Characterization of cell death induced by vinflunine, the most recent Vinca alkaloid in clinical development

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Vinflunine, the most recent Vinca alkaloid in clinical development, demonstrated superior antitumour activity to other Vincas in preclinical tumour models. This study aimed to define its molecular mechanisms of cell killing in both parental sensitive and vinflunine-resistant P388 leukaemia cells. Vinflunine treatment of these cells resulted in apoptosis characterized by DNA fragmentation and proteolytic cleavage of poly-(ADP-ribose) polymerase. Apoptosis-inducing concentrations of vinflunine caused c-Jun N-terminal kinase 1 stimulation, as well as caspases-3/7 activation. This activation of caspases and the induction of apoptosis could be inhibited by the caspase inhibitor acetyl-Asp-Glu-Val-Asp-aldehyde. Interestingly, the apoptosis signal triggered by vinflunine in these P388 cells was not mediated through Bcl-2 phosphorylation. In addition, when vinflunine resistance was developed in P388 cells, it was associated with resistance to vinflunine-induced apoptosis, as reflected by a loss of capacity to induce DNA fragmentation and PARP degradation, and characterized by increased levels of Bcl-2 and Bfl-1/A1. Therefore, these data indirectly implicate Bcl-2 and Bfl-1/A1 in vinflunine-induced cell death mechanisms.

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Vinflunine or 20', 20'-difluoro-3',4'-dihydrovinorelbine, is a novel Vinca alkaloid selectively fluorinated by superacid chemistry in a rarely exploited region of the velbanamine moiety (Fahy et al., 1997). It was selected for clinical development on account of its markedly superior antitumour efficacy in vivo in preclinical studies, in a series of experimental models compared to other Vinca alkaloids (Kruczynski et al., 1998a; Hill et al., 1999). Promising results have recently been obtained in Phase I clinical trials, now completed in Europe with three partial responses being identified in patients with advanced breast cancer or renal cell carcinoma (Fumoleau et al., 2000). Phase II clinical evaluations in melanoma and renal carcinoma are now on going.

In vitro studies have confirmed its mitotic-arresting and tubulin-interacting properties (Kruczynski et al., 1998b) and identified differences in tubulin binding relative to the other Vincas (Kruczynski et al., 1998b; Lobert et al., 1998). However, the molecular mechanisms of cell killing by vinflunine remain to be characterized.

Anticancer agents are known to trigger apoptotic mechanisms in tumour cells, especially those of haematological origin, although the relative contribution of apoptosis to drug-induced cell death in advanced solid tumours remains controversial (Brown and Wouters, 1999).

Apoptosis, a genetically regulated process triggered by various biological signals, can be transduced through numerous pathways (Wang et al., 1999b). However, cells which die by apoptosis exhibit typical morphological changes, DNA fragmentation (Wyllie, 1997) and activate a cascade of aspartate-specific cysteine proteinases, namely the caspases. Caspase activity is responsible, either directly or indirectly, for the cleavage of cellular proteins which are characteristically proteolysed during apoptosis (Miller, 1997). For example, once activated, caspase-3 cleaves specific substrates including the nuclear protein poly(ADP-ribose) polymerase (PARP), involved in DNA repair and genome maintenance (Kaufmann et al., 1993). Microtubule targeting agents, including taxanes and Vinca alkaloids, have been shown to promote apoptosis in cancer cells through a complex process involving many protein kinase signalling pathways (Wang et al., 1999b), including activation of c-Jun N-terminal kinases (JNKs) (Stone and Chambers, 2000). Furthermore, a critical role for the Bcl-2 protein family in cell death induced by microtubule damaging agents has been suggested (Wang et al., 1999b).

The aim of this study was to determine whether vinflunine-induced cell death in murine P388 leukaemia cells exhibited biochemical characteristics of apoptosis. The contributions of JNK1 signalling, caspases and of Bcl-2 protein family members to vinflunine-induced cell death were also investigated. Furthermore, we examined whether vinflunine resistance developed in vivo in P388 cells (P388/VFL) was associated with changes in vinflunine-activated programmed cell death.

MATERIALS AND METHODS

Cells

Murine sensitive P388 (National Cancer Institute, Tumour Repository, Frederick, MD, USA) and the in vivo established vinflunine-resistant P388/VFL (Etievant et al., 1998) leukaemia cells were collected from the peritoneal cavities of DBA/2 mice (DBA/2Jico, Iffa Credo, L’Arbresle, France), where they were maintained in vivo, and adapted to in vitro culture conditions in RPMI 1640 medium supplemented with 10% heat-inactivated horse serum, 4 mM L-
glutamine, 1.25 μg ml⁻¹ fungizone, 100 μg ml⁻¹ penicillin-streptomycin, and 20 μM β-mercaptoethanol. The vinflunine-resistant P388/VFL cells established in vivo initially were subsequently characterized in vitro as showing a 17-fold level of resistance to vinflunine, with marked overexpression of P-glycoprotein (P-gp) associated with reduced accumulation of [³H]-vinflunine (Etievant et al., 1998).

Nuclear staining and apoptotic cell percentage determination

After 24 h of treatment with vinflunine, P388 cells collected by centrifugation were fixed with methanol:acetic acid (3:1) for 30 min prior to staining using a Diff-Quik kit (Maurepas, France), based on the Giemsa-May-Grunwald technique, before examination under light microscopy. At least 300 cells were scored for the incidence of apoptosis, in five randomly selected fields and the per cent of apoptotic cells with fragmented nuclei and condensed chromatin relative to the total was calculated.

DNA fragmentation assay

DNA fragmentation was quantitated according to Bertrand et al. (1991). Briefly, ¹⁴C-prelabelled thymidine cells, after exposure to vinflunine, were pelleted and lysed for 30 min at 4°C in 1 ml ice-cold PBS buffer containing 0.5% (v:v⁻¹) Triton-X-100 and 20 mM EDTA, pH 8. Cellular lysates were centrifuged at 12,000 g for 30 min at 4°C to separate low molecular weight DNA fragments (supernatant) from intact chromatin or high molecular weight DNA (pellet). Radioactivity was measured in each collected fraction and the amount of fragmented [¹⁴C]DNA released into the supernatant was expressed as a percentage of the total. Results are expressed as the drug-specific percentage of DNA fragmented using the formula: \( \frac{(F - F_0\times 100 - F_0)}{100} \), where \( F \) and \( F_0 \) represent percentages of DNA fragmentation in drug-treated and control cells, respectively.

Determination of caspase activation in cellular extracts

After in vitro vinflunine treatment for 24 h, P388 cells were washed with ice-cold PBS and then lysed for 10 min on ice with 50 mM HEPES, pH 7.4, 0.1% Chaps, 1 mM DTT and 0.1 mM EDTA. Caspase activity in the supernatant (100 μl) was determined using the caspases-3/7 specific colorimetric substrate, acetyl-Asp-Glu-Val-Asp-\( p \)-nitroaniline (Ac-DEVD-\( p \)-NA) (Biomol, Plymouth, USA). Briefly, 20 μg protein extract were incubated with 200 μM substrate peptide in 50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% Chaps, 10 mM DTT, 1 mM EDTA and 10% glycerol. When using the caspases-3/7 inhibitor, Ac-DEVD-CHO (Biomol), cell free extracts were incubated with the inhibitor (200 μM final) for 10 min at 37°C prior to substrate addition. Production of cleaved \( p \)-nitroaniline from the tetrapeptide substrate Ac-DEVD-\( p \)-NA was monitored using a Dynatech...
Determination of JNK activation in cell extracts

JNK activity was determined by an immunoprecipitation assay (Wang et al, 1998). After in vitro vinflunine treatment, P388 cells were washed in PBS and lysed for 10 min on ice with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP40, 5 mM NaF, 1 mM sodium orthovanadate. After centrifugation, the cellular extract (supernatant, 1 mg protein) was mixed with 20 µl anti-JNK1 antibody coupled to agarose (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and incubated overnight. The immunocomplex was recovered by sedimentation for 5 min at 20 000 g, washed three times in 1% (v v–1) sodium orthovanadate. The immunoprecipitate was resuspended in 50 µl JNK reaction buffer containing 25 mM HEPES, pH 7.5, 25 mM MgCl2, 25 mM β-glycerophosphate, and 0.1 mM sodium orthovanadate. The immunoprecipitate was resuspended in 50 µl JNK reaction buffer supplemented with 10 µM ATP, 5 µCi of 33P-ATP (2500 Ci mmol–1; Amersham, Les Ulis, France) and 0.5 µg GST-c-Jun substrate (amino acids 1–79)-agarose (Santa Cruz Biotechnology) and incubated for 30 min at 30°C. Samples were analyzed by spotting an aliquot of the reaction mixture onto a P81 phosphocellulose membrane (Whatman, Maidstone, UK) which was allowed to dry and then washed three times in 1% (v v–1) phosphoric acid in 50% (v v–1) ethanol. Radioactivity associated with c-Jun was assessed by scintillation counting. Assays were performed in duplicate, on at least three separate occasions, and results are expressed relative to the activity of controls.

Western blot analyses

Following in vitro treatment with vinflunine, whole cell extracts were subjected to SDS–PAGE before transfer onto nitrocellulose membranes. After blocking non-specific sites, this was probed overnight with an anti-PARP (Serotec, Oxford, UK), an anti-Bcl-2 (Interchim, Montluçon, France), an anti-Bcl-xL (Tebu, Le Perray-en-Yvelines, France), an anti-Bfl-1/A1 (Tebu, Le Perray-en-Yvelines, France), or an anti-Bax antibody (Euromedex, Soufflewersheim, France), followed by a 1-h incubation with appropriate secondary antibody conjugated to peroxidase (Jackson Immunoresearch Labs, West Grove, PA, USA). When the β-actin loading control was included, probing with an anti-β-actin (Tebu, Le Perray-en-Yvelines, France) or an anti-Bcl-2 family protein antibody was carried out separately. Proteins of interest were visualised by enhanced chemiluminescence (Pierce, Rockford, IL, USA) and quantified using a Bio-Rad (Ivry-sur-Seine, France) MolecularImager.
RESULTS

Does vinflunine induce apoptosis in P388 leukaemia parental cells?

Vinflunine-induced DNA fragmentation Initially, the chromatin precipitation assay was used to assess the cell death response of P388 leukaemia cells to vinflunine treatment. Vinflunine induced fragmentation in P388 cells in a dose- (Figure 1A) and time- (Figure 1B) dependent manner. DNA fragmentation became apparent following 24 h continuous exposure to 0.1 – 0.5 \( \mu M \) vinflunine (Figure 1A) and was detectable as early as 3 h with 0.3 \( \mu M \) vinflunine (Figure 1B). Vinflunine-induced DNA fragmentation was also observed using the TUNEL assay (data not shown).

Vinflunine-activated JNK1 A 6-h treatment with 0.025, 0.25 or 0.5 \( \mu M \) vinflunine resulted in the activation of JNK1 in these P388 cells in a dose-dependent manner, as illustrated in Figure 2.

Vinflunine-stimulated caspase protease activities and caused PARP degradation

Vinflunine, over a concentration range of 0.05 – 0.3 \( \mu M \), stimulated caspases-3/7 activities in P388 cells, in a dose-dependent manner (Figure 3A). The inhibitor, Ac-DEVD-CHO was able to inhibit vinflunine-induced DEVD-specific protease activation, confirming that caspases-3/7 were specifically activated by vinflunine (Figure 3A). Their activation is known to lead to the cleavage of several proteins, with PARP (116-kD) being cleaved to produce an 85-kD fragment during apoptosis (Kaufmann et al, 1993). As shown in Figure 3B, PARP and its 85-kD cleaved fragment were detected in P388 cells by immunoblotting, following incubation with vinflunine. A dose-, as well as a time-dependent, cleavage of PARP was observed, with up to 59% PARP cleavage product being recorded after a 24-h exposure to 0.5 \( \mu M \) vinflunine (Figure 3B1), and this was detected as early as 3 h with 0.3 \( \mu M \) vinflunine (Figure 3B2). Overall these results suggest that at least caspases-3/7 play a role in the apoptotic signalling pathway induced by vinflunine in P388 leukaemia cells.

Inhibition of vinflunine-induced apoptosis by Ac-DEVD-CHO

The caspases 3/7 inhibitor Ac-DEVD-CHO inhibited vinflunine-induced apoptosis as demonstrated by a determination of apoptotic cell number. Optimal inhibition of 86% was noted at a concentration of 300 \( \mu M \) (Figure 4). These results also provide evidence that at least caspases-3/7 mediate vinflunine-induced apoptosis in P388 cells.

Effects of vinflunine on Bcl-2 phosphorylation

Neither vinflunine (0.1 – 0.5 \( \mu M \)) nor taxol (0.04 and 0.4 \( \mu M \)), at equi-cytotoxic concentrations, induced any alteration of the Bcl-2 electrophoretic pattern in P388 cells after a 24-h exposure (Figure 5A). Taxol was included as a reference since it has been shown to induce Bcl-2 phosphorylation in human leukaemias and solid tumour cells (Haldar et al, 1997). Furthermore, a time-course study including treatment of these P388 cells with 0.3 \( \mu M \) vinflunine for 3 to 48 h also failed to result in any modification of Bcl-2 status. A similar study was then conducted with another cell type, namely human CEM leukaemia cells. After a 24-h exposure to either vinflunine or taxol, immunoblot analyses revealed a dose-dependent modification of the Bcl-2 electrophoretic pattern in these CEM cells, characterized by the appearance of a slower mobility form of Bcl-2 (Figure 5B), similar to that reported after serine-phosphorylation of Bcl-2 (Haldar et al, 1995). These findings indicate therefore that microtubule damaging agent-induced Bcl-2 phosphorylation appeared related to the cell type studied.

Is vinflunine-induced resistance associated with changes in vinflunine-activated programmed cell death or with changes of apoptosis-related protein status?

PARP degradation and DNA fragmentation in P388/VFL resistant cells In order to detect PARP cleavage and DNA fragmentation after a 24-h exposure of P388/VFL resistant cells to vinflunine, it was necessary to increase the vinflunine concentrations 10-fold (i.e., 2 – 5 \( \mu M \)) relative to those required to induce comparable effects in the sensitive cells (Figure 6A,B). Therefore, these P388/VFL resistant cells exhibited a 10-fold level of resistance to vinflunine-induced apoptosis, consistent with their 17-fold level of resistance to vinflunine-induced cytotoxicity.

Status of Bcl-2, Bcl-xL, Bfl-1/A1 and Bax proteins in P388 sensitive and P388/VFL resistant cells Western blot analyses were performed to determine whether vinflunine resistance in P388 leukaemia cells was associated with changes in relative endogenous Bcl-2, Bfl-1/A1, Bax, and Bcl-xL expression. As shown in Figure 7, the basal level of expression of Bcl-2 was 3.7-fold increased in P388/VFL resistant cells relative to the sensitive cells. In addition, a 1.9-fold increase was detected in the basal level of Bfl-1/A1, another anti-apoptotic protein in these resistant cells relative to their sensitive counterparts (Figure 7). The pro-apoptotic protein Bax and the anti-apoptotic protein Bcl-xL however were not differentially expressed in sensitive and resistant cells (Figure 7).

DISCUSSION

This study aimed to identify the cell killing mechanisms of vinflunine, selected for clinical development on account of its marked in
vivo antitumour activity and good overall tolerance in preclinical models (Kruczynski et al, 1998a; Hill et al, 1999) and its quantitatively distinct tubulin interacting properties, compared to other Vincas (Kruczynski et al, 1998b; Lobert et al, 1998).

Our results suggest that vinflunine treatment of P388 leukaemia cells initiates a series of events leading to apoptosis, including DNA fragmentation accompanied by cellular morphological changes specific for apoptosis observed by electron microscopy and formation of apoptotic bodies containing genetic materials identified using a Giemsa-based staining technique (data not shown). Caspase activation and subsequent cleavage of functionally-essential key enzymes also play a central role in the biological processing of apoptosis (Miller, 1997). Caspase-3, required for DNA fragmentation and for some of the typical morphological changes associated with apoptosis (Jänicke et al, 1998), cleaves several important cellular targets including PARP (Kaufmann et al, 1993). The results presented here show that vinflunine treatment of P388 cells stimulated caspases-3/7 activities and resulted in PARP degradation in a dose- and time-dependent manner. Furthermore, Ac-DEVD-CHO, a caspases 3/7 inhibitor inhibited vinflunine-induced DEVD-specific caspase activity and apoptosis. Therefore, overall these data suggest that vinflunine triggers apop-

Figure 5 Effects of vinflunine on Bcl-2 phosphorylation. (A) Neither vinflunine, nor taxol induced Bcl-2 phosphorylation in P388 leukaemia cells. Western blot analyses of P388 cells after a 24-h exposure to either 0.1 – 0.5 \(\mu\text{M}\) vinflunine (A1) or 0.04 – 0.4 \(\mu\text{M}\) taxol (A2), or to 0.3 \(\mu\text{M}\) vinflunine for 3 – 24 h (A3). (B) Vinflunine and taxol induced Bcl-2 phosphorylation in human CEM leukaemia cells. Western blot analyses of CEM cells after a 24-h exposure to either 0.08 – 1.6 \(\mu\text{M}\) vinflunine or 0.005 – 0.1 \(\mu\text{M}\) taxol.
totic mechanisms involving caspases-3/7 in these P388 leukaemia cells. Involvement of caspases from the caspase-3-like family, as well as PARP cleavage in drug-induced apoptosis has been demonstrated previously for several other microtubule damaging agents, including cryptophycin (Mooberry et al, 1997), taxol, vinblastine, vincristine (Srivastava et al, 1998) and vinorelbine (Toh et al, 1998).

A great deal of evidence implicates JNKs signalling in apoptosis occurring in response to diverse cellular stress stimuli, although it probably has a multifunctional role, varying with the stimulus, the cell type, the duration of enzyme activation, or the specific JNKs isoform(s) involved (Stone and Chambers, 2000). This study reveals that vinflunine induced a dose-dependent JNK1 activation in P388 leukaemia cells. Earlier reports showed that several structurally-distinct anticancer compounds, including vinblastine, doxorubicin and etoposide activated JNKs in human KB-3 carcinoma cells (Osborn and Chambers, 1996). Subsequently confirmation of JNKs activation by vincristine, vinblastine, taxol and colchicine in a variety of cell lines, suggested that this may represent a general stress response to microtubule dysfunction (Wang et al, 1998; Stone and Chambers, 2000). Our data showing that JNK1 activation is associated with vinflunine-induced cell death in P388 cells, support these reports.

Mechanisms of drug-induced cell death though appear to be multiple and context-dependent. Thus, vinflunine may trigger other cell death mechanisms under differing experimental conditions. For example, it was suggested (Torres and Horwitz, 1998) that taxol-induced cell killing may result from two mechanisms, involving different mediators depending on the concentration used. A more general review of cell death mechanisms mediated by microtubule damaging agents supports the concept that these are complex processes involving many protein kinase pathways (Wang et al, 1999b). The Bcl-2 gene family has been shown to play a crucial role and among multiple genes involved in the regulation of programmed cell death, this gene family stands out for its ability to block apoptosis (Hockenbery et al, 1990). Several studies have associated the disorganization of microtubule structure by drugs with mitotic arresting- and apoptosis-inducing activities such as taxol, taxotere, vinblastine, vincristine or the dolastatins with Bcl-2 phosphorylation in various human tumour cell lines (Haldar et al, 1997). Bcl-2 can be phosphorylated at serine residues 70 and
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