Cell Biology and Toxicology. 2000; **16**: 235–241.

In vitro inhibition of murine hematopoietic progenitors and stromal cells by vinorelbine

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Received 11 November 1999; accepted 26 May 2000

Keywords: cytotoxicity, hematopoietic progenitors, murine bone marrow, stromal cell layers, vinorelbine

Abstract

Hematopoietic progenitor colony assays were used to establish the effects of the vinca alkaloid vinorelbine (VRB) on murine bone marrow. The *in vitro* growth of colony-forming units–granulocyte/macrophage (CFU-GM), burst forming units–erythroid (BFU-E) and colony-forming units–mix (CFU-mix) was dose-dependently inhibited by VRB. The highest dose assayed (0.02 μg/ml) suppressed all of the different progenitor cells by 100%. A comparison of the dose–response curves showed that CFU-GM, BFU-E, and CFU-mix exhibited similar patterns of sensitivity to the cytotoxic action of VRB. Long-term bone marrow cultures have provided a valuable *in vitro* model for studying the role of the microenvironment of bone marrow. Cellularity of stromal layers was reduced with increasing doses of VRB. The appearence of these layers was altered minimally with the lowest dose used; a gradual loss of cellularity was seen in cultures exposed to 0.05 and 0.075 μg/ml; and a marked loss at the dose of 0.1 μg/ml. Our results show that VRB has an important effect on hematopoietic progenitors at the highest dose tested, while the stromal cells were not affected at a similar dose (0.025 μg/ml), suggesting that the stroma is more resistant to this drug.

Abbreviations: BFU-E, burst forming units–erythroid; CFU-GM, colony-forming units–granulocyte/macrophage; CFU-mix, colony-forming units–mix; ID, inhibitory dose; LTBMC, long-term bone marrow cultures; VRB, vinorelbine

Introduction

VRB, a semisynthetic vinca alkaloid, has been shown to have a increased antitumor activity and a reduced neurotoxicity compared with the naturally occurring compounds vinblastine and vincristine (Burris and Fields, 1994). Antitumor effects of VRB were seen in patients with several tumor types, including non-small-cell lung cancer, breast cancer, lymphoma, Hodgkin disease, and head and neck squamous cell carcinomas. In preclinical toxicology studies, the dose-limiting effect of VRB was neutropenia and leukopenia (Budman, 1997). The profound effect upon hematopoiesis limited intensification of the chemotherapy dose and excludes optimal treatment scheduling for cancer patients (Johnston and Crawford, 1998).

Different strategies have been followed to modify these hematotoxic side-effects. For example, human hematopoietic colony-stimulating factors have proven to be a useful adjunct to chemotherapy because they accelerate marrow recovery after intensive treatments (Yalcin et al., 1996). In phase I studies, granulocyte colony-stimulating factor decreased hematological toxicity induced by high doses of VRB in combination with carboplatin (Crawford and O'Rourke, 1994) or ifosfamide (Masters et al., 1996).

In the study reported here we analyzed the *in vitro* effect of VRB on the hematopoietic myeloid and erythroid progenitors and stromal cells derived from murine bone marrow.

Materials and methods

Cells

Bone marrow cells were harvested by flushing femurs from BALB/c J mice (8–12 weeks old, 22–25 g), suspended in Iscove's Modified Dulbecco's Medium (IMDM), and counted.

Drug

VRB (5′-noranhydrovinblastine; CAS no. 71486-22-1) was commercially obtained as Navelbine (Rontag, Argentina). To assess colony growth inhibition for VRB, the drug was added to dishes on day 1 at concentrations of 0.0025, 0.005, 0.01, 0.015, or 0.02 μg/ml. To evaluate the effect on long-term bone marrow cultures, 0.025, 0.05, 0.075, or 0.1 μg/ml of VRB was added to the cultures prior to confluency of adherent cell layers (approximately three weeks).

Budman (1997) reported that the half-life of this drug is 44.7 h in human plasma. Patients undergoing chemotherapy receive a weekly dose of 30 mg/m^2; this dose is equivalent to 10 μg/ml.

Progenitor cell assay

Mononucleated cells were plated for colony-forming units–granulocyte/macrophage (CFU-GM) assay according to the Iscove method (Isocove et al., 1971): 2×10^5 cells/ml were plated in 35 mm Petri dishes in IMDM medium containing 0.3% agar, 10% fetal calf serum (FCS, Gibco), and 10% conditioned medium from cultures of human bladder carcinoma cell line 5637 as source of colony-stimulating factor (Welte et al., 1985).

Burst-forming units–erythroid (BFU-E) assay and colony-forming units–mix (CFU-mix) were performed according to the Messner (1984) technique: 2×10^5 cells/ml were plated in a semisolid medium containing 0.8% methylcellulose, 10% FCS, 1% human serum albumin, 5×10^{-5} mol/L 2-mercaptoethanol, 10% conditioned medium from murine spleen cells cultured in the presence of pokeweed mitogen, and 1 U/ml recombinant human erythropoietin (Sidus, Buenos Aires, Argentina).

Dishes were incubated at 37°C under 5% CO_2 in a humidified atmosphere. For each dose, duplicate cultures were prepared. The colonies were scored after 7 days for CFU-GM and after 14 days for BFU-E and CFU-mix using an inverted microscope. The experiments were repeated four times.

The percentage of colony growth inhibition was calculated as

$$100 - 100 \times (\text{colony number in treated culture/colony number in control culture}).$$

Long-term bone marrow cultures (LTBMC)

The culture technique described by Dexter (1982) was used to establish the bone marrow-adherent cell layers. Briefly, 2×10^6 cells/ml were cultured in 25 cm^2 plastic tissue culture flasks containing IMDM medium with 2 mmol/L L-glutamine, 12.5% horse serum (Gibco), 12.5% FCS, and 10^{-6} mol/L hydro-

cortisone sodium succinate (Cilag, Buenos Aires, Argentina). Half of the supernatant was replaced weekly with the same volume of fresh medium. VRB at different doses was added to the cell layers. After incubation for 14 days at 37°C in a humidified atmosphere supplemented with 5% CO_2, the cultures were examined using an inverted microscope. Four replicate cultures were set up for each individual data point. The layers were assessed for cellularity and photographed.

Statistical analysis

Statistical significance of differences between treatment groups was assessed by the Kruskal–Wallis test. The dose–response relationships were determined by means of the regression coefficients.

Results

The *in vitro* colony-forming ability of control and VRB-treated bone marrow cultures is shown in Table 1. The addition of VRB to the cultures induced significant dose-dependent decreases of CFU-GM ($p = 0.002$), BFU-E ($p = 0.012$), and CFU-mix ($p = 0.003$). The highest dose tested (0.02 μg/ml) suppressed all of the different hematopoietic progenitor cells by 100%. These results were used to calculate the 50% growth-inhibitory dose (ID_{50}) of colony formation. The ID_{50} values estimated from the graph were approximately 0.0042 μg/ml for BFU-E, 0.005 μg/ml for CFU-GM, and 0.0055 μg/ml for CFU-mix (Figure 1).

The effects of different doses of VRB on bone marrow adherent cell layers are presented in Table 2 and Figure 2. The appearance of the stromal layer of LTBMC was altered minimally with the lowest dose used (0.025 μg/ml); a gradual loss of stromal cellularity was seen in cultures exposed to 0.05 and 0.075 μg/ml; and a marked loss at the dose of 0.1 μg/ml.

Discussion

Neutropenia and leukopenia caused by VRB are the major dose-limiting factors in the management of treatment for cancer patients (Vokes et al., 1996). The antineoplastic activity of VRB is caused mainly by its ability to bind to tubulin, inhibiting tubulin polymerization and assembly of mitotic spindle microtubules (Budman, 1997).

Previous studies have shown the mitotic arresting activity induced by VRB on mouse and human cell lines (Pauwels et al., 1995), on Chinese hamster ovary cells (González Cid et al., 1997a), and on human lymphocytes (González Cid et al., 1997b).

In the present study hematopoietic progenitor colony assays were used to establish the effects of VRB on murine bone marrow.

Table 1. Effect of VRB on CFU-GM, BFU-E, and CFU-mix from murine bone marrow cells

Drug	Dose (μg/ml)	CFU-GM (mean ± SE)	BFU-E (mean ± SE)	CFU-mix (mean ± SE)
Control		185.0 ± 33.3	111.0 ± 55.6	15.5 ± 2.6
VRB	0.0025	138.0 ± 41.4	106.0 ± 65.7	10.7 ± 2.9
VRB	0.005	91.5 ± 13.8**	40.3 ± 18.4	8.3 ± 0.9**
VRB	0.01	66.5 ± 14.6**	24.5 ± 11.6	4.0 ± 1.7**
VRB	0.015	36.3 ± 10.9**	9.3 ± 4.2*	0.0
VRB	0.02	0.0	0.0	0.0

*$p < 0.04$, **$p < 0.02$ vs. control, Kruskal–Wallis test.

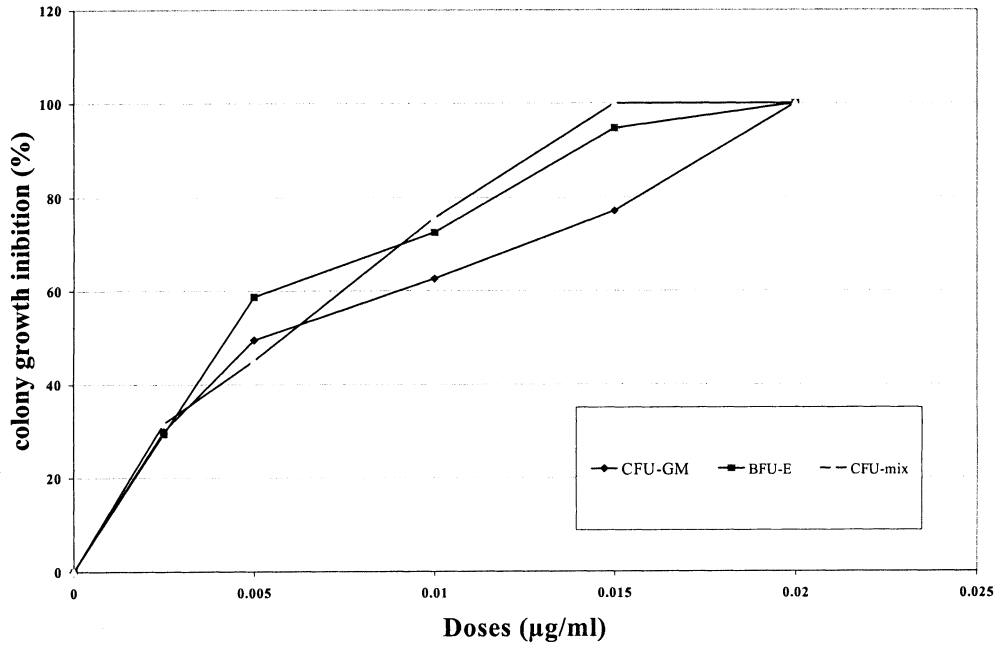

Figure 1. Inhibition of *in vitro* growth of CFU-GM, BFU-E, and CFU-mix from murine bone marrow cells exposed to VRB.

Table 2. Appearance of adherent layers from murine bone marrow cells

Drug	Dose (µg/ml)	Cellularity of adherent cells in Dexter layers
Control		++++
VRB	0.025	+++ –
VRB	0.05	++ – –
VRB	0.075	+ – – –
VRB	0.1	± – – –

The results obtained demostrate that VRB is able to induce inhibition of colony formation in a dose-dependent manner. Mitotic spindle poisons such as vincristine (Yalowich et al., 1985), docetaxel (Botta et al., 1999), and paclitaxel (Pannacciulli et al., 1999) are known to exhibit *in vitro* inhibitory effects on human hematopoietic progenitors. The ID_{50} of CFU-GM was 0.31 µmol/L for vincristine after 1 h of incubation; 0.04 µmol/L for docetaxel and 0.005 µmol/L for paclitaxel, both during 24 h of incubation; and 0.006 µmol/L (0.005 µg/ml) for VRB, indicating that this drug induces a bone marrow toxicity similarly to the other spindle poisons, in spite of the different incubation period.

A comparison of the dose–response curves shows that CFU-GM, BFU-E, and CFU-mix exhibited similar patterns of sensitivity to the cytotoxic action of VRB. However, previous *in vivo* and *in vitro* studies have shown that CFU-GM were less sensitive to the antiretroviral nucleoside derivatives azidothymidine (Ganser et al., 1989; Scheding et al., 1994; Lerza et al., 1997), and 2′,3′-dideoxycytidine (Johnson et al., 1988), and to the anticancer drug suramin (Estrov et al., 1995) than were BFU-E of murine and human origin.

Direct and indirect interactions between hematopoietic cells and the bone marrow stroma appear to play important roles in the regulation of hematopoiesis *in vivo* (Hasthorpe et al., 1992; Fukushima et al., 1994).

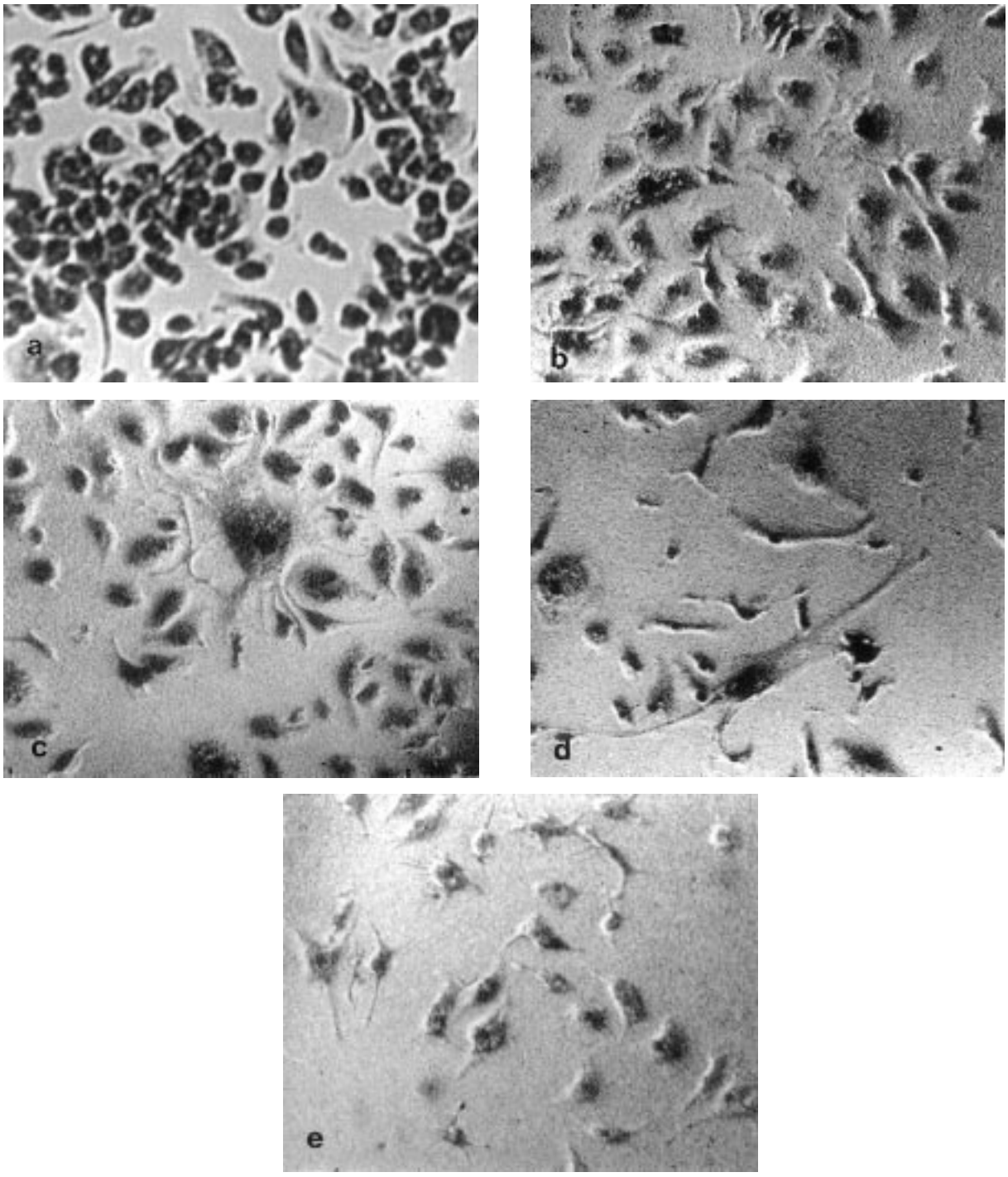

Figure 2. Bone marrow cells treated with different doses of VRB: (a) control; (b) 0.025 μg/ml; (c) 0.05 μg/ml; (d) 0.075 μg/ml; (e) 0.1 μg/ml.

Long-term marrow cultures have provided a valuable *in vitro* model for studying the role of the microenvironment of bone marrow in mammals. These cultures show that the stroma consists of a heterogeneous cell population that forms an *in vitro* microenvironment capable of supporting hematopoiesis (Boettiger and Dexter, 1984).

The stromal layers treated with increasing doses of VRB showed less cellularity compared to control layers. Furthermore, the dose of VRB to induce a marked stromal hypocellularity was 5 times higher than the dose that produced a total growth inhibition of CFU-derived colonies. Similarly, stromal cells in murine long-term bone marrow cultures did not show evidence of substantial cell death after exposure to relatively high doses of ionizing radiation (Gualtieri and McGraw, 1985; Zuckerman et al., 1986). It was observed that the hematopoietic stroma *in vivo* is more resistant to the destructive effects of ionizing radiation than are the hematopoietic stem cells (Bierkens et al., 1989). This could be due to the fact that the hematopoietic stromal microenvironment consists of a more static organ with a very slow turnover of both cellular and extracellular components (Zuckerman et al., 1986).

Our *in vitro* results show that VRB has an important effect on hematopoietic progenitors at the highest dose tested (0.02 μg/ml), while the stromal cells were not affected at a similar dose (0.025 μg/ml), suggesting that the stroma is more resistant to this drug.

The myelotoxicity induced by VRB in a murine bone marrow system permits study of the sensitivity of the different hematopoietic cells. The impairment of hematopoiesis in patients after VRB treatment could be due to the toxicity induced mainly on hematopoietic progenitors.

Acknowledgments

The authors thank Mrs M.T. Santarelli for statistical analysis. This work was supported by grants from the National Research Council (CONICET).

References

Bierkens JG, Hendry JH, Testa NG. The radiation response and recovery of bone marrow stroma with particular reference to long-term bone marrow cultures. Eur J Haematol. 1989; 43:95–107.

Boettiger D, Dexter TM. Long-term marrow cultures in the analysis of viral leukaemogenesis. In: McCulloch EA, ed, Clinics in haematology. Cell culture techniques. Philadelphia: WB Saunders; 1984:349–70.

Botta M, Arboscello E, Lerza R, Mencoboni M, Bogliolo G, Pannacciulli I. Effects of docetaxel on the *in vitro* growth of human myeloid progenitors. Anticancer Res. 1999;19:1855–8.

Budman DR. Vinorelbine (Navelbine): A third-generation vinca alkaloid. Cancer Invest. 1997;15:475–90.

Burris HA III, Fields S. Summary of data from *in vitro* and phase I vinorelbine (Navelbine) studies. Semin Oncol. 1994;21:14–20.

Crawford J, O'Rourke MA. Vinorelbine (Navelbine)/carboplatin combination therapy: dose intensification with granulocyte colony-stimulating factor. Semin Oncol. 1994; 21:73–8.

Dexter TM. Stromal cells associated hemopoiesis. J Cell Physiol. 1982;1:87–94.

Estrov Z, Talpaz M, Estey EH, Strassmann G. Role of suramin as an IL-1 inhibitor in suppression of acute myelogenous leukemia progenitor proliferation. Exp Hematol. 1995;23: 1080–7.

Fukushima N, Ueno K, Ohkawa H, Hayashi Y. Donor stromal cells support hematopoiesis in CFU-S colonies. Exp Hematol. 1994;22:1210–6.

Ganser A, Greher J, Völkers B, Staszewski S, Hoelzer D. Inhibitory effect of azidothymidine, 2′,3′-dideoxyadenosine, and 2′,3′-dideoxycytidine on *in vitro* growth of hematopoietic progenitor cells from normal persons and from patients with AIDS. Exp Hematol. 1989;17:321–5.

González Cid M, Cuello MT, Larripa I. Mitotic arrest and anaphase aberrations induced by vinorelbine in hamster cells *in vitro*. Anti-Cancer Drugs. 1997a;8:529–32.

González Cid M, Larripa I, Slavutsky I. Vinorelbine: cell cycle kinetics and differential sensitivity of human lymphocyte subpopulations. Toxicol Lett. 1997b;93:171–6.

Gualtieri RJ, McGraw J. Bone marrow stromal cells survive mega-dose irradiation and continue to produce hematopoietic growth factor(s). Blood (supplement 1) 1985;66:152.

Hasthorpe S, Bogdanovski M, Rogerson J, Radley JM. Characterization of endothelial cells in murine long-term

marrow culture. Implication for hemopoietic regulation. Exp Hematol. 1992;20:476-81.
Iscove NN, Senn JS, Till JE, McCulloch EA. Colony formation by normal and leukemic human marrow cells in culture: effect of conditioned medium from human leukocytes. Blood. 1971;37:1–5.
Johnson M, Caiazzo T, Molina JM, Donahue R, Groopman J. Inhibition of bone marrow myelopoiesis and erythropoiesis *in vitro* by anti-retroviral nucleoside derivatives. Br J Haematol. 1988;70:137–41.
Johnston EM, Crawford J. Hematopoietic growth factors in the reduction of chemotherapeutic toxicity. Semin Oncol. 1998; 25:552–61.
Lerza R, Castello G, Mela GS, et al. *In vitro* synergistic inhibition of human bone marrow hemopoietic progenitor growth by a 3′-azido-3′-deoxy-thymidine, 2′,3′-dideoxycytidine combination. Exp Hematol. 1997;25:252–5.
Masters GA, Hoffman PC, Drinkard LC, et al. Preliminary report on a phase I study of ifosfamide and vinorelbine (Navelbine) in advanced non-small cell lung cancer. Semin Oncol. 1996;23:11–8.
Messner HA. Human stem cells in culture. In: McCulloch EA ed, Clinics in haematology. Cell culture techniques. Philadelphia: WB Saunders; 1984:393–404.
Pannacciulli I, Ballarino P, Castello G, et al. *In vitro* toxicity of taxol based anticancer drug combinations on human hemopoietic progenitors. Anticancer Res. 1999;19:409–12.
Pauwels O, Kiss R, Pasteels JL, Atassi G. Cytotoxicity, cell cycle kinetics and morphonuclear-induced effects of Vinca alkaloid anticancer agents. J Pharm Pharmacol. 1995;47: 870–5.
Scheding S, Media JE, Nakeff A. Acute toxic effects of 3′-azido-3′-deoxythymidine (AZT) on normal and regenerating murine hematopoiesis. Exp Hematol. 1994;22:60–5.
Vokes EE, Haraf DJ, Masters GA, et al. Vinorelbine (Navelbine), cisplatin, and concomitant radiation therapy for advanced malignancies of the chest: a phase I study. Semin Oncol. 1996;23:48–52.
Welte K, Platzer E, Lu L, et al. Purification and biochemical characterization of human pluripotent hematopoietic colony-stimulating factor. Proc Natl Acad Sci USA. 1985; 82:1526–30.
Yalcin S, Guler N, Kansu E, et al. Granulocyte-colony stimulating factor (G-CSF) administration for chemotherapy-induced neutropenia. Hematology. 1996;1:155–61.
Yalowich JC, Zucali JR, Gross M, Ross WE. Effects of verapamil on etoposide, vincristine, and adriamycin activity in normal human bone marrow granulocyte-macrophage progenitors and in human K562 leukemia cells *in vitro*. Cancer Res. 1985;45:4921–4.
Zuckerman KS, Prince CW, Rhodes RK, Ribadeneira M. Resistance of the stromal cells in murine long-term bone marrow cultures to damage by ionizing radiation. Exp Hematol. 1986;14:1056–62.

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