SPANDIDOS PUBLICATIONS

Effect of Vinorelbine on cell growth and apoptosis induction in human osteosarcoma *in vitro*

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Received July 8, 2005; Accepted August 23, 2005

Abstract. Vinorelbine (VNR) is a semi-synthetic vinca alkaloid known to exert its antitumour activity by interfering with the polymerisation of tubulin. It has shown a broad spectrum of activity in some advanced carcinomas of lung, breast and ovary. This report demonstrates for the first time the antiproliferative effect of VNR and its molecular mechanism in human osteosarcoma *in vitro*. TP53 wild-type HOS cells and TP53 mutated MG-63 cells were chosen for this study. In each cell line, VNR caused a significant dose- and time-dependent growth inhibition and induced apoptotic death independent of TP53 status. Phosphorylation and/or alteration of Bcl-2 were not induced by VNR, thereby indicating a new pathway utilised by the drug to induce apoptosis in this tumour *in vitro*. VNR produced a down-regulation of cyclin D1 and an up-regulation of p53 expression in TP53 wild-type HOS cells, whereas no alteration in cyclin D1 expression was evident in the TP53 negative MG-63 cells. These data suggest a new potential use for Vinorelbine as a therapeutic agent against human osteosarcoma.

Introduction

Osteosarcoma (OS) is the most frequent malignant bone tumour with a peak incidence in the second and third decade of life (1). As a result of the introduction of neoadjuvant chemotherapy, an improvement in the long-term survival rate from 10% to nearly 70% has been achieved (2). At present, osteosarcoma patients receive full neoadjuvant multi-agent chemotherapy immediately after diagnosis. Initial tumour size and detectable metastases at diagnosis are prognostic factors and serve together with the response to chemotherapy as a basis for the risk adapted postoperative therapy factor (3,4). Despite the improved survival rate, drug resistance is still one of the major problems in the treatment of this cancer (5). Alterations in the TP53 gene are frequent in human osteosarcoma cells and have been associated with resistance to chemotherapy as well as with poor prognosis in this malignancy (6,7). Pompetti *et al* (8) correlated TP53 mutations with a lack of therapy-induced apoptosis, while studies by Goto *et al* (9) demonstrated an association between loss of heterozygosity at TP53 locus and chemoresistance in human osteosarcoma.

Different studies indicate that cells lacking a p53 function may present a higher sensitivity to anticancer drugs that induce DNA damage and to the cytotoxic effects of anti-microtubule agents (10-12). Vinorelbine (VNR) is a semi-synthetic vinca alkaloid which binds to α and ß tubulin, thus inhibiting microtubule assembly and impairing metaphasic tumour cell division. Compared with different vinca alkaloids, VNR shows markedly improved clinical efficacy and reduced toxicity (13). This drug is now widely used and licensed for the treatment of non-small cell lung cancer, breast cancer and ovarian cancer (14) and shows promise in relapsed or refractory Hodgkin's disease (15) and prostatic carcinoma (16). Like other antimicrotubule agents, VNR is known to be a promoter of apoptosis in cancer cells. The precise mechanism by which this process occurs is complex and needs to be fully elucidated. Disorganisation of the microtubule structure has a number of effects such as the induction of tumour suppressor gene, TP53, and activation/inactivation of a number of protein kinases involved in key signalling pathways, including p21 WAF1/CIP1 and Ras/Raf, PKC/PKA (17). These molecular changes result in phosphorylation and inactivation of the apoptosis inhibitor, Bcl-2 (18).

The present study was designed to investigate the effects of Vinorelbine on the growth of human osteosarcoma *in vitro* in relation to TP53 status. For this purpose, a TP53 wild-type (HOS) and a TP53 mutated (MG-63) osteosarcoma cell line were chosen. VNR caused a significant dose- and time-dependent growth inhibition and induced apoptotic death in

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Key words: osteosarcoma, Vinorelbine, apoptosis, cyclin D1, p53

each cell line, independent of TP53 status. In the p53-positive HOS cells, VNR induced a down-regulation of cyclin D1 expression and an up-regulation of p53. No alteration of Bcl-2 protein expression was induced here by VNR, indicating a different pathway followed by the drug to induce growth inhibition in osteosarcoma cells in respect to breast, ovary and lung carcinoma (17,18).

Materials and methods

Cell cultures. Human osteosarcoma cell lines, MG-63 and HOS, were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated foetal bovine serum (FBS), 2 mM L-glutamine, 50 IU/ml penicillin, and 50 μg/ml streptomycin (Gibco, Invitrogen, Milano, Italy). Cultures were maintained at 37˚C in a humidified atmosphere containing 5% CO_2. The Mycoplasma Plus PCR primer set (Stratagene, CA) was employed for mycoplasma testing.

Drug. Vinorelbine (Navelbine®, Pierre Fabre Pharma, Italy) was prepared as a 1 mg/ml stock solution in phosphate-buffered saline (PBS) and stored at -20˚C. The drug was diluted freshly to the appropriate concentration in the culture medium before each experiment.

Growth inhibition assay. Growth inhibition was determined using the SRB (sulforhodamine B) test according to the method of Skehan *et al* (19). Briefly, cells were seeded in 96-well, flat-bottomed tissue culture plates (Corning Inc., NY) at a density of 6000 cells/well. At the end of drug exposure, cells were fixed with 50% trichloroacetic acid (TCA) at 4˚C (final concentration 10%) for 1 h. TCA fixed cells were then washed with bidistilled water, air-dried and stained at room temperature for 30 min with 0.4% sulforhodamine B (Sigma-Aldrich, St. Louis, MO) dissolved in 1% acetic acid. Finally, the plates were washed with 1% acetic acid, air-dried and the protein bound-dye was solubilized by adding 100 μl of 10 mM unbuffered Tris base per well. Absorbance was measured at 550 nm in a 96-well microplate reader (Bio-Rad, CA). Cell viability was assessed by the trypan blue dye exclusion method.

The concentration causing 50% growth inhibition (IC_{50}) was determined by linear regression method. All experiments were performed in triplicate, unless otherwise indicated, and mean values were presented as ± SE.

Cell death assays. Two different assays were used to determine cell death, DNA fragmentation and DAPI staining. For DNA fragmentation, following incubation with the designated concentrations of the drug, $1x10^6$ cells were pelleted and the total genomic DNA was extracted and purified as previously described (20). Apoptosis was assessed by DNA fragmentation using an ApoAlert LM-PCR ladder assay kit according to the manufacturer's instructions (Clontech, CA). After electrophoresis in 1.2% agarose gel, DNA was stained with 0.25 μg/ml ethidium bromide and the resulting DNA fragmentation pattern was visualised by UV light. For DAPI staining, the cells grown on cover slips were fixed with 4% paraformaldehyde in PBS for 30 min at 4˚C, washed in PBS and then stained with DAPI (4',6-diamidino-2-phenylindole) (10 μg/ml) for 5 min. Cells which detached from the bottom of the culture flask were collected by centrifugation of the culture medium at 500 rpm for 10 min. They were then fixed in suspension with 4% paraformaldehyde in PBS for 30 min, centrifuged at 500 rpm and resuspended in 100 μl of DAPI (10 μg/ml) for 5 min. The cover slips and suspensions of the detached cells were mounted on slides and examined by a fluorescence microscope (Nikon Eclipse E 600) equipped with a digital camera (Nikon DMX 1200). Apoptotic cells were identified by condensation and fragmentation of nuclei.

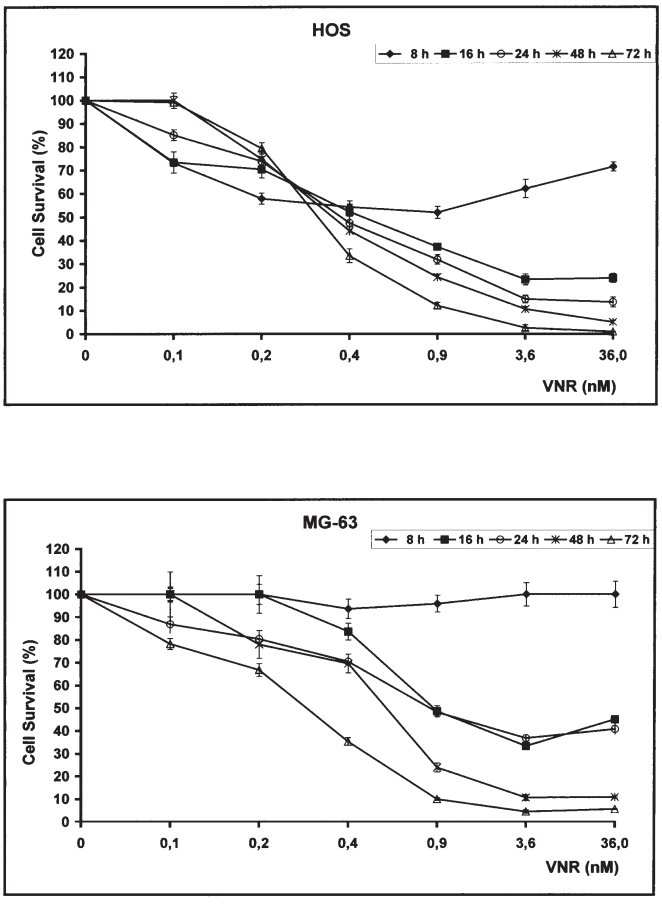

Figure 1. Effect of Vinorelbine on the growth of osteosarcoma cells. Cells were treated with varying concentrations of VNR for 8-72 h. Each point represents the mean ± SE from three different experiments and is expressed as percent of control.

Western blot analysis. Cells were lysed by buffer containing: 1 mM Na_3VO_4, 1 mM $Na_4P_2O_7.10H_2O$, 10 mM NaF, 1 mM 4-(2-aminoethil)-benzenesulfonylfluoride, 1% Nonidet P-40, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. The protein concentration was quantified by bicinchoninic acid (BCA, Pierce) protein assay (21). Proteins were separated by 10% SDS-PAGE followed by electrotransfer onto a PVDF (polyvinylidene difluoride) membrane (Immobilon-P, Millipore, MA) by semi-dry blotting. Following protein transfer, the membranes were placed in blocking buffer 8% non-fat dried milk in PBS-T (0.14 M NaCl, 5 mM NaH_2PO_4 pH 7.2 and 0.1% Tween-20) for 1 h prior to addition of the primary antibody. The antibodies used were p53, Bcl-2, Bax, cyclin D1 and PCNA (Santa Cruz Biotechnology, Santa Cruz, CA).

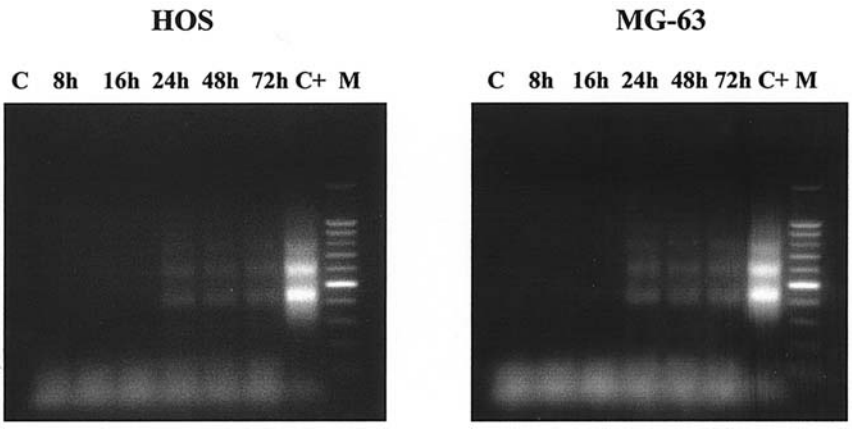

Figure 2. DNA fragmentation assay: time course of VNR-induced apoptosis. Osteosarcoma cells were treated with VNR for 8-72 h. C, untreated cells; C+, apoptotic DNA from calf thymus; M, 100-bp ladder DNA.

Incubation with the primary antibody was carried out at 4˚C overnight and then the membranes were incubated with horseradish peroxidase-labelled secondary antibody for 1 h at room temperature. Immunological complexes were visualised by an ECL (Amersham Biosciences, UK) detection system and quantified by a DU-640 Beckman Spectrophotometer-Densitometer (Beckman Instruments, CA).

Results

Cell growth inhibition by Vinorelbine. The effect of VNR on the growth of HOS and MG-63 osteosarcoma cell lines was tested by SRB assay. The drug concentration utilised ranged from 0.1 to 36 nM. VNR was found to inhibit the growth of each cell line in a dose- and time-dependent manner after exposure times ranging from 8 to 72 h (Fig. 1). In the HOS cell line, the IC_{50} value was 0.6±0.3 nM after 24 and 48 h and 0.4±0.2 nM after 72 h of treatment. In the MG-63 cell line, the IC_{50} values were 3.2±0.8 nM, 0.8±0.5 nM and 0.4±0.3 nM after 24, 48 and 72 h of exposure, respectively. Cell growth inhibition was confirmed by trypan blue dye exclusion test in each cell line (data not shown).

Induction of cell death by Vinorelbine. Induction of apoptosis by VNR was observed by two independent methods. Cells were exposed to 0.9 nM VNR for 8-72 h and apoptosis was evident in the 'laddering formation' after 24-h treatment in each cell line (Fig. 2). Optical images, by DAPI analysis, showed the progressive nuclear changes in HOS and MG-63 cell lines treated with VNR: distinct nuclear shapes were observed in adherent cells at an early phase of apoptosis. After this change, the cells lost their adhesiveness and the detached cells represented a late apoptotic population which contained both hallmarks of apoptosis, nuclear condensation and nuclear fragmentation (Fig. 3).

Effect of Vinorelbine on Bcl-2, Bax, cyclin D1 and p53 protein expression. In order to evaluate the mechanism of apoptosis induced by VNR in osteosarcoma cell lines, we examined the changes in apoptotic and cell-cycle protein expression including Bcl-2, Bax, cyclin D1 and p53. Reduced cyclin D1 protein expression after 16-24 h of treatment and increased p53 expression after 48 h of treatment were observed in the HOS cell line exposed to Vinorelbine, whereas no change in cyclin D1 protein expression was evident in the MG-63 cell line. VNR induced no phosphorylation and/or changes in Bcl-2 and Bax protein expression in either of the cell lines analysed (Fig. 4).

Discussion

Osteosarcoma is a highly malignant tumour of the bone which occurs predominantly in the second and third decade of life. Neoadjuvant chemotherapy with subsequent surgical resection has produced a great improvement in prognoses in the last 20 years, with progression to a long-term survival rate of nearly 70%; however, drug resistance is still one of the main problems in the treatment of this tumour (5). Mutations in TP53 may change the individual's sensitivity to anti-cancer therapeutic drugs (6-9). Interestingly, the growth-inhibitory activity of microtubule-interfering agents tends to be independent of p53 status (10-12). Vinorelbine is a semi-synthetic vinca alkaloid which reversibly binds to tubulin, thus inhibiting microtubule assembly and impairing metaphasic tumour cell division. Currently, VNR is clinically used in some advanced carcinomas of the breast, non-small cell lung cancer and ovary (14) and has been reported to actively reduce the tumour burden in patients with relapsed or refractory Hodgkin's disease (15). Compared with different vinca alkaloids, VNR has a lower blocking activity against axonal microtubules, suggesting a potentially lower neurotoxicity (13).

In the present study, we demonstrate for the first time that VNR has an inhibitory effect on human osteosarcoma growth *in vitro*, at pharmacologically achievable doses, by inducing apoptotic death independent of TP53 status. Apoptosis was observed in the MG-63 cell line (p53 null) and in the HOS cell line (p53 positive) after 24-h treatment with the drug. A weak increase of p53 expression was evident in the HOS cell line after a 48-h treatment with VNR, whereas no phosphorylation and/or changes in Bcl-2 and Bax protein expression were present. This may suggest that p53 is not involved in the apoptotic pathway, even in the p53 positive osteosarcoma

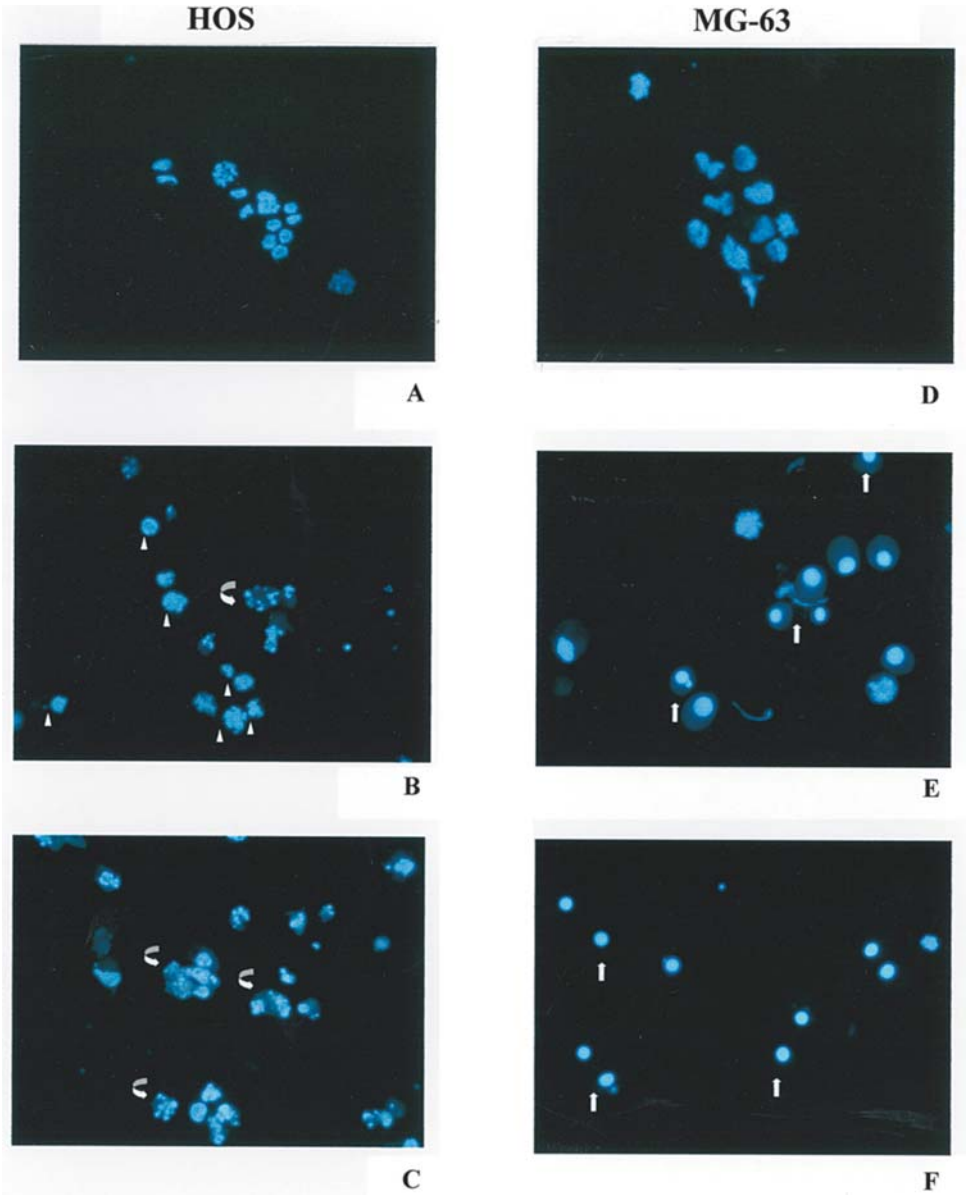

Figure 3. Microscopic images of osteosarcoma cells after DAPI staining. HOS floating cells after VNR treatment: chromatin condensation (arrow head) and nuclear fragmentation (round arrow) are visible, both features of apoptosis. Metaphase chromosomes in (A) control cells, (B) 24-h treated cells, and (C) 48-h treated cells. Morphological shape resembling apoptotic bodies (arrow) is observed in floating MG-63 cells after VNR treatment. Metaphase chromosomes in (D) control cells, (E) 24-h treated cells, and (F) 48-h treated cells.

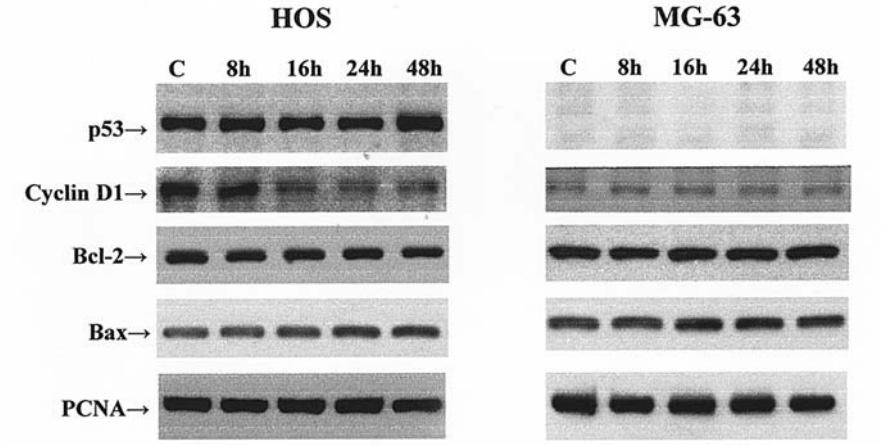

Figure 4. Western blot analysis of p53, cyclin D1, Bcl-2 and Bax proteins in osteosarcoma cells treated with VNR. C, untreated cells. Proliferating cell nuclear antigen (PCNA) was used as a control for loading.

These findings confirm that p53 status is not an indicator of cyclin-inhibitory activity for anti-microtubule drugs, as previously demonstrated by O' Connor *et al* (12) for paclitaxel and vincristine.

In this study, VNR exposure induced a reduction of cyclin D1 protein expression in p53 positive HOS cells, whereas no alteration was present in the p53 negative MG-63. The down-regulation of the cyclin D1 function is considered to be a mechanism of growth suppression in cancer cells, due to the inhibition of cell cycle progression at G1 to S transition (22,23).

Our findings demonstrate that VNR exhibits antiproliferative activity in human osteosarcoma *in vitro* by inducing a TP53-independent apoptotic death and suggest a mechanism of action mediated by the down-regulation of cyclin D1 to induce growth suppression in p53-positive osteosarcoma cells. The present data may suggest a role for VNR in the treatment of human osteosarcomas, including those in which the lack of therapy-induced apoptosis is correlated to TP53 mutations.

Acknowledgements

This work was supported by grants from CNR-Oncology Strategic Project SP4, MIUR, Carisbo Foundation, Pallotti's Legacy for Cancer Research and University of Bologna (Funds for Selected Research Topics).

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