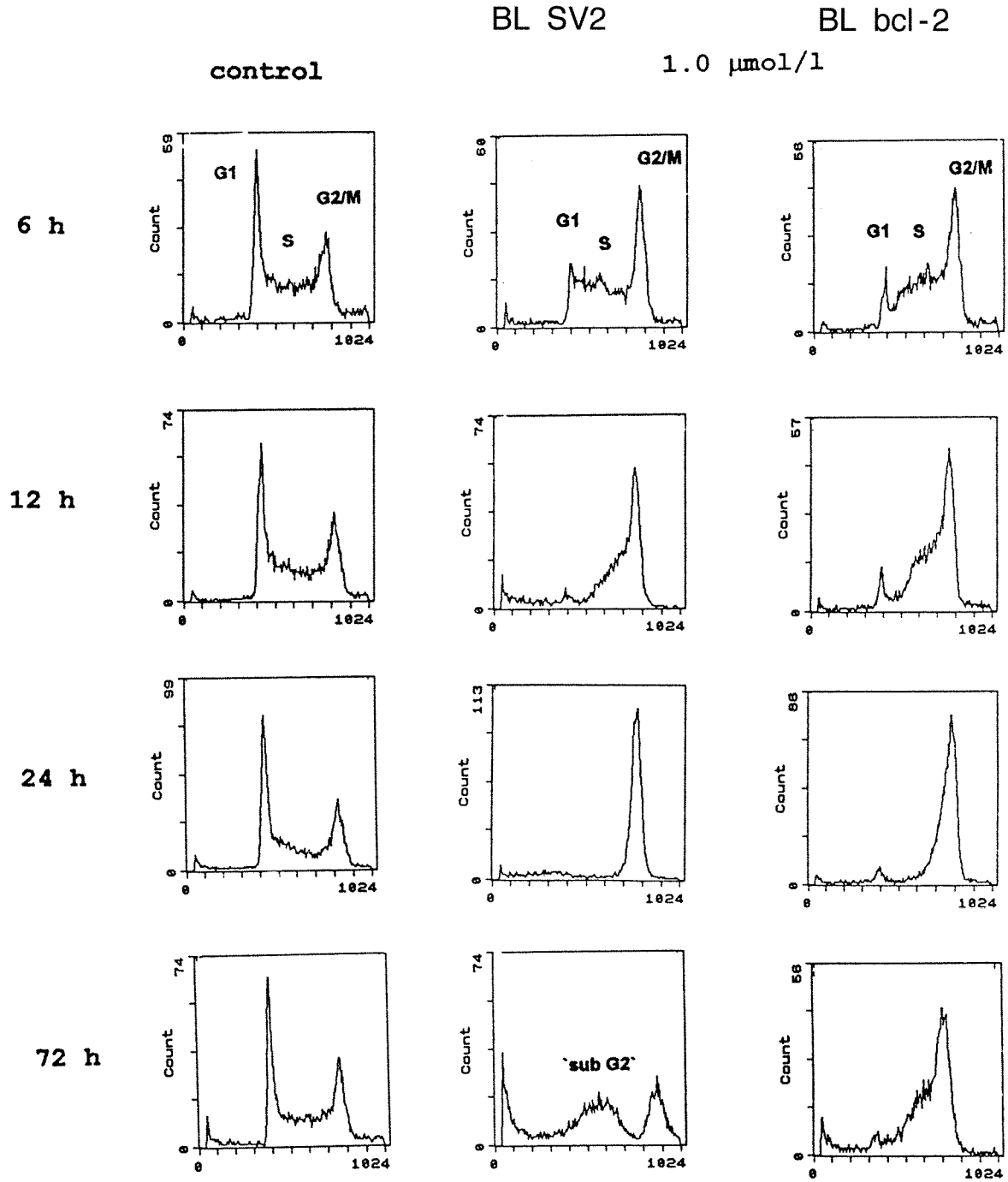

Fig. 4. Progression of BL cells through the cell cycle phases in the absence and presence of oracin. DNA histograms of oracin-treated cells at 24 h show accumulation in G2 phase. The 'sub-G2' peak representing the apoptotic cells is seen at 72 h incubation time.

significantly reduced in the bcl-2 transfectants. The sensitivity of SV2 cells was clearly demonstrated after exposure to 10 μmol/l oracin (Fig. 2B). Cell viability sharply decreased by 2 orders of magnitude within 48 h after treatment while the bcl-2 viable cells remained nearly unaffected during the same period. However, unlike the 1.0 μmol/l condition, viable cell number sharply reduced on days 3 and 4. After the first 12 h of drug treatment, the level of apoptotic cells was unchanged in both cell lines (Fig. 3). A significant change in percentage of apoptotic cells in SV2 was observed 24 h after drug removal (26%), whereas bcl-2 transfected cells exhibited only basal level of apoptosis (6%). 72 h after removal of oracin the level of apoptosis in SV2 transfectants was approx. 70% and viability of cell culture had fallen to 4% (Fig. 3). In contrast, the bcl-2 cells continued to show high cell viability (86%) and an unchanged level of apoptotic cells (7%). An increase in the size of cells treated with oracin in comparison with untreated cells was also observed after 24 h (data not shown).

The DNA histograms of SV2 and bcl-2 cells exposed continuously to 1.0 μmol/l of oracin together with the untreated control are shown in Fig. 4. Analysis of the cell-cycle profiles showed a significant decrease in the G1 cell population and an increase in the number of cells in the S and G2 phases after 6 h of oracin treatment. After 24 h most cells were arrested in G2 with no significant changes in cell viability (82 and 93% for SV2 and bcl-2, respectively). After 72 h the viability of SV2 culture had fallen to 5%, leading to the appearance of a subpopulation of reduced DNA stainability to the left of the G2 peak on the PI histogram. This sub-G2 peak was a characteristic of the SV2 cells but not of the bcl-2 cells, indicating that bcl-2 expression had protected transfected cells from dying by apoptosis after cell arrest in the G2 phase induced by oracin. The bcl-2 cells remained highly viable within the G2 phase at 72 h with few cells actually dying by apoptosis. These cells eventually died after extended culture without progressing to mitosis. Cell size was increased as a consequence of arrest in the G2 phase of the cell cycle with a very close correlation between the two parameters (data not shown). The inability to recover from G2 arrest may be attributed to the lack of ability to repair DNA damage [17]. Similar results have been reported in which X-ray irradiation of L929 and HL-60 cell lines resulted in G2 arrest accompanied by apoptosis and a reduced number of kinetochores in contrast to mitotic cells, suggesting processes different from those that operate in mitosis [18]. It is interesting to mention that the Burkitt's lymphoma cells used in this study were also blocked in G2 phase after exposure to γ-irradiation but the bcl-2-rescued cells underwent G1 arrest [19]. The progression of cells through mitosis after exposure to cytotoxic agents has been suggested to be facilitated by the presence of functional p53, whereas p53-deficient cells are stalled in G2 thereby triggering apoptosis [20]. The BL cell lines are heterozygous-mutant with respect to the p53 configuration and therefore more likely to be p53-deficient. Our results may therefore be in agreement with the finding of Wahl et. al. [20] that the loss of normal p53 function has increased sensitivity to the anticancer agent paclitaxel by increasing G2/M arrest and apoptosis.

4. Conclusion

The results indicate that oracin acts as a mitosis inhibitor and its site of action is probably at the G2 checkpoint. Arresting cells at this point can induce apoptosis which is prevented by the overexpression of bcl-2. bcl-2-rescued cells, however, do not progress through mitosis and consequently die after extended longevity which may be attributed to p53 deficiency.

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