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Original Paper

Vincristine- and Cisplatin-induced Apoptosis in Human Retinoblastoma. Potentiation by Sodium Butyrate

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Chemotherapy alone has largely been unsuccessful in controlling retinoblastoma growth, and has traditionally been limited in use as an alternative to irradiation for the treatment of retinoblastoma. Recently, clinical studies combining chemotherapy with local therapies, including radiotherapy, laser therapy or cryotherapy and in some cases, cyclosporine A, have been effective in treating retinoblastoma. Differentiating agents may also be combined with chemotherapy to enhance the action of cytotoxic drugs on tumor cell growth, although this approach has not been fully investigated in retinoblastoma. In this study, we evaluated the cytotoxic response of human retinoblastoma cell lines (Y79 and WERI-Rb1) to two chemotherapy agents commonly used in treating retinoblastoma, vincristine (VCR) and cisplatin (CDDP). Retinoblastoma cells have been shown to be sensitive to the differentiating agent sodium butyrate, and cell lines were also treated with a combination of VCR or CDDP with sodium butyrate, and the effects on retinoblastoma viability assessed. Both VCR and CDDP induced dose-dependent death of Y79 and WERI-Rb1 cells, accompanied by nuclear and cytoplasmic condensation and DNA laddering, features characteristic of apoptosis. Inhibitors of macromolecular synthesis, cycloheximide and actinomycin-D, significantly reduced VCR- and CDDP-induced apoptosis, although putative endonuclease inhibitors zinc sulphate and aurintricarboxylic acid had no apparent effect. Treatment with 0.5 mM or 1 mM sodium butyrate combined with VCR or CDDP significantly increased induction of apoptosis by these agents. This augmentation of chemotherapy-induced apoptosis may have implications for retinoblastoma therapy. © **1998 Elsevier Science Ltd. All rights reserved.**

Key words: apoptosis, cell death, chemotherapy, differentiation, retinoblastoma
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INTRODUCTION

Retinoblastoma, the most common intraocular malignancy of childhood, has traditionally been treated by enucleation of the affected eye and/or combinations of radiation therapy, cryotherapy or focal laser therapy. The morbidity associated with enucleation and the side-effects of therapies, particularly external beam radiation therapy (EBR), has prompted the development of alternative treatments for retinoblastoma. Patients with a germline *Rb* gene mutation treated with EBR have an increased risk of dying from a second malignancy of 6–35% within 40 years of treatment [1].

Chemotherapy alone has been largely unsuccessful for retinoblastoma, possibly related to the expression of the multidrug resistance P-glycoprotein (Pgp) observed in many untreated retinoblastoma tumours and in retinoblastoma where treatment has failed [2, 3]. Recent clinical studies combining systemic chemotherapy with focal therapy (photocoagulation, radiotherapy or cryotherapy) and in some cases, high-doses of cyclosporine A (a known multidrug-resistance reversal agent), indicate an emerging role for chemotherapy combined with local tumour ablation in controlling retinoblastoma tumour growth [4–7]. Short term, high-dose systemic cyclosporine A combined with chemotherapy has been very successful in treating retinoblastoma, with 86% success at 3.5 years for the worst tumours with vitreous seeds [6, 8]. Clinically used cytotoxic drugs induce apoptosis *in vitro* and *in vivo* in a variety of tumours [9–11], and modulation of apoptosis by these agents appears to be critical in the response of tumours to chemotherapy [11]. Further,

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measurement of apoptosis induction has been suggested as a useful predictor of *in vitro* tumour chemosensitivity [12]. Several *in vitro* studies have examined the chemosensitivity of human retinoblastoma cell lines to various cytotoxic agents [2, 13, 14], but the induction of apoptosis in retinoblastoma by cytotoxic drugs, particularly vincristine (VCR) and cisplatin (CDDP), remains to be fully characterised [13].

Differentiating agents have also been used in combination with chemotherapy to enhance the cytotoxicity of chemotherapy drugs [15, 16]. For example, all *trans* retinoic acid and sodium butyrate have been observed to increase chemotherapy-induced cell death in several tumours [15–17]. Previous studies suggest that cellular differentiation may influence the expression of apoptosis-related genes and the process of apoptosis [16, 17], whilst other studies suggest that some differentiating agents may enhance cytotoxicity by reducing Pgp expression [18, 19]. The effects of sodium butyrate, a non-toxic [20] cell growth inhibitor and differentiating agent, combined with chemotherapy on retinoblastoma cell survival, have yet to be determined; we have observed that sodium butyrate alone can modulate cell growth and apoptotic cell death in human retinoblastoma cell lines, with minimal effects on normal retinal cells [21].

In the present study, we assessed the effects of chemotherapy drugs, VCR and CDDP on retinoblastoma cell lines, using morphology, DNA stains (acridine orange/ethidium bromide) and DNA electrophoresis. Further, the *in vitro* potential of sodium butyrate to enhance chemotherapy-induced apoptosis in retinoblastoma was investigated.

MATERIALS AND METHODS

Cell lines

Y79 (Corriell Institute, New Jersey, U.S.A.) and WERI-Rb1 (American Tissue Culture Collection, Rockville, Maryland, U.S.A.) cells were grown as suspension cultures in RPMI 1640 medium with 10% fetal bovine serum, 2 mM glutamine and 50 μg/ml penicillin/streptomycin (Trace Pty Ltd, Sydney, Australia). The cultures were maintained in a humidified atmosphere of 5% CO_2/95% air at 37°C.

Dose response: VCR and CDDP

Cells were seeded into 24 well plates (Costar Corporation, Cambridge, Massachusetts, U.S.A.) at 5×10^4 cells/ml. After 2 days, the medium was replaced with fresh medium containing a range of concentrations (0.001–100 μM) of VCR or CDDP (Sigma Pty Ltd, Sydney, Australia). The dose response of the two cell lines to treatment with VCR or CDDP was determined after 48 h with trypan blue and haemocytometer counting. All experiments were carried out with parallel untreated controls for each cell line. For both cell lines, a lethal dose for 50% of cells (LD_{50}) for VCR and CDDP of between 1 and 10 μM was observed (see Results). All further experiments evaluating the effects of VCR and CDDP on cell viability used a 10 μM concentration, unless otherwise stated.

Analysis of cell death using acridine orange/ethidium bromide staining

In a separate series of experiments, retinoblastoma cells were seeded as above and treated with either 10 μM VCR or CDDP. At 0, 24, 48 and 72 h after treatment, cells were stained with acridine orange/ethidium bromide, and the apoptotic index (AI) calculated as described previously [11, 21]. Briefly, apoptotic cells displayed well-defined condensed chromatin or peripheral nuclear crescents, which clearly stained with acridine orange; these cells were further classified as either viable ethidium bromide negative (EB −) or non-viable ethidium bromide positive (EB +), depending on the integrity of the cytoplasmic membrane. Necrotic cells were invariably EB + and displayed obviously dispersed nuclear chromatin. Another group of cells classified as degenerate cells [21], displayed advanced nuclear degeneration and stained poorly with acridine orange and ethidium bromide.

Light and electron microscopy

Retinoblastoma cells treated with 10 μM VCR or CDDP for 48 h and controls were prepared for light and electron microscopy as described previously [21].

Assessment of DNA degradation

DNA degradation in lysates from 10 μM VCR or CDDP treated cells (48 h) and controls, was assessed using the method of Smith and colleagues [22] with some modifications. Briefly, 10^6 cells were washed, then centrifuged at 2500 rpm for 5 min. Cell pellets were resuspended in 20 μl lysis buffer: 1 mM ethylene diamine tetra-acetic acid (EDTA), 10 mM Tris (pH 8.0), 1% w/v sodium dodecyl sulphate (Boehringer Mannheim, Sydney, Australia), and 1 mg/ml proteinase K (Boehringer Mannheim). After a 1 h incubation at 37°C, 2 μl of 10 μg/ml RNase (Boehringer Mannheim) was added and incubation continued for a further 1 h. Cell lysate samples of 20 μl/well were subsequently run at 120 V on a 1% (w/v) agarose gel containing 0.1 mg/ml ethidium bromide. Gels were examined on an ultraviolet light source and photographed.

Inhibition of protein and RNA synthesis, and endonuclease activity

Cells were treated with 10 μM VCR or CDDP and either putative inhibitors of protein or mRNA synthesis: cycloheximide (10 μM) (Sigma) or actinomycin-D (5 μg/ml) (Sigma), or putative inhibitors of endonuclease activity: $ZnSO_4$ (0.1 mM, 1 mM; Sigma) or aurintricarboxylic acid (200 μM; Sigma). Control cells treated with either VCR or CDDP or inhibitors alone were also examined. After 48 h, cells were assessed with acridine orange/ethidium bromide staining as described above.

Sodium butyrate and chemotherapy

After treatment for up to 72 h with 10 μM VCR or CDDP, a small population of viable cells survived, particularly for the WERI-Rb1 cell line (see Results). Previous studies have suggested that the sensitivity of tumour cells to chemotherapy agents may be enhanced by differentiating agents, many of which are reported to modulate apoptosis [16, 17], and we have previously reported that sodium butyrate can induce apoptosis in retinoblastoma cell lines [21]. Given these observations, the effect of sodium butyrate combined with either VCR or CDDP on cell viability was also assessed. VCR or CDDP were used at concentrations of either 1 or 10 μM (the LD_{50} for VCR and CDDP was between 1 and 10 μM; see Results). For experiments involving combined treatments, Y79 or WERI-Rb1 cells were seeded into 24 well plates as described above, and treated simultaneously with either 1 or 10 μm VCR or CDDP, and 0.5 or 1.0 mM sodium butyrate. To more closely simulate clinical exposure to chemotherapy agents (usually an exposure time of a few hours), a further set

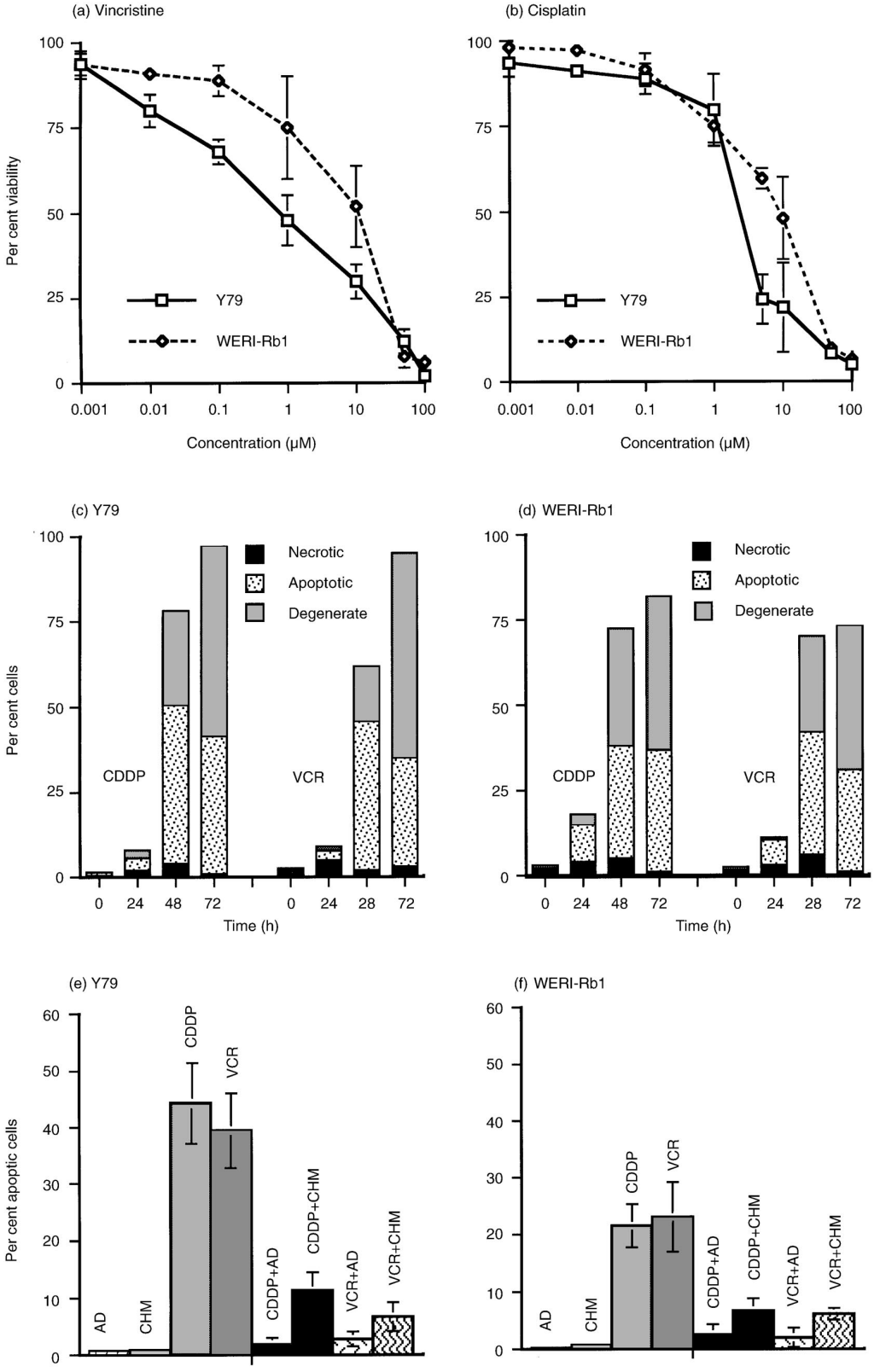

Figure 1. Dose–response, determined by trypan blue staining, of Y79 and WERI-Rb1 cells treated with (a) vincristine (VCR) and (b) cisplatin (CDDP) for 48 h (0.001–100 μM; $n = 3$). For cell death, the frequency of necrotic, apoptotic and degenerate cells within Y79 and WERI-Rb1 cultures treated with either 10 μM CDDP or 10 μM VCR over 72 h, expressed as the percentage of the total cells counted are shown in (c) and (d), respectively (acridine orange/ethidium bromide staining; $n = 3$). The effects of inhibitors of macromolecular synthesis on either 10 μM CDDP- or 10 μM VCR-induced apoptosis in (e) Y79 and (f) WERI-Rb1 cells, respectively. Y79 and WERI-Rb1 cells treated with inhibitors only (actinomycin-D (AD) and cycloheximide (CHM)) had levels of apoptosis similar to controls (not shown). (Error bars = standard deviations).

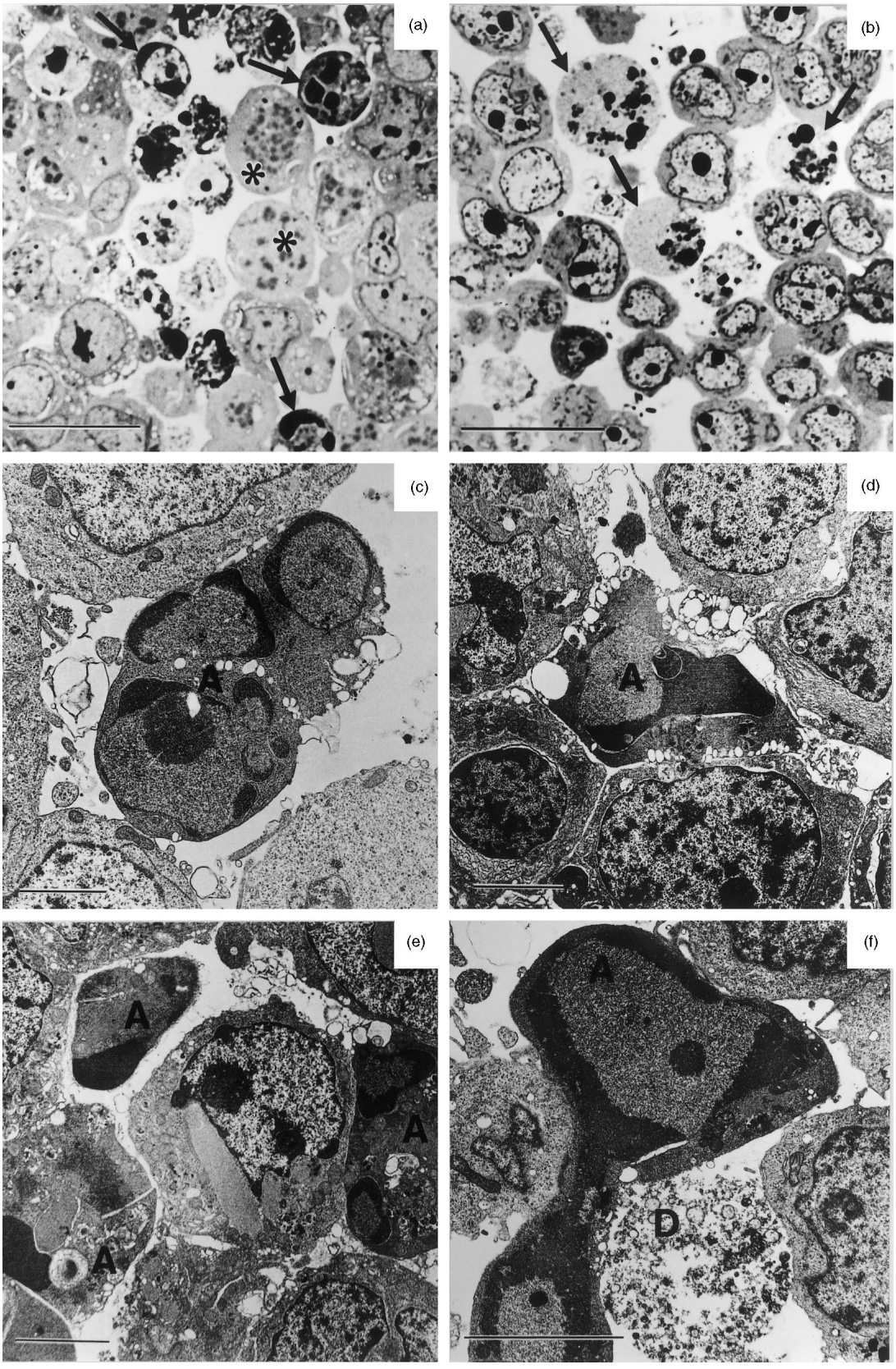

Figure 2. Light micrographs of Y79 cells treated with (a) 10 μM vincristine (VCR) and (b) 10 μM cisplatin (CDDP) after 48 h showing cells at various stages of apoptosis (arrows). Arrested mitotic cells (*) are indicated in VCR treated cultures. Treated WERI-Rb1 cells had a similar light microscopic appearance to treated Y79 cells. Electron micrographs of Y79 cells treated with (c) 10 μM CDDP or (d) 10 μM VCR and WERI-Rb1 cells treated with (e) 10 μM CDDP or (f) 10 μM VCR after 48 h. Characteristic condensation of the cytoplasm and nuclear chromatin is visible in apoptotic cells (A). An example of a degenerate cell (D) is seen in (f). (a,b bar = 20 μm, toluidine blue; c–f bar = 5 μm).

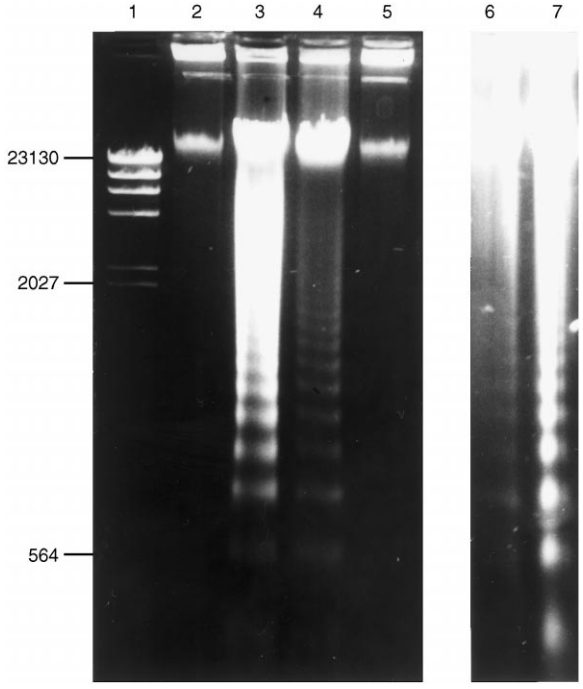

Figure 3. Agarose gel electrophoresis of lysed Y79 and WERI-Rb1 cells 48 h after treatment with various agents. Lane 1, DNA size marker; lane 2, control (untreated) Y79; lane 3, Y79+10 μM cisplatin (CDDP); lane 4, Y79+10 μM vincristine (VCR); lane 5, control (untreated) WERI-Rb1; lane 6, WERI-Rb1+10 μM CDDP; lane 7, WERI-Rb1+10μM VCR.

of experiments was conducted in which cells were initially pulse-treated with either 10 μM VCR or CDDP for 1.5 h at 37°C, rinsed and then treated continuously for 72 h with 1 mM sodium butyrate. Viability was determined by trypan blue exclusion 48 h after treatment, and compared with cells treated with either VCR, CDDP or sodium butyrate at appropriate concentrations alone.

RESULTS

Dose response

For both cell lines and both drugs (VCR and CDDP), viability assessed by trypan blue exclusion displayed a dose-dependent decrease over 48 h (Figure 1a,b). After 10 μM VCR treatment for 48 h, mean viability (± standard deviation (SD)) was 29.9 ± 9.4% for Y79 cells and 51.0 ± 11.9% for WERI-Rb1 cells ($n = 3$). Treatment with 10 μM CDDP resulted in a reduced viability compared with VCR in both cell lines (Y79 = 21 ± 13.1%, WERI-Rb1 = 47.2 ± 13.6%; $n = 3$), although this difference was not statistically significant ($P > 0.05$; paired t-test). After 72 h, mean viability of Y79 cells treated with 10 μM VCR or 10 μM CDDP was 1.3 ± 0.3% and 6.9 ± 3.0%, respectively. In contrast, mean viability of WERI-Rb1 cells treated with 10 μM VCR or 10 μM CDDP was 26 ± 1.5% and 20 ± 2.0%, respectively (Figure 1a,b).

Cell death: acridine orange/ethidium bromide

Acridine orange/ethidium bromide staining revealed that apoptosis was the predominant form of cell death induced in chemotherapy treated cultures. The time course for induction of apoptosis in Y79 and WERI-Rb1 cell lines following treatment with 10 μM VCR or CDDP is shown in Figure 1(c) and (d). A maximal AI was observed after 48 h (Y79: AI ± SD 10 μM CDDP = 46.5 ± 6.5%, 10 μM VCR = 43.7 ± 3.0%; WERI-Rb1: AI ± SD 10 μM CDDP = 33.0 ± 7.0%; 10 μM VCR = 36 ± 4.0%). In parallel control cultures, the AI remained less than 1.7% for both lines at all time points examined (not shown).

The frequency of degenerate cells increased markedly over the time course of the experiments, reaching maximal levels 72 h after treatment with VCR or CDDP in both cell lines (Figure 1c,d). Necrotic cells were also identified, but the frequency of these cells remained < 6% over the time course of treatments (Figure 1c,d).

Effects of VCR and CDDP on cell morphology

Apoptotic or necrotic cells were rarely observed in control Y79 and WERI-Rb1 cultures (not shown). However, light microscopy of cultures treated with either 10 μM VCR or CDDP (48 h) revealed numerous cells at varying stages of apoptotic involution (Figure 2a,b). Electron microscopy confirmed these observations and cells treated with 10 μM VCR or CDDP (48 h) displayed features characteristic of apoptosis, including condensed peripheral nuclear chromatin and cytoplasm (Figure 2c–f). Degenerate cells lacking distinct nuclear morphology were also observed in treated cultures, particularly after 48 h (Figure 2a,b,d). In VCR treated cultures, numerous early mitotic figures displaying condensed chromosomes were observed (Figure 2a).

DNA changes associated with VCR and CDDP treatment

Agarose gel electrophoresis of cell lysates obtained from 10 μM VCR or CDDP treated Y79 and WERI-Rb1 (48 h) revealed DNA laddering characteristic of apoptosis (Figure 3). A comparison with DNA molecular weight markers indicated that the fragments were multiples of 180–200 base pairs in size. Control (untreated) cells displayed no evidence of DNA laddering (Figure 3).

Effects of putative inhibitors of macromolecular synthesis and endonuclease activity

When Y79 and WERI-Rb1 cells were pretreated with either 10 μM cycloheximide or 5 μg/ml actinomycin-D and then exposed to 10 μM CDDP or VCR, the levels of apoptosis induced after 48 h as assessed by acridine orange/ethidium bromide staining, was significantly reduced ($P < 0.05$, paired t-test, $n = 3$), compared with levels of apoptosis in Y79 and WERI-Rb1 cells treated with either CDDP or VCR alone (Figure 1e,f). Neither $ZnSO_4$ nor aurintricarboxylic acid significantly affected CDDP- or VCR-induced apoptosis compared with controls (data not shown).

Effect of sodium butyrate treatment on VCR/CDDP induced cytotoxicity

Simultaneous treatment with 0.5 mM and 1 mM sodium butyrate enhanced the cytotoxicity of 1 μM and 10 μM VCR or CDDP at 48 h. For 10 μM VCR or CDDP combined with sodium butyrate, viability was significantly reduced compared with chemotherapy alone for both cell lines ($P < 0.05$; Figure 4). Sodium butyrate combined with 1 μM VCR or CDDP also significantly reduced viability for WERI-Rb1 cells ($P < 0.05$; Figure 4), although Y79 cells were not as responsive.

Pulsed treatment for 1.5 h with either 10 μM VCR or CDDP followed by continuous treatment with 1 μM sodium

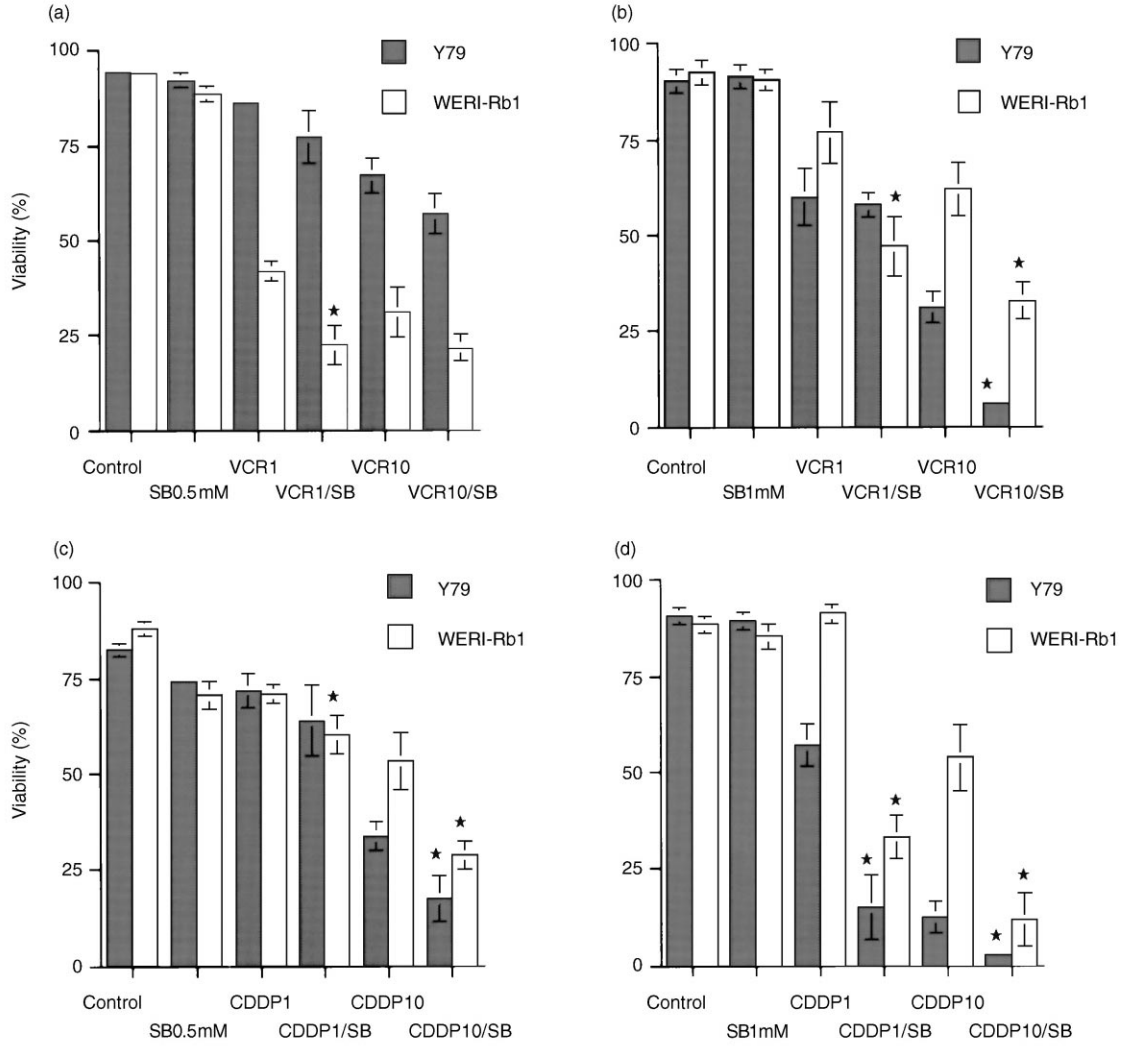

Figure 4. Effect of combined treatment with (a) vincristine (VCR)+0.5 mM sodium butyrate, (b) VCR+1 mM sodium butyrate, (c) cisplatin (CDDP)+0.5 mM sodium butyrate and (d) CDDP+1 mM sodium butyrate on viability of Y79 cells and WERI-Rb1 cells, 48 h post-treatment. (*P<0.05, unpaired t-test, VCR or CDDP versus VCR or CDDP+sodium butyrate.)

butyrate also enhanced induction of cell death compared with cells pulse-treated with either 10 μM VCR or 10 μM CDDP alone (Table 1). This difference was statistically significant for both cell lines following CDDP and sodium butyrate but only for WERI-Rb1 cells following VCR and sodium butyrate treatment (Table 1)

DISCUSSION

In the present study, we observed that VCR or CDDP treated retinoblastoma cell lines displayed morphological features and DNA laddering characteristic of apoptotic involution [11]. We observed that inhibitors of macromolecular synthesis (actinomycin-D and cycloheximide) significantly reduced chemotherapy-mediated apoptosis in retinoblastoma cell lines. It is possible that retinoblastoma cells treated with cytotoxic drugs may be primed to undergo apoptosis, and that cycloheximide or actinomycin-D delays cell cycle progression, as suggested in other cell lines where susceptibility to apoptosis appears to be related to the cell cycle [23]. Alternatively, another study has reported that, in some instances, chemotherapy-induced apoptosis may involve modulation of gene expression, requiring active participation of the target cell and a dependence on macromolecular synthesis, which may be partially or completely inhibited by cycloheximide and actinomycin-D treatment [24].

In vitro, apoptotic cells often undergo secondary degeneration, rather than uptake by neighbouring viable cells observed following apoptotic involution *in vivo* [11, 21, 25]. Consistent with these observations, the degenerate cells seen following treatment of retinoblastoma cells with cytotoxic agents most probably represent secondary degeneration of apoptotic cells; the incidence of these cells was temporally related to the induction of apoptotic cells, accumulating to high levels by 72 h (see Figure 1c,d). Further, the frequency of necrotic cells observed with acridine orange/ethidium bromide staining remained low throughout the experiments and was apparently unaltered by VCR/CDDP treatment (approximately 1–6% of non-viable cells throughout the experiments).

At 24 h post-treatment in the present study, mean viability for both cell lines was between 70 and 90%; at 48 h an LD_{50} between 1 and 10 μM CDDP or VCR was found for both cell lines. Viability assessed by trypan blue exclusion represents a limitation of the present study; although this does provide an

Table 1. *Viability: retinoblastoma cells pulsed with vincristine (VCR)* 10 μM *or cisplatin (CDDP)* 10 μM *(*1.5 *h) followed by sodium butyrate* 1 *mM (*48 *h)*

Treatment ($n = 3$)	Y79 viability (%)* (mean ± S.D.)	*P* value†	WERI-Rb1 viability (%)* (mean ± S.D.)	*P* value†
Control (untreated)	90.9 ± 3.8		92.5 ± 5.4	
Sodium butyrate (1 mM)	91.2 ± 0.5		89.9 ± 3.2	
VCR (10 μM)	43.9 ± 13.4		92.2 ± 5.0	
VCR (10 μM) + sodium butyrate (1 mM)	37.1 ± 8.3	0.6	48.2 ± 1.7	0.005
CDDP (10 μM)	79.5 ± 7.9		90.7 ± 4.9	
CDDP (10 μM) + sodium butyrate (1 mM)	6.0 ± 8.9	0.01	9.0 ± 13.4	0.02

*Cell viability determined by trypan blue staining 48 h after exposure to VCR or CDDP. †Paired *t*-test: VCR or CDDP versus VCR or CDDP + sodium butyrate 1 mM. S.D., standard deviation.

opportunity to estimate cell damage caused by cytotoxic drugs, this assay does not provide a measure of long term cell survival, which has clinical significance. We did observe survival of a population of WERI-Rb1 cells in the short term, in particular, and clonogenic survival of retinoblastoma cell lines treated with chemotherapy drugs is currently being studied. Several previous studies have examined the response of retinoblastoma cell lines to chemotherapy drugs [2, 13, 14] by various methods, including clonogenic assays, and as such were not directly comparable with the present results. Other *in vitro* studies of cytotoxicity in embryonic tumours, such as testicular germ cell tumours and some paediatric sarcomas and lymphomas, to a variety of chemotherapy agents including CDDP and VCR, have reported an LD_{50} of between 5 and 15 μM after only 24 h treatment [26]. This may suggest an extended mitotic phase is necessary prior to induction of apoptosis in retinoblastoma cell lines; a previous study observed that induction of apoptosis in retinoblastoma cell lines was delayed for approximately 40 h following treatment with etoposide [14]. Alternatively, retinoblastoma cell lines may have a reduced sensitivity to VCR or CDDP compared with other paediatric tumours (see above). Although the mechanisms of drug resistance have been studied in detail, the reasons for groups of tumours to be inherently less sensitive to chemotherapy compared with others remains obscure and does not appear to be correlated with differences in cell cycle kinetics, DNA damage and repair or ability to mutate to a drug resistant genotype [26–28].

Modulation of chemotherapy-induced cell death by sodium butyrate observed in the present study, has not previously been reported in retinoblastoma. Sodium butyrate can induce diverse effects in transformed cells, including morphological changes and differentiation, growth arrest at G1 in the cell cycle, changes in expression of cell surface antigens and modulation of expression of genes including *c-myc*, *c-fos*, *p53*, *bcl-2*, and cyclins [29, 30]. However, the intracellular signals by which sodium butyrate can induce these varied effects remains incompletely understood [31, 32].

The mechanism(s) involved in sodium butyrate-enhanced cytoxicity of retinoblastoma were not specifically addressed, but several possibilities may be considered. Variable effects of sodium butyrate on Pgp expression and activity, which may result in accumulation of chemotherapy agents, have been reported [18, 19]. In colon carcinoma cells and a leukaemia cell line, increased expression of Pgp has been described, although this was not associated with increased Pgp function [18, 32]. However, in another study, sodium butyrate-induced Pgp phosphorylation reduction was associated with increased accumulation of vinblastine and doxorubicin [19]. Recently, cyclosporine A, an inhibitor of Pgp, has been combined with chemotherapy in the treatment of intra-ocular retinoblastoma, and we are currently investigating the *in vitro* effects of this agent on the effectiveness of sodium butyrate to enhance chemotherapy. Development of chemoresistance via expression of apoptosis-suppressing gene products such as bcl-2 and related proteins has also been observed [33, 34], and down-modulation of bcl-2 expression associated with increased apoptosis has been reported in several tumour cell lines [17, 35]. Preliminary studies indicate that constitutive expression of bcl-2 protein in retinoblastoma cell lines (Y79 and WERI-Rb1) is down-modulated to almost negligible levels 3 days after exposure to 1 mM sodium butyrate (data not shown). We are currently investigating the potential role(s) of bcl-2 family proteins in retinoblastoma following treatment with sodium butyrate and other differentiating agents. The chemosensitising effect of sodium butyrate on chemotherapy-induced apoptosis suggests additional approaches to controlling retinoblastoma growth.

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