Preclinical Studies in Support of Defibrotide for the Treatment of Multiple Myeloma and Other Neoplasias

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Abstract

Purpose of the Study: Defibrotide, an orally bioavailable polydisperse oligonucleotide, has promising activity in hepatic veno-occlusive disease, a stem cell transplantation–related toxicity characterized by microangiopathy. The antithrombotic properties of defibrotide and its minimal hemorrhagic risk could serve for treatment of cancer-associated thrombotic complications. Given its cytoprotective effect on endothelium, we investigated whether defibrotide protects tumor cells from cytotoxic antitumor agents. Further, given its antiadhesive properties, we evaluated whether defibrotide modulates the protection conferred to multiple myeloma cells by bone marrow stromal cells.

Methods-Results: Defibrotide lacks significant single-agent *in vitro* cytotoxicity on multiple myeloma or solid tumor cells and does not attenuate their *in vitro* response to dexamethasone, bortezomib, immunomodulatory thalidomide derivatives, and conventional chemotherapeutics, including melphalan and cyclophosphamide. Importantly, defibrotide enhances *in vivo* chemosensitivity of multiple myeloma and mammary carcinoma xenografts in animal models. In cocultures of multiple myeloma cells with bone marrow stromal cells *in vitro*, defibrotide enhances the multiple myeloma cell sensitivity to melphalan and dexamethasone, and decreases multiple myeloma–bone marrow stromal cell adhesion and its sequelae, including nuclear factor-κB activation in multiple myeloma and bone marrow stromal cells, and associated cytokine production. Moreover, defibrotide inhibits expression and/or function of key mediators of multiple myeloma interaction with bone marrow stromal cell and endothelium, including heparanase, angiogenic cytokines, and adhesion molecules.

Conclusion: Defibrotide's *in vivo* chemosensitizing properties and lack of direct *in vitro* activity against tumor cells suggest that it favorably modulates antitumor interactions between bone marrow stromal cells and endothelia in the tumor microenvironment. These data support clinical studies of defibrotide in combination with conventional and novel therapies to potentially improve patient outcome in multiple myeloma and other malignancies.

Translational Relevance

Thrombotic and microangipathic complications of malignancy are well established, and constitute a major source of morbidity and mortality in cancer patients both from treatment and the disease process itself. Therapeutic strategies targeting endothelial injury may have the dual benefit of reducing toxicity and enhancing efficacy in patients. Defibrotide constitutes an orally bioavailable novel agent with antithrombotic properties and minimal toxicity. Our study shows that defibrotide modulates the interaction of stromal cells with tumor cells in a manner that increases the responsiveness of the latter to existing antineoplastic therapies. These data indicate that the defibrotide has considerable potential as part of combination treatment in specific settings, including multiple myeloma and solid tumors, such as breast cancer.

Thrombotic and microangiopathic complications of malignancy are well established, and constitute a major source of morbidity and mortality in cancer patients because of the disease process itself and/or because of complications of treatment (1, 2). Therapeutic strategies targeting endothelial injury may be beneficial in preventing or helping the treatment of cancer-associated thrombotic complications. Defibrotide is a novel agent that fits such a profile. This polydisperse polydeoxyribonucleotide, derived from mammalian tissue (porcine mucosa) by controlled depolymerization (3), exhibits diverse biological properties, including antithrombotic, thrombolytic, and antiadhesive effects, and can also protect endothelial cells from chemotherapy-mediated cytotoxicity (3, 4). It is orally bioavailable and, importantly, because it confers no significant hemorrhagic risk (5), defibrotide is an attractive therapeutic option for clinical settings where cytoprotective and antithrombotic interventions are needed with a small margin for compromise of hemostasis. Hepatic veno-occlusive disease is one such clinical setting where defibrotide has been successfully evaluated as an important therapeutic option (6–12). This clinical syndrome of painful hepatomegaly, jaundice, ascites, fluid retention, and otherwise unexplained weight gain is a common (present in up to 60% of patients) regimen-related complication of hematopoietic stem cell transplantation, with severity ranging from mild, reversible disease to a severe syndrome associated with multiorgan failure and death (13–16). Established severe veno-occlusive disease has a mortality rate approaching 100% by 100 days post–stem cell transplantation (13, 14, 16, 17). Veno-occlusive disease is currently considered to result from conditioning regimen-induced injury to the hepatic sinusoidal endothelium (18), leading to hepatocellular injury, stellate cell activation, and subendothelial edema, which eventually result in a triad of sinusoidal obstruction, hepatocellular necrosis, and venous occlusion, which are the basic pathophysiologic processes contributing to the clinical presentation of veno-occlusive disease (as reviewed in ref. 19). Many of the pleiotropic biological effects of defibrotide directly counteract the fundamental pathophysiologic sequelae of veno-occlusive disease and potentially confer protection to hepatic microvasculature against chemotherapy-mediated injury (3, 4). These considerations have provided a basis for the use of defibrotide for both the treatment and prevention of veno-occlusive disease (6–12).

The clinical efficacy and safety profile of defibrotide in veno-occlusive disease prompted us to hypothesize that it could be used for the prevention and/or treatment of cancer-associated thromboembolic events, such as those emerging in the field of multiple myeloma with the use of certain types of novel agents (2). It was important, however, to first evaluate whether defibrotide administration could interfere with the antitumor activity of certain conventional or novel therapeutics for both hematologic malignancies and solid tumors. We specifically studied two distinct questions, both of which derive from our current understanding of its properties. First, we sought to determine whether defibrotide could attenuate the antitumor activity of various therapeutic agents, including cytotoxic chemotherapy, glucocorticoids, or novel therapies, including the first-in-class proteasome inhibitor bortezomib. Given its cytoprotective properties, specifically its ability to protect endothelial cells from chemotherapy-mediated cytotoxicity (4), it was critical to assess whether defibrotide could similarly protect tumor cells from cytotoxic injury, because defibrotide use might theoretically compromise the benefit derived from the treatment regimen. Second, we wanted to evaluate whether defibrotide could interfere with tumor-stromal interactions and their impact on the response of malignant cells to various therapeutic agents. Extensive data in various disease settings, most importantly in multiple myeloma as well as in other hematologic malignancies (e.g. various forms of leukemia) and bone metastases of various solid tumors (e.g. breast or prostate carcinomas; as reviewed in refs. 20, 21), have indicated that adhesion of malignant cells to bone marrow stromal cells can protect the tumor cells from the effects of conventional therapeutics such as cytotoxic chemotherapeutics or, in the case of multiple myeloma, dexamethasone. Given the antiadhesive properties of defibrotide in other model systems (22–26), we hypothesized that defibrotide could play a similar role in

abrogating the tumor-stromal adhesive interaction and thus enhance the responsiveness of malignant cells to various antitumor agents.

In this current study, we observed that in conventional *in vitro* assays, defibrotide has no direct anti–multiple myeloma activity against multiple myeloma, breast cancer, and colon cancer cells, and does not attenuate the antitumor activity of several different classes of antineoplastic drugs. Interestingly, with *in vitro* cocultures of multiple myeloma cells with bone marrow stromal cells, defibrotide enhances the anti–multiple myeloma activity of certain drugs, such as melphalan or dexamethasone. This effect is associated with suppressed adhesive interaction between the two cell compartments, abrogation of nuclear factor-κB (NF-κB) activation triggered by multiple myeloma–bone marrow stromal cell adhesion, decreased production of proliferative/antiapoptotic cytokines, and perturbed expression and/or function of key mediators of multiple myeloma–microenvironment interactions, such as heparanase, angiogenic cytokines, and adhesion molecules.

Importantly, in *in vivo* animal models of xenografted tumor cells (from multiple myeloma or from breast carcinoma), administration of defibrotide increased the antitumor activity of cytotoxics, including cyclophosphamide. Furthermore, pharmacokinetic analyses revealed that the levels of defibrotide achieved *in vivo* in mice treated with defibrotide are consistent with the levels required (based on *in vitro* studies) for the antitumor properties of defibrotide.

These results suggest that defibrotide administration is not only compatible with conventional and many novel antitumor treatment strategies, but also indicate that defibrotide exhibits microenvironment-modulatory properties that serve to attenuate key aspects of tumor pathophysiology and drug resistance (e.g. tumor cell adhesion to stroma and ensuing production of antiapoptotic cytokines). This therefore allows defibrotide to enhance the responsiveness of tumor cells to certain conventional therapies. These results, coupled with the particular role of tumor-stromal interactions in the pathophysiology of multiple myeloma, provide the basis for further preclinical and clinical evaluation of defibrotide as an adjuvant agent for the management of multiple myeloma and other malignancies, including breast cancer.

Materials and Methods

Cell lines and primary cells. Our studies included the human breast adenocarcinoma cell line MCF-7 (American Type Culture Collection); the human colorectal adenocarcinoma cell line HT-29 (purchased from the American Type Culture Collection); and a series of human multiple myeloma cell lines, including MM-1S, MM-1R (kindly provided by Dr. Steve Rosen, Feinberg School of Medicine, Northwestern University, Chicago, IL), RPMI-8226 and Dox40 (kindly provided by William Dalton, Moffitt Cancer Center, Tampa, FL), U266 (purchased from the American Type Culture Collection), and OPM-1. Human dermal microvascular endothelial cells were kindly provided by the Centers for Disease Control and Prevention. Human neonatal dermal microvascular endothelial cells and human umbilical vein endothelial cells were purchased from Cambrex and propagated in EGM2-MV medium (Cambrex). Primary multiple myeloma tumor cells were obtained as previously described (27). Bone marrow stromal cells were cultured as previously described (27). All cultures were done at 37°C in a humidified atmosphere and in medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Invitrogen, Inc.).

Compounds. Defibrotide was provided by Gentium. Carboplatin, melphalan, paclitaxel, vinblastine, vincristine, doxorubicin, dexamethasone, cyclophosphamide, BCNU, and monocrotaline (28) were purchased from SIGMA-Aldrich. Bortezomib (PS-341, Velcade) was obtained from Millennium Pharmaceuticals. CC4047 (Actimid) and CC5013 (Lenalidomide, Revlimid) were obtained from Celgene.

Cell viability assays. MM1-S and MM1-R cells were detached from the flask using a cell scraper and were washed once in RPMI-2%FBS. Two thousand cells were plated per well in a 96-well format in RPMI-2%FBS. The cells were allowed to recover for 24 h after which they were exposed to the drugs in RPMI-2%FBS for 96 h. MCF-7 cells and HT-29 cells were detached from the flask using 0.25% trypsin in EDTA (Invitrogen, Inc.) and washed once in RPMI-2%FBS. Two thousand cells were plated per well in a 96-well format in RPMI-2%FBS. The cells were allowed to recover for 24 h after which they were exposed to the drugs in RPMI-2%FBS for 96 h. Human neonatal dermal microvascular endothelial cells and human umbilical vein endothelial cells were detached from the flask using 0.25% trypsin in EDTA (Invitrogen, Inc.) and washed once in EGM2-MV. Two thousand cells were plated per well in a 96-well format in EGM2-MV. The cells were allowed to recover for 24 h after which they were exposed to the drugs in EGM2-MV for 96 h. The final volume of each well during the 96-h incubation with the drugs was 100 μL and the incubation took place at 37°C in a humidified atmosphere. Following the 96-h drug exposure, 10 μL of the WST-1 reagent (BioVision Research Products) were added to each well and incubated for 30 min at 37°C. WST-1 is a tetrazolium salt that is cleaved to formazan by mitochondrial dehydrogenases (29). Increased cell number results in increased mitochondrial dehydrogenase activity and therefore increased formazan dye formation. Measurement of the absorbance at 440 nm allowed quantification of the formazan dye. A reference wavelength of 650 nm was used per recommendations from the manufacturer of the reagent. The 650-nm reference absorbance value was subtracted from the 440-nm absorbance value. Background was measured using an empty well containing medium and no cells.

For *in vitro* coculture assays of multiple myeloma cells with bone marrow stromal cells, primary multiple myeloma tumor cells were cultured in the presence or absence of bone marrow stromal cells, and exposed to defibrotide, dexamethasone or melphalan, or combination of defibrotide with dexamethasone or melphalan. Treatment-induced myeloma cell death was measured by flow cytometry using the Apo2.7 mitochondrial early apoptosis marker. Multiple myeloma cells were distinguished from bone marrow stromal cells by staining for CD38 (30).

In vivo *studies of defibrotide as single agent and in combination with other therapeutics.* To evaluate the potential impact of defibrotide on *in vivo* growth of tumors, we carried out studies in the following different animal models: a SCID/NOD mouse model of s.c. plasmacytoma xenografts of MM-1S cells; a SCID/NOD mouse model of diffuse multiple myeloma bone lesions generated by MM-1S cells; and a rat 13762 mammary carcinoma model.

In the SCID/NOD mouse model of s.c. plasmacytoma xenografts of MM-1S cells, 60 male SCID/NOD mice (6-8 wk old) were irradiated (450 rads) and, 24 h later, injected s.c. with 5×10^6 MM-1S human multiple myeloma cells. Upon formation of palpable tumors, the mice were randomly assigned to 6 cohorts (10 mice each) receiving (*a*) vehicle; (*b*) defibrotide i.v. 450 mg/kg b.i.d.; (*c*) melphalan 2.5 mg/kg i.p. once weekly; (*d*) cyclophosphamide 50 mg/kg i.p., on days 8, 10, 12, 20, 22, and 24; (*e*) and (*f*) combinations of defibrotide (300 mg/kg i.v.) with melphalan or cyclophosphamide, respectively. Mice were monitored every 3 d for body weight, potential toxicity, and electronic caliper-based tumor volumes.

In the SCID/NOD mouse model of diffuse multiple myeloma bone lesions, 20 male SCID/NOD mice (6-8 wk old) were irradiated (450 rads) and, 24 h later, injected i.v. with 1×10^6 MM-1S human multiple myeloma cells, leading to formation of diffuse multiple myeloma tumors, as previously described (31). The mice were then randomly assigned to four cohorts (five mice each) receiving (*a*) vehicle; (*b*) defibrotide orally 45 mg/kg b.i.d.; (*c*) melphalan 2.5 mg/kg i.p. once weekly; and (*d*) combinations of defibrotide (45 mg/kg) with melphalan. The mice were monitored for body weight and potential toxicity. If they developed signs of

morbidity (e.g. hind limb paralysis due to tumor growth in the spine, infection, major bleeding, etc.), they were sacrificed by CO_2 inhalation according to Animal Care Use Committee guidelines. The primary end point for this model was the overall survival of mice (defined as time between initiation of treatment and sacrifice).

Rat mammary adenocarcinoma 13762 is a carcinogen-induced tumor syngeneic for the female Fisher 344 rat. For experiments, 13762 mammary carcinoma cells (2×10^6) prepared from a brei of donor tumors were implanted s.c. into a hind leg of female Fisher 344 rats weighing between 140 and 160 g (Taconic Farms). The animals were housed and handled in accordance with Animal Care and Use Committee guidelines. The 13762 tumor grew to 100 mm^3 in about 8 d (32, 33). Each group consisted of five rats. Defibrotide administration was initiated either at the same time as cytotoxic treatment or 2 d after the first dose of cytotoxic treatment. Monocrotaline (350 mg/kg) or BCNU (150 mg/kg) was administered by i.p. injection on days 8 and 18 post–tumor cell implant (28). Defibrotide (200 mg/kg) was administered twice per day by i.v. injection on days 8 through 26 or on days 10 through 26. Tumor volumes were calculated using the formula $(w^2 \times l)/2$ where w is the width of the tumor and l is the length of the tumor. Individual rats were weighed and tumor measurements taken by calipers twice weekly. The antitumor activity of the treatments was determined by calculating tumor growth delay (T-C) in days. Tumor growth delay was obtained by determining the difference between treatment and control group mean tumor growth in days at a predetermined tumor volume. The data are presented as means ± SE. caliper-based tumor volumes.

Pharmacokinetic studies. Eight-week-old male Fisher 344 rats (Charles River Laboratories) were treated (10 mice per treatment cohort), either orally or i.v., with defibrotide (48 mg/kg). Peripheral blood plasma samples were serially collected (0-8 h) via preinserted jugular vein catheter for determination of pharmacokinetic profile by high-pressure liquid chromatography analyses and validation by agarose gel determination, as previously described. Briefly, blood samples taken before the administration (basal condition) of defibrotide and at 1, 3, 5, 10, 20, 30, 40 min and 1, 2, and 4 h after administration. The anticoagulated blood was centrifuged to obtain the plasma samples and 1 mL of N perchloric acid was added to each 1 mL of plasma. After stirring, the sample was heated for 15 min in water bath at 70°C degrees. The samples were centrifuged at 3,000 *g*. The pellet was discarded and 1 mL of diphenylamine reagent [100 mL of glacial acetic acid + 1.5 g diphenylamine + 1.5 mL conc. sulfuric acid and 0.6 mL of 0.16% (w/v) solution of acetaldehyde in water] was added to 1 mL of supernatant. The samples were incubated for 17 to 20 h at 25°C to 30°C. The absorbance of the samples at 600 nm was read in spectrophotometer. A standard curve was also prepared with defibrotide dissolved in plasma taken from control animals. For the high-pressure liquid chromatography determination, plasma samples were heated to 70°C for 3 min and were then left to cool to 37°C. After adding 12 mg trypsin, samples were kept in water bath at 37°C for 5.5 h and then diluted with 10 mmol/L phosphate buffer, pH 7.0-Mobile phase of Chromatography, injected into liquid phase chromatograph. For agarose gel determination, plasma samples were heated to 70°C for about 3 min, then left cool to 37°C. After addition of trypsin, the samples were kept in water bath at 37°C for 5.5 h and then were diluted with Tris-acetate buffer, pH 7.8, to give concentrations of defibrotide from 10 to 50 ug/mL. The samples were run in 2-mm thick 0.5% agarose gel in 5% sorbitol in acetate buffer, pH 7.8 at >4°C, 90V, 30 to 35mA for 75 min, in a LKB model 2117 multiphor II electrophoretic chamber (from reference materials). The gels were stained with acridine orange (0.006% w/v), in 0.01 mol/L phosphate buffer, pH 7.0. The gel slabs, after drying, were analyzed by a thin-layer chromatography scanner photo densitometer. The measurements with carried at 470 nm.

Molecular profiling studies and functional experiments. mRNA levels of heparanase, vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF-2), ICAM-1, and E-selectin were measured through

Syber-green Real-Time PCR of cDNA prepared from target cell populations (multiple myeloma cells, endothelial cells) treated with and without defibrotide using the following primers heparanase: F- TCACCATTGACGCCAACCT and R- CTTTGCAGAACCCAGGAGGAT; VEGF: F- CTACCTCCACCATGCCAAGT and R- GCAGTAGCTGCGCTGATAGA; FGF-2: F- CCACTTCAAGGACCCCAAG and R- ATAGCCAGGTAACGGTTAGC; ICAM-1: F- CTGTTCCCAGGACCTGGCAAT and R- AGGCAGGAGCAACTCCTTTTTA; E-selectin: F- CTCTGACAGAAGAAGCCAAG and R- ACTTGAGTCCACTGAAGCCA; and β-actin: F- TCACCCACACTGTGCCCATCTACGA and R- CAGCGGAACCGCTCATTGCCAATGG (housekeeping gene). Intracellular levels of heparanase were measured by intracellular flow cytometry through FACSCalibur flow cytometer and the CellQuest analysis program (Becton Dickinson) using goat polyclonal antihuman heparanase antibodies (Santa Cruz Biotecnology Inc). Omitting the first antibody served as a negative control to detect unspecific fluorescence. Heparanase enzymatic activity was measured in extracts of defibrotide-treated versus control cells by a commercial heparan degrading enzymatic kit (Takara-bio Inc.).

Adhesion assays of multiple myeloma cells on bone marrow stromal cells were done by using carboxyl fluorescent succinimidyl ester (CFSE) labeling of multiple myeloma cells with the CellTrace CFSE Cell Proliferation Kit (Molecular Probes) according to the instructions of the manufacturer. After CFSE labeling, multiple myeloma cells were plated in optical 96-well plates on which bone marrow stromal cells had been preseeded and defibrotide or control medium. At the conclusion of the cocultures, each well was processed by gentle aspiration of their supernatant and washing with 1× PBS. After repeating the aspiration and addition of 1×PBS twice, the plates were read in a fluorescence plate reader (excitation and emission wavelengths of 485 and 520 nm, respectively) to evaluate the percentage of fluorescent multiple myeloma cells that remained adhered to bone marrow stromal cells in the presence versus absence of defibrotide treatment.

NF-kB–binding ELISAs for evaluation of transcription factor activity were carried out with NF-kB transcription factor ELISA kits (Active Motif) as previously described. Interleukin 6 (IL-6) and VEGF protein levels were measured with corresponding ELISA kits (R&D Diagnostics) according to the instructions of the manufacturer.

Tumor cell invasion assays were done to assess *in vitro* the invasive potential of multiple myeloma cells. Briefly, multiple myeloma cells overexpressing heparanase or supplemented with heparanase recombinant enzyme (GenWay Biotech Inc.) treated with and without defibrotide were layered on the top chamber of a dual-chamber *in vitro* culture system (Becton Dickinson). The two chambers were separated by a polyethylene terephthalate membrane and matrigel, and the number of multiple myeloma cells that invaded the matrigel and the polyethylene terephthalate membrane towards the lower chamber were evaluated by fluorescence microscopy and fluorescence plate readers. The invasive potential of the multiple myeloma cells was assessed on the basis of the number of fluorescent (calcein-labeled) multiple myeloma cells penetrating the lower chamber. In order to have multiple myeloma cells overexpressing heparanase, heparanase cDNA (HPSE1) was subcloned into the pcDNA3.1/CT-GFP (Invitrogen) vector to give an in-frame COOH-terminal green fluorescent protein fusion construct. The pcDNA3.1/CT-GFP/HPSE1 construct was transfected into the RPMI 8226 human multiple myeloma cells using Lipofectin reagents (Invitrogen) following the manufacturer's instructions.

Statistical methods. In the cell viability assays, each experimental point was set up in duplicate wells and each assay was repeated identically and independently at least once and, for each experiment, two repetitions of the assay were combined into a single data set in which each experimental point was represented by four values. The mean and SD of these four values were determined for each experimental

point. The final data were expressed as a percentage of the proliferation that took place in control wells where cells were not exposed to any drugs. IC_{50} values were determined for each experiment. In the *in vivo* models, the overall survival of mice was evaluated with Kaplan-Meier survival analyses, and differences between the various cohorts of each experiment were assessed by log-rank tests.

Results

Assessment of direct effects of defibrotide on* in vitro *viability of tumor cells and endothelial cells. We did colorimetric survival assays to test the *in vitro* effects of defibrotide (0-1,000 µg/mL for 96 hours) on human multiple myeloma cell lines. We observed that exposure to defibrotide did not significantly affect the *in vitro* viability of these cell lines (except for a modest decline in viability of MM-1R cells treated with the highest concentration of defibrotide tested, i.e. 1,000 µg/mL; Fig. 1A and Supplementary Fig. S1). We extended these observations to other tumor types (e.g. the breast carcinoma cell line MCF-7 and the colon cancer cell line HT-29; Supplementary Fig. S1C and S1D, respectively); to two different endothelial cell models (human neonatal dermal microvascular endothelial cells and human umbilical vein endothelial cells; Supplementary Fig. S1E and S1F, respectively); and to additional well-characterized human multiple myeloma cell line models (e.g. OPM-1, Dox40, and RPMI-8226/S; Supplementary Fig. S1G).

Fig. 1.

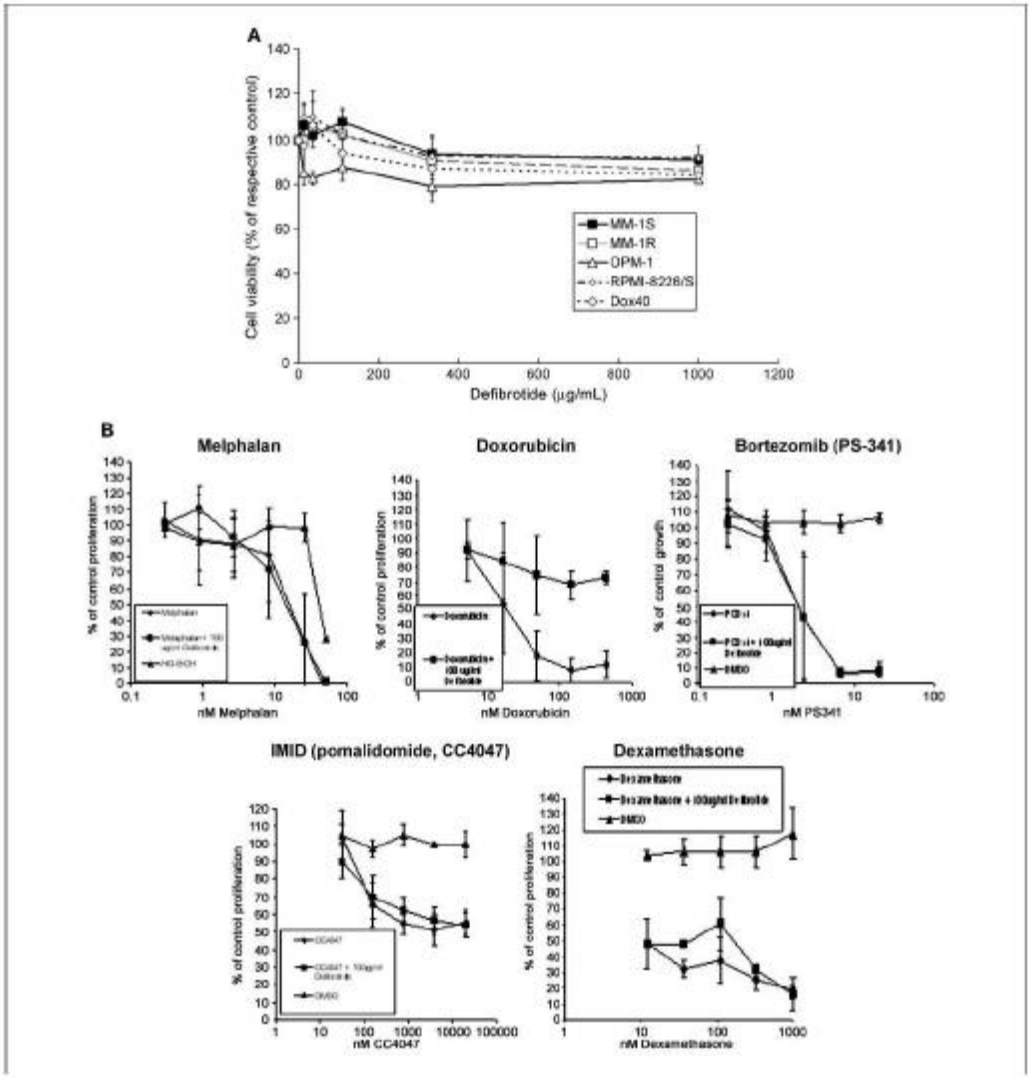

Fig. 1. *In vitro* viability of multiple myeloma cell lines treated with defibrotide alone or its combination or novel anti – multiple myeloma agents. *A*, results of *in vitro* defibrotide treatment of multiple myeloma cell lines, including MM-1S, its dexamethasone-resistant subline MM-1R, the chemosensitive RPMI-8226/S and its doxorubucin-resistant subline Dox40, as well as the OPM-1 cells. *B*, WST-1 assays were done to assess the putative impact of defibrotide on the *in vitro* response of multiple myeloma cells to diverse agents, including melphalan, doxorubicin, bortezomib (PS341), the IMID pomalidomide (CC-4047), and dexamethasone. These results represent the combination of two separate independent experiments.

In vitro viability of multiple myeloma cell lines treated with defibrotide alone or its combination or novel anti–multiple myeloma agents. *A*, results of *in vitro* defibrotide treatment of multiple myeloma cell lines, including MM-1S, its dexamethasone-resistant subline MM-1R, the chemosensitive RPMI-8226/S and its doxorubucin-resistant subline Dox40, as well as the OPM-1 cells. *B,* WST-1 assays were done to assess the putative impact of defibrotide on the *in vitro* response of multiple myeloma cells to diverse agents, including melphalan, doxorubicin, bortezomib (PS341), the IMID pomalidomide (CC-4047), and dexamethasone. These results represent the combination of two separate independent experiments.

Evaluation of* in vitro *effects of combinations of defibrotide with diverse antitumor agents. Colorimetric survival assays (with WST-1) were done to evaluate whether defibrotide can modulate the activity of various therapeutics against multiple myeloma cells, cell lines from other tumor models, or endothelial cells. We specifically evaluated the *in vitro* effects of combinations of defibrotide with a series of antitumor agents, including dexamethasone, the proteasome inhibitor bortezomib (PS-341), the immunomodulatory derivatives CC-5013 (lenalidomide, Revlimid), and CC-4047 (pomalidomide, Actmid), as well as carboplatin, melphalan, paclitaxel, vinblastine, vincristine, and doxorubicin. When used as single agents, carboplatin,

melphalan, paclitaxel, vinblastine, vincristine, doxorubicin, bortezomib, CC-4047, and dexamethasone significantly decreased the numbers of viable MM-1S (Fig. 1, Supplementary Fig. S2 and Supplementary Table S1). When these MM-1S cells were exposed to a combination of 100 μg/mL defibrotide with increasing concentrations of carboplatin, melphalan, paclitaxel, vinblastine, vincristine, bortezomib, CC-4047, CC-5013, or dexamethasone, the effect of each one of these combinations on the viability of MM-1S was similar to the effect observed with each agent alone in the absence of defibrotide (Fig. 1, Supplementary Fig. S2, and Supplementary Table S1). We then extended these combination studies to other types of tumor cells and to endothelial cells and observed that defibrotide also did not sensitize MCF-7, HT-29, human neonatal dermal microvascular endothelial cells, or human umbilical vein endothelial cells to any of these agents or antagonize the activity of any of these agents (Supplementary Table S1). The only exception to that general pattern was observed in combinations of defibrotide with doxorubicin: when defibrotide was used (at a fixed concentration of 100 μg./mL) in combination with doxorubicin, the decrease in MM-1S cell viability was significantly attenuated, with an IC_{50} of 20 nmol/L in the absence of defibrotide and of >400 nmol/L in the presence of defibrotide (Fig. 1, and Supplementary Table S1). Similarly, defibrotide significantly increased the IC_{50} value of doxorubicin against MCF-7 cells (IC_{50} value of 20 nmol/L in the absence of defibrotide versus 550 nmol/L in the presence of defibrotide), HT-29 cells (IC_{50} value of 60 versus 25,000 nmol/L, respectively), human neonatal dermal microvascular endothelial cells (200 versus 2900 nmol/L respectively), and human umbilical vein endothelial cells (200 versus 1,100 nmol/L, respectively; Supplementary Table S1).

We also evaluated whether defibrotide could revert the resistance to dexamethasone. To this end, we treated the MM-1R cells (a dexamethasone-resistant subline of the MM-1S cells) with dexamethasone alone versus a combination of dexamethasone and defibrotide. We observed that dexamethasone alone failed (at concentrations up to 1 μmol/L) to inhibit the survival of MM-1R cells (whereas it inhibited MM-1S cell viability with an IC_{50} of 12 nmol/L) and that in the presence of defibrotide, the IC_{50} for inhibition of MM-1R cell viability by was still >1 μmol/L, suggesting that defibrotide does not overcome the dexamethasone resistance of MM-1R cells (Supplementary Fig. S2F and Supplementary Table S1).

In vivo *antitumor activity of defibrotide.* The *in vivo* antitumor activity of defibrotide was evaluated in a series of different animal models of tumor growth, including a SCID/NOD mouse model of s.c. plasmacytoma xenografts of MM-1S cells, a SCID/NOD mouse model of diffuse multiple myeloma bone lesions generated by MM-1S cells, and a rat 13762 mammary carcinoma model. In each one of these models, tumor-bearing mice were treated with defibrotide alone, with various antitumor therapeutics (the choice of which depended on the particular tumor type that was studied), with combinations of defibrotide with these antitumor therapeutics, or with the respective vehicles. In the first of these models, SCID/NOD mice bearing s.c. plasmacytoma xenografts of MM-1S cells were randomly assigned to cohorts treated with vehicle, melphalan alone, cyclophosphamide alone, defibrotide alone, defibrotide plus melphalan, and defibrotide plus cyclophosphamide. Defibrotide, either as single agent or in combination with melphalan or cyclophosphamide, was well tolerated without hemorrhagic complications or body weight loss ($P > 0.05$) in all groups. The major end points for efficacy were (*a*) tumor volume changes and (*b*) overall survival (time-to-sacrifice, done when tumor diameters >2 cm). Defibrotide treatment resulted in significantly lower tumor volumes than in control mice ($P < 0.05$ for all comparisons by ANOVA and posthoc tests). The combination of defibrotide with melphalan or cyclophosphamide induced significantly lower tumor volumes than the respective single-agent cytotoxic chemotherapy (Fig. 2A and B ; $P < 0.05$ for all comparisons). Kaplan-Meier survival analyses showed that defibrotide administration, either as single agent or in combination with cytotoxic chemotherapy (melphalan or cyclophosphamide), was associated with statistically significant prolongation of overall survival, in comparison with vehicle-treated control

group or melphalan- or cyclophosphamide-treated groups, respectively ($P < 0.001$ for all comparisons, log-rank test).

Fig. 2.

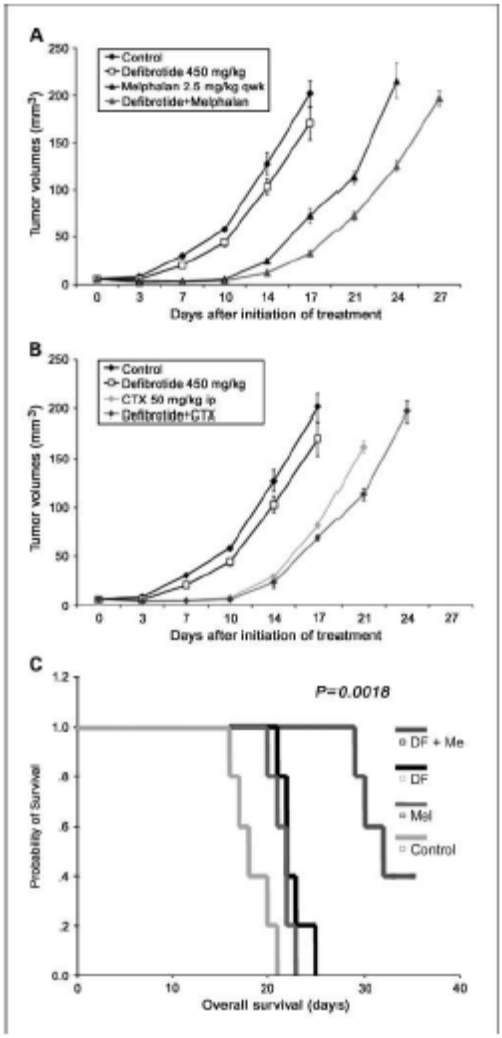

Fig. 2. *In vivo* studies of defibrotide in combination with other antitumor agents. Changes in tumor volume over time in mice xenografted with human MM-1S cells (s.c. xenograft model in *A* and *B*, and model of diffuse multiple myeloma lesions in *C*) and treated with defibrotide either alone or in combination with melphalan (*A* and *C*) or cyclophosphamide (*B*).

In vivo studies of defibrotide in combination with other antitumor agents. Changes in tumor volume over time in mice xenografted with human MM-1S cells (s.c. xenograft model in *A* and *B*, and model of diffuse multiple myeloma lesions in *C*) and treated with defibrotide either alone or in combination with melphalan (*A* and *C*) or cyclophosphamide (*B*).

In the second model, SCID/NOD mice injected i.v. with multiple myeloma cells to generate diffuse multiple myeloma bone lesions were randomly assigned to cohorts treated with vehicle, melphalan alone, defibrotide alone, and defibrotide plus melphalan. In this model, the combination of defibrotide plus melphalan led to significant prolongation in overall survival of mice compared with each drug alone (Fig. 2C; $P = 0.0018$, log-rank test).

In the third tumor model of rat 13762 mammary carcinoma, tumor-bearing animals were treated with monocrotaline alone, BCNU alone, or with combinations thereof with defibrotide. The primary endpoint in

this model was the growth delay that was achieved in each treatment cohort, defined as the difference in the number of days necessary for mice in the control cohort to reach tumor volumes of 500 mm^3 compared with mice in the respective treatment cohort (day 0 was the day of tumor cell implantation and chemotherapy was administered on days 8 and 18; Supplementary Table S2). In this model, the growth delay achieved in the cohorts of mice treated with the combination of defibrotide with either monocrotaline or BCNU was longer than the growth delay in the cohorts treated with the respective cytotoxic chemotherapeutic alone.

Preclinical pharmacokinetic studies of defibrotide. We evaluated the *in vivo* pharmacokinetic profile of defibrotide in one of our *in vivo* animal models. Specifically, blood samples were collected at specific intervals (as described in Materials and Methods) from preinserted jugular vein catheters in Fisher male rats treated with defibrotide (48 mg/kg single dose) either through oral or i.v. route (Supplementary Fig. S3). Plasma levels of defibrotide were evaluated by high-pressure liquid chromatography and agarose gel determination and provided highly consistent pharmacokinetic data between the two techniques (with mean and median variability of measurement of 8.07% and 7.89%, respectively). With i.v. and oral administration, peak defibrotide levels (C_{max}) were 1,253 and 474 μg/mL, and area under the curve ($AUC_{0\text{-}240\ min}$) 14.71 and 17.19 μg × min/L, respectively. Time to C_{max} was ~30 min for oral administration and returned to undetectable levels by 240 min posttreatment for both oral and i.v treatment (Supplementary Fig. S3). These results suggest that the levels of defibrotide that are required for some of the mechanistic sequelae of defibrotide in our preclinical *in vitro* models are achievable *in vivo* and could be involved in the *in vivo* mechanism(s) whereby defibrotide sensitizes multiple myeloma tumor cells to certain antitumor therapies.

Effect of defibrotide on* in vitro *cocultures of multiple myeloma cells with bone marrow stromal cells. Adhesion to bone marrow stromal cells attenuates the response of multiple myeloma cells to conventional antineoplastic agents, e.g. dexamethasone and cytotoxic chemotherapeutics (as reviewed in ref. 21). Because of the proposed antiadhesive properties of defibrotide in the context of vascular biology (22–26), we hypothesized that defibrotide may have a similar effect in the setting of tumor-stromal interactions in multiple myeloma. We thus evaluated whether defibrotide can modify the response of primary multiple myeloma cells to such conventional therapeutics in the context of stromal coculture. Primary multiple myeloma tumor cells (CD38 positive) isolated from multiple myeloma patients were cocultured with bone marrow stromal cells (CD38 negative) and flow cytometry was used to evaluate the fraction of dead cells (Apo2.7-PE positive) within the CD38-positive multiple myeloma cells. Treatment with defibrotide (100 μg/mL) alone did not trigger significant changes in multiple myeloma cell viability compared with defibrotide-free control cultures, either in the presence or absence of bone marrow stromal cells (Fig. 3 and Supplementary Fig. S4). Furthermore, in the absence of bone marrow stromal cells, defibrotide administration did not significantly increase the percentage killing of multiple myeloma cells exposed to dexamethasone (Fig. 3A and Supplementary Fig. S4A) or melphalan (Fig. 3B and Supplementary Fig. S4B), consistent with the colorimetric survival assay results obtained with multiple myeloma cell lines. When multiple myeloma cells were treated with dexamethasone or melphalan alone in the presence of bone marrow stromal cells, the percentage multiple myeloma cell killing was in most cases significantly lower compared with single-agent dexamethasone- or melphalan-induced cell killing in the absence of bone marrow stromal cells. However, in the context of multiple myeloma cell coculture with bone marrow stromal cells, the combination of defibrotide with dexamethasone (Fig. 3A and Supplementary Fig. S4A) or melphalan (Fig. 3B and Supplementary Fig. S4B) significantly increased in a large proportion of cases the percentage cell killing of multiple myeloma cells compared with treatment with dexamethasone or melphalan alone.

Fig. 3.

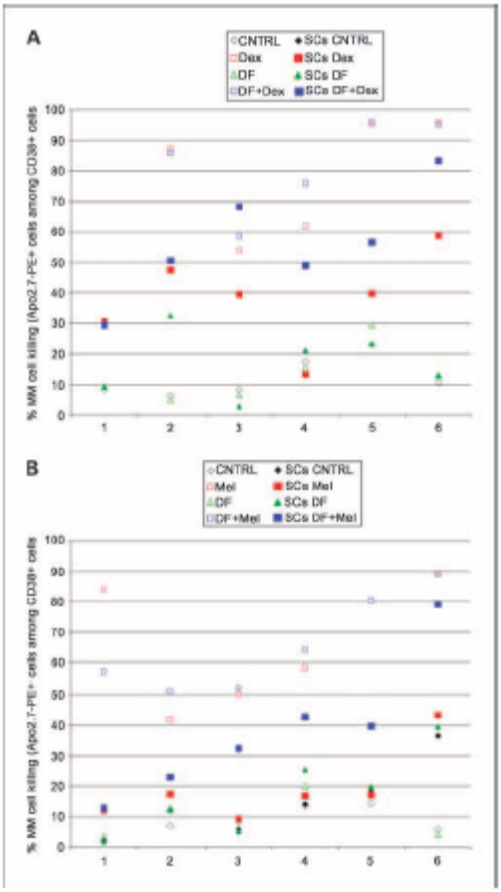

Fig. 3. Defibrotide (*DF*) treatment enhances the anti – multiple myeloma activity of conventional therapeutics in the context of multiple myeloma – bone marrow stromal cell interactions. Illustrative examples of results from treatment of primary multiple myeloma cells with dexamethasone (*Dex, A*) or melphalan (*Mel, B*), in the presence or absence of bone marrow stromal cells (*SCs*), with or without treatment with defibrotide. Among multiple myeloma samples that exhibit constitutive responsiveness to these agents in the absence of stroma, but significantly less pronounced response in multiple myeloma – bone marrow stromal cell cocultures, defibrotide is able to lead to variable degrees of sensitization to these agents in the presence of stromal cells. In each panel, primary samples 3 to 6 are examples of how defibrotide treatment can increase the presence of stromal cells higher response to treatment with dexamethasone or melphalan, respectively.

Defibrotide (*DF*) treatment enhances the anti–multiple myeloma activity of conventional therapeutics in the context of multiple myeloma–bone marrow stromal cell interactions. Illustrative examples of results from treatment of primary multiple myeloma cells with dexamethasone (*Dex, A*) or melphalan (*Mel, B*), in the presence or absence of bone marrow stromal cells (*SCs*), with or without treatment with defibrotide. Among multiple myeloma samples that exhibit constitutive responsiveness to these agents in the absence of stroma, but significantly less pronounced response in multiple myeloma–bone marrow stromal cell cocultures, defibrotide is able to lead to variable degrees of sensitization to these agents in the presence of stromal cells. In each panel, primary samples 3 to 6 are examples of how defibrotide treatment can increase the presence of stromal cells higher response to treatment with dexamethasone or melphalan, respectively.

Cellular and molecular sequelae of defibrotide treatment in the multiple myeloma-bone marrow stromal cell coculture. To evaluate the mechanisms whereby defibrotide can attenuate protective effects conferred with multiple myeloma cells by bone marrow stromal cells, we did *in vitro* cell adhesion assays and observed that defibrotide significantly decreased the adhesion of multiple myeloma cells to bone marrow

stromal cells (Fig. 4A). The multiple myeloma cell adhesion to bone marrow stromal cells has been previously associated with increased transcriptional activity of NF-κB in both cellular compartments (34, 35), leading to increased secretion of antiapoptotic cytokines (such as IL-6) and proangiogenic growth factors (such as VEGF; ref. 27). We therefore hypothesized that the decrease in multiple myeloma–bone marrow stromal cell adhesion could have an impact on these molecular sequelae. Indeed, separate assays in the multiple myeloma cell compartment versus bone marrow stromal cells of the *in vitro* coculture model showed that defibrotide treatment decreased the NF-κB transcriptional activity in both multiple myeloma cells (Supplementary Fig. S5A) and bone marrow stromal cells (Supplementary Fig. S5B). This finding was also associated with suppression in secretion of IL-6 (Fig. 4B) and VEGF (Fig. 4C), consistent with the antiadhesive properties of defibrotide and with the impact of multiple myeloma–bone marrow stromal cell adhesion on secretion of these cytokines.

Fig. 4.

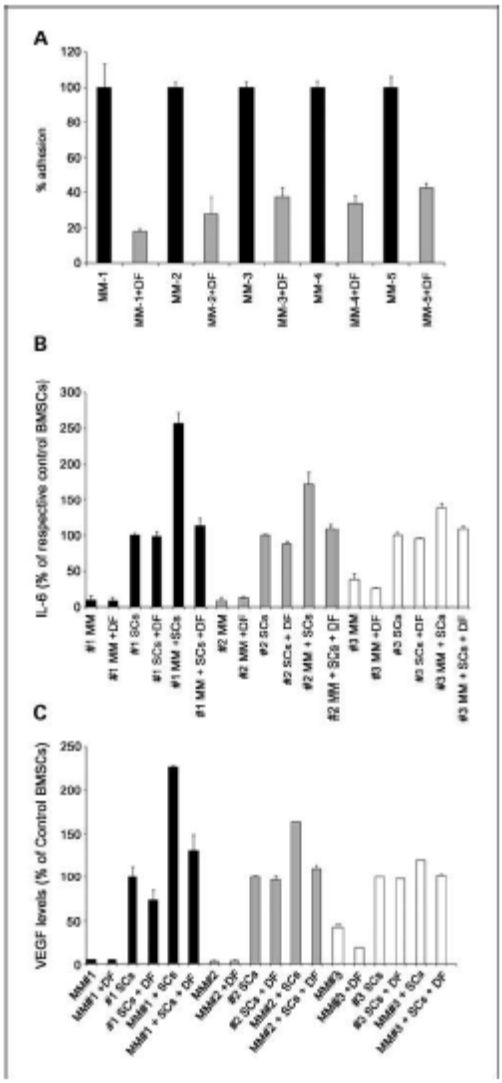

Fig. 4. Defibrotide (*DF*) modulates multiple myeloma (*MM*) cell adhesion to bone marrow stromal cells (*BMSC* or *SCs*) and its sequelae. *In vitro* adhesion assays (with CFSE labeling of multiple myeloma cells and quantification of adhesion with fluorescence plate reader) show that defibrotide treatment decreases the adhesion of primary multiple myeloma tumor cells to bone marrow stromal cells (*A*). Furthermore, defibrotide treatment suppresses the increase, triggered by multiple myeloma – bone marrow stromal cell interaction, in secretion of IL-6 (*B*) or VEGF (*C*). Cytokine levels in *B* and *C* are expressed, in each experiment, as percentage of levels in cultures of stromal cells alone.

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Defibrotide modulates the expression and function of heparanase. Heparanase is an enzyme that cleaves heparan sulfate chains of proteoglycans, and its expression has been associated with increased growth, metastasis, and angiogenesis of several tumor types, including multiple myeloma (36–40). Because of the increasing interest in the potential role of heparanase as a mediator of tumor-microenvironment interactions in multiple myeloma, we evaluated whether defibrotide modulates the biological activity of

heparanase. We first confirmed that defibrotide treatment suppresses the expression of heparanase transcripts (assessed by real-time reverse transcription-PCR) in RPMI-8226 and U266 multiple myeloma cells (Fig. 5A and Supplementary Fig. S6A); and decreases the intracellular protein expression of heparanase (as evaluated by intracellular flow cytometry; Fig. 5B). These effects correlated with defibrotide-induced decreases in heparanase activity in cultures of RPMI-8226 cells (Fig. 5C) and U266 cells (Supplementary Fig. S6B). To further probe the functional significance of these observations, we evaluated the impact of defibrotide on an *in vitro* tumor invasion assay in dual-chamber culture system, in which multiple myeloma cells labeled with calcein were incubated in the upper chamber of the system. Exogeneous addition of recombinant human heparanase in the culture (Fig. 6A) or transfection of RPMI-8226 cells with heparanase construct (Fig. 6B) increased the invasion potential of the multiple myeloma cells, but in both cases defibrotide treatment abrogated that effect (Fig. 6A and B).

Fig. 5.

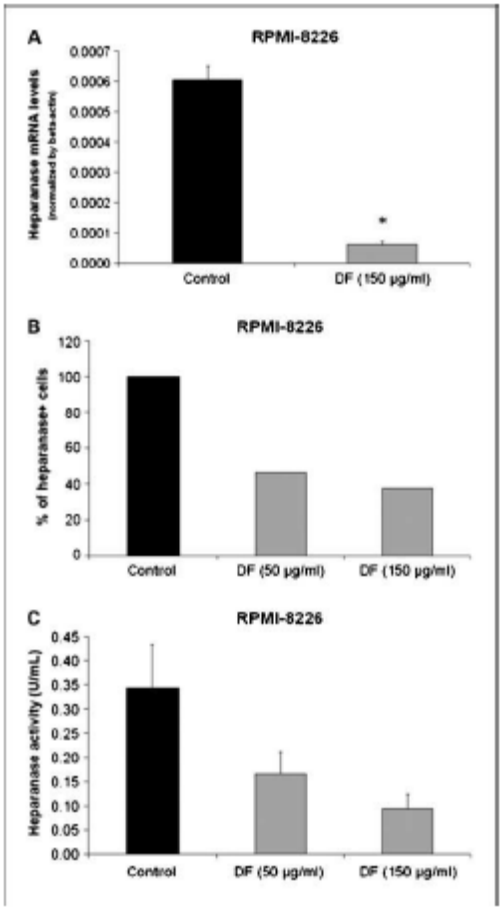

Fig. 5. Defibrotide (*DF*) treatment suppresses the expression and function of heparanase. Reverse transcription-PCR was done as described in Material and Methods. Results are expressed as mean mRNA heparanase level normalized by β-actin housekeeping gene. Defibrotide treatment suppresses the levels of heparanase transcript in RPMI-8226 (*A*) multiple myeloma cells. Furthermore, defibrotide suppresses the intracellular protein expression of heparanase, measured by flow cytometric analysis (*B*), as well as the heparanase activity in cellular extracts of RPMI-8226 (*C*) multiple myeloma cells. Student's test: $^{*}P < 0.05$ and $^{**}P < 0.01$.

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Fig. 6.

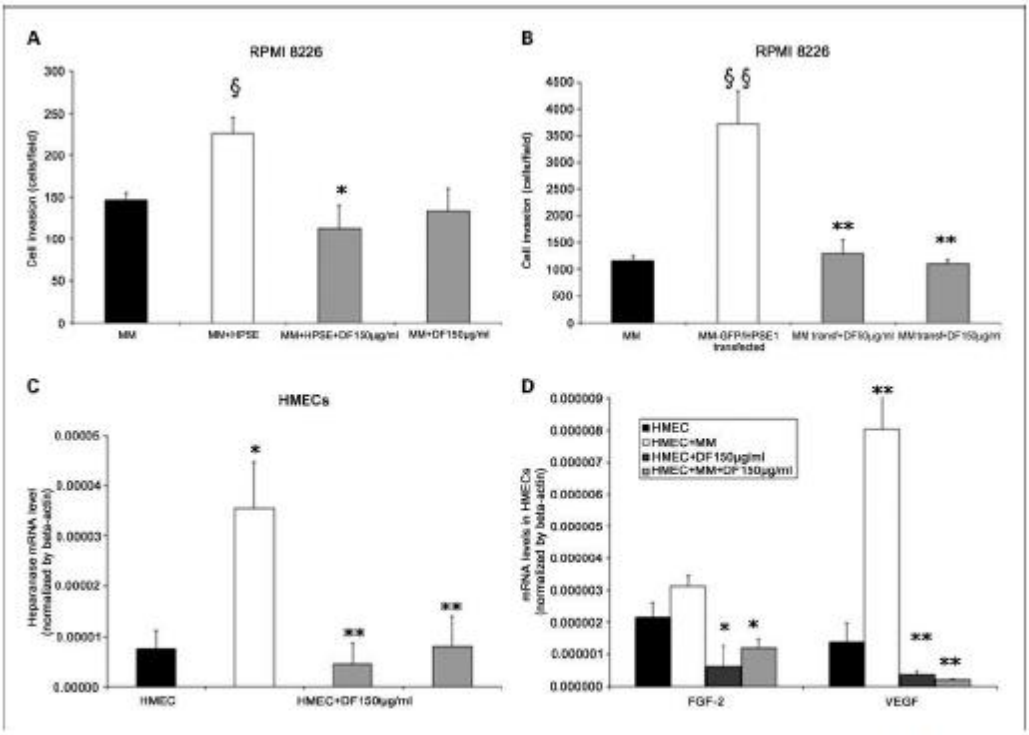

Fig. 6. Defibrotide (*DF*) modulates the invasive potential of multiple myeloma cells and the molecular sequelae of their interactions with endothelial cells. The invasive potential of multiple myeloma cells was assessed *in vitro* on the basis of their ability to invade from the top to the bottom chamber of a dual-chamber *in vitro* culture system when these two chambers are separated by matrigel and a polyethylene terephthalate membrane. The invasive potential of the multiple myeloma cells was enhanced by either exogenous addition of heparanase (*HSPE*) in the culture (*A*) or multiple myeloma cell transfection with heparanase construct (*B*), but was suppressed, in both cases, by defibrotide treatment. The interaction with multiple myeloma cells triggers in human microvascular endothelial cells (*HMECs*) the up-regulation of transcripts for heparanase (*C*); VEGF and FGF-2 (*D*), but these events are suppressed by defibrotide treatment. Student's test: $^{\S,*}P < 0.05$ and $^{\S\S,**}P < 0.01$.

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Defibrotide modulates the molecular events triggered by interaction of endothelial cells with multiple myeloma cells. We established an *in vitro* system where multiple myeloma cells and human dermal microvascular endothelial cells were cultured in close proximity to each other, with the human dermal microvascular endothelial cells cultured in the lower chamber of a dual-chamber *in vitro* system, while the upper chamber contained either medium only (control) or medium with multiple myeloma cells. In that system, the human dermal microvascular endothelial cells were cultured (for 48 hours) and after removal

of the upper chamber inset, the human dermal microvascular endothelial cells and the multiple myeloma cells were separately harvested and processed for mRNA extraction and Real-Time PCR analysis for heparanase transcripts. In this experiment, interaction with multiple myeloma cells triggered in human dermal microvascular endothelial cells a significant increase in heparanase expression (Fig. 6C), which was abrogated by defibrotide. In this same system, the interaction of human dermal microvascular endothelial cells with multiple myeloma cells triggered increased gene expression of VEGF, FGF-2 (Fig. 6D), ICAM-1 (Supplementary Fig. S7A), and E-selectin (Supplementary Fig. S7B) in human dermal microvascular endothelial cells; as well as increased expression of heparanase, VEGF, and FGF-2 in multiple myeloma cells (Supplementary Fig. S7C). Importantly, treatment with defibrotide was able to suppress these events triggered by human dermal microvascular endothelial cell–multiple myeloma interaction (Fig. 6D and Supplementary Fig. S7).

Discussion

The key focus of this study was to evaluate whether defibrotide can be administered concomitantly with various antineoplastic agents currently used for the management of multiple myeloma and other neoplasias. Defibrotide has clinical activity for the treatment of severe veno-occlusive disease (6, 7, 10, 41), a clinical setting which historically has >90% mortality rate (42). Among the diverse properties of defibrotide, its antithrombotic effects, and relative lack of severe hemorrhage or other serious treatment-related toxicity, led us to hypothesize that defibrotide may be an attractive agent for prevention and/or treatment of thromboembolic complications associated with cancer treatment (2). Specifically, we hypothesized that the pleiotropic features of defibrotide that confer protection of endothelial cells from diverse forms of injury could be useful in treating vascular damage associated with neoplasia-related prothrombotic states. For extensive clinical evaluation of defibrotide in cancer-related clinical settings to occur, it was essential to address whether its administration is compatible with anticancer treatments. In particular, given the known protective effect of defibrotide on the endothelium, it would be potentially detrimental if defibrotide were to extend similar protective effects to neoplastic tissues. Specifically, if neoplastic cells were among those tissues protected by defibrotide, its administration (e.g. as prophylaxis against development of veno-occlusive disease following high-dose chemotherapy and stem cell transplantation), might theoretically counteract the antitumor effects of the cytotoxic conditioning regimen, thus defeating the purpose of the stem cell transplantation approach.

Our present study indicates that defibrotide does not counteract the antitumor activity of several cytotoxic chemotherapy agents commonly used in the management of hematologic malignancies (including multiple myeloma) and/or solid tumors. This observation was made in *in vitro* experiments with cell lines from multiple myeloma, and colon and breast carcinoma. We also confirmed that defibrotide does not blunt the anti–multiple myeloma effect of dexamethasone or the proteasome inhibitor borterzomib (PS-341). Notably, among the diverse classes of anticancer drugs that we tested, defibrotide attenuated the antitumor effect (in multiple myeloma or other tumor models) of doxorubicin. Further studies in our group are addressing whether this effect represents a specific feature of doxorubicin or a phenomenon pertinent to the entire class of anthracyclines. It is possible that the three-dimensional structure of defibrotide retains sufficient similarity to the “stacked ring” structure of its precursor DNA to allow for anthracyclines to intercalate with defibrotide, similar to anthracycline intercalation with DNA strands in target cells. For the moment, based on these results, we would suggest caution in terms of concurrent use of defibrotide with doxorubicin or other anthracyclines.

While evaluating the possible impact defibrotide on antitumor activities of various classes of agents, we observed no inhibition of their antineoplastic activity. However we also did not observe a pronounced sensitization of tumor cells to these drug classes (an exception included a modest sensitization of some, but not all, solid tumor cell lines to certain platinum agents). Furthermore, we observed that defibrotide had no significant effect on tumor cell survival as a single agent. These results taken together suggest that, in respect to the tumor models and majority of cancer drug classes that we tested, defibrotide does not exhibit direct antitumor effects but also does not function to decrease drug responsiveness in these neoplastic cells.

However, conventional assays for *in vitro* drug sensitivity of tumor cells cultured in isolation have the key limitation that they do not take into account the interactions of tumor cells with their local microenvironment. It is known that responses of multiple myeloma and other tumor types to various anticancer therapies can be attenuated by cell adhesion–mediated and cytokine/growth factor–driven interactions of tumor cells with their local microenvironment (reviewed in ref. 21). For instance, multiple myeloma cell adhesion to bone marrow stromal cells stimulates paracrine (bone marrow stromal cell–derived) and/or autocrine (multiple myeloma cell–derived) production of anti-apoptotic cytokines, such as IL-6; as well as triggers direct cell-to-cell contact–mediated antiapoptotic signaling cascades in multiple myeloma cells (21). The composite effect of these interactions is to attenuate the response of multiple myeloma cells to various conventional treatments, such as dexamethasone or cytotoxic chemotherapeutics (21). Because this effect is mediated at least in part by adhesion of multiple myeloma cells on bone marrow stromal cells, we hypothesized that the antiadhesive properties of defibrotide (22) could potentially serve to modulate multiple myeloma–bone marrow stromal cell interactions and so sensitize multiple myeloma cells to some of these therapeutics against which the bone marrow milieu and its stromal cells confer protection.

Our *in vitro* and *in vivo* studies support this hypothesis. In *ex vivo* cocultures of multiple myeloma cells with bone marrow stromal cells, defibrotide had no significant single-agent effect on multiple myeloma cell viability, either in the presence or absence of bone marrow stromal cells, and had no sensitizing effect on multiple myeloma cells exposed to melphalan in the absence of bone marrow stromal cells. However, the protection conferred by bone marrow stromal cells to multiple myeloma cells against dexamethasone or melphalan was significantly suppressed by defibrotide. Further studies at a mechanistic level showed that defibrotide treatment of multiple myeloma–bone marrow stromal cell cocultures is associated with decreased adhesion of multiple myeloma cells to bone marrow stromal cells and suppression of several key molecular events triggered by multiple myeloma–bone marrow stromal cell adhesion, including IL-6 and VEGF secretion, and NF-κB activation in both the multiple myeloma and bone marrow stromal cell compartments. IL-6 secretion and NF-κB activation play well-characterized roles in conferring decreased sensitivity of multiple myeloma cells (or other tumor models) to proapoptotic agents, including dexamethasone and/or cytotoxic chemotherapy.

Our *in vitro* studies support the notion that the effects of defibrotide extend to other aspects of tumor-microenvironment functional interface, such as the interaction of multiple myeloma cells with human dermal microvascular endothelial cells and the invasiveness of multiple myeloma cells. Although interaction of multiple myeloma cells with human microvascular endothelial cells up-regulates the expression of heparanase, VEGF, FGF-2, ICAM-1, and E-selectin in human dermal microvascular endothelial cells, and of VEGF and FGF-2 in multiple myeloma cells, these events are suppressed by defibrotide treatment. In addition, defibrotide suppresses in multiple myeloma cell cultures the transcript and protein expression of heparanase, as well as its enzymatic activity. Recent studies in multiple myeloma and solid tumors have

identified heparanase as an important regulator of tumor cell proliferation, invasiveness, and metastatic potential (36, 38, 40). To further evaluate the functional significance of the effects of defibrotide on heparanase, we did *ex vivo* tumor cell invasion assays, which showed that defibrotide treatment suppressed the increase in multiple myeloma cell invasive potential triggered by either exogenous addition of heparanase in the culture or multiple myeloma cell transfection with heparanase construct.

Importantly, defibrotide exhibits antitumor activity *in vivo*. We observed in three different animal models that defibrotide can sensitize multiple myeloma or solid tumor cells to various cytotoxic agents, without significant side effects. In addition, we did *in vivo* pharmacokinetic studies of defibrotide (both in oral and i.v. administration) that suggest that the defibrotide levels required for *in vitro* tumor sensitization to dexamethasone and cytotoxic chemotherapy are achievable *in vivo*.

Our *in vitro* results indicate that the ability of defibrotide to enhance the antitumor activity of dexamethasone or chemotherapeutics in the context of multiple myeloma cell interactions with their milieu is multifactorial. Specifically, defibrotide modulates tumor cell interactions with different nonmalignant accessory cell compartments of the local milieu (e.g. bone marrow stromal cells and endothelial cells), influences the protection that these cells can confer to multiple myeloma cells against conventional antitumor agents, interferes with the ability of tumor cells and their microenvironment to trigger cytokine production necessary for recruitment of new blood vessels without significant direct cytotoxic effect on endothelial cells, and targets heparanase and its role in facilitating the invasiveness potential of tumor cells. These tumor-microenvironment interactions are not exclusive to multiple myeloma, but are features of a broad spectrum of hematologic malignancies and solid tumors (43–45). Therefore, the activity of defibrotide in both multiple myeloma and solid tumor models suggests that its antitumor properties may have applications in a broad spectrum of neoplasias. With its proven chemoprotective activity towards normal tissues, clinically leveraged to treat veno-occlusive disease (6–9, 11) and its activity in sensitizing tumor cells to anticancer therapeutics, defibrotide presents an encouraging profile of biological effects that warrant further evaluation in clinical trials.

Our results do not preclude additional potential mechanism(s) of antitumor effects of defibrotide. For instance, the effects of defibrote on veno-occlusive disease seem to occur via cytoprotective effects on liver sinusoidal endothelium and are related to the antithrombotic and profibrinolytic properties of defibrotide (as reviewed in ref. 3). Because there is evidence that procoagulation mechanisms, and in particular thrombin, may promote tumor progression and neoangiogenesis (46, 47), the demonstration that defibrotide antagonizes thrombin (48) suggests that thrombin inhibition may also contribute to the anticancer effect of defibrotide.

The present study aimed at determining whether defibrotide would interfere with the anticancer effect of chemotherapy if administered concurrently with it. In view of the emerging role of defibrotide for the management of veno-occlusive disease and other vascular complications of cancer treatment, this question has direct implication to the future use of this agent in the context of patients with neoplasias. The results of this study show that defibrotide does not adversely interfere with the antitumor activity of many important classes of antineoplastic agents, with the exception of protection of tumor cells against doxorubicin. Interestingly, our results indicate that defibrotide has intriguing anticancer properties in its own right: in multiple *in vivo* tumor models that were tested, defibrotide confers a modest tumor growth delay as a single agent but also enhances the antitumor activity of several chemotherapeutic agents, including melphalan and cyclophosphamide. These results provide a rationale for clinical trials of defibrotide in the treatment of multiple myeloma and other neoplasias, as well as to a multicenter phase

I/II study of melphalan, prednisone, thalidomide, and defibrotide in advanced multiple myeloma patients (49, 50).

References

1. *Lee AY. Management of thrombosis in cancer: primary prevention and secondary prophylaxis. Br J Haematol 2005;128:291–302.*
2. *Knight R, DeLap RJ, Zeldis JB. Lenalidomide and venous thrombosis in multiple myeloma. N Engl J Med 2006;354:2079–80.*
3. *Pescador R, Porta R, Ferro L. An integrated view of the activities of defibrotide. Semin Thromb Hemost 1996;22 Suppl 1:71–5.*
4. *Eissner G, Multhoff G, Gerbitz A, et al. Fludarabine induces apoptosis, activation, and allogenicity in human endothelial and epithelial cells: protective effect of defibrotide. Blood 2002;100:334–40.*
5. *Palmer KJ, Goa KL. Defibrotide. A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic use in vascular disorders. Drugs 1993;45:259–94.*
6. *Richardson PG, Elias AD, Krishnan A, et al. Treatment of severe veno-occlusive disease with defibrotide: compassionate use results in response without significant toxicity in a high-risk population. Blood 1998;92:737–44.*
7. *Richardson PG, Murakami C, Jin Z, et al. Multi-institutional use of defibrotide in 88 patients after stem cell transplantation with severe veno-occlusive disease and multisystem organ failure: response without significant toxicity in a high-risk population and factors predictive of outcome. Blood 2002;100:4337–43.*
8. *Abecasis MM, Conceicao Silva JP, Ferreira I, Guimaraes A, Machado A. Defibrotide as salvage therapy for refractory veno-occlusive disease of the liver complicating allogeneic bone marrow transplantation. Bone Marrow Transplant 1999;23:843–6.*
9. *Chopra R, Eaton JD, Grassi A, et al. Defibrotide for the treatment of hepatic veno-occlusive disease: results of the European compassionate-use study. Br J Haematol 2000;111:1122–9.*
10. *Richardson P, Guinan E. Hepatic veno-occlusive disease following hematopoietic stem cell transplantation. Acta Haematol 2001;106:57–68.*
11. *Corbacioglu S, Greil J, Peters C, et al. Defibrotide in the treatment of children with veno-occlusive disease (VOD): a retrospective multicentre study demonstrates therapeutic efficacy upon early intervention. Bone Marrow Transplant 2004;33:189–95.*
12. *Chalandon Y, Roosnek E, Mermillod B, et al. Prevention of veno-occlusive disease with defibrotide after allogeneic stem cell transplantation. Biol Blood Marrow Transplant 2004;10:347–54.*
13. *McDonald GB, Sharma P, Matthews DE, Shulman HM, Thomas ED. Venocclusive disease of the liver after bone marrow transplantation: diagnosis, incidence, and predisposing factors. Hepatology 1984;4:116–22.*
14. *McDonald GB, Hinds MS, Fisher LD, et al. Veno-occlusive disease of the liver and multiorgan failure after bone marrow transplantation: a cohort study of 355 patients. Ann Intern Med 1993;118:255–67.*
15. *Bearman SI. The syndrome of hepatic veno-occlusive disease after marrow transplantation. Blood 1995;85:3005–20.*
16. *Bearman SI, Anderson GL, Mori M, Hinds MS, Shulman HM, McDonald GB. Venoocclusive disease of the liver: development of a model for predicting fatal outcome after marrow transplantation. J Clin Oncol 1993;11:1729–36.*

17. *Jones RJ, Lee KS, Beschorner WE, et al. Venoocclusive disease of the liver following bone marrow transplantation. Transplantation 1987;44:778–83.*
18. *King PD, Perry MC. Hepatotoxicity of chemotherapeutic and oncologic agents. Gastroenterol Clin North Am 1995;24:969–90.*
19. *Richardson P. Hemostatic complications of hematopoietic stem cell transplantation: from hemorrhage to microangiopathies and VOD. Pathophysiol Haemost Thromb 2003;33 Suppl 1:50–3.*
20. *Bogdanos J, Karamanolakis D, Tenta R, et al. Endocrine/paracrine/autocrine survival factor activity of bone microenvironment participates in the development of androgen ablation and chemotherapy refractoriness of prostate cancer metastasis in skeleton. Endocr Relat Cancer 2003;10:279–89.*
21. *Mitsiades CS, Mitsiades NS, Munshi NC, Richardson PG, Anderson KC. The role of the bone microenvironment in the pathophysiology and therapeutic management of multiple myeloma: Interplay of growth factors, their receptors and stromal interactions. Eur J Cancer 2006;42:1564–73. Epub 2006 Jun 9.*
22. *Carlo-Stella C, Di Nicola M, Magni M, et al. Defibrotide in combination with granulocyte colony-stimulating factor significantly enhances the mobilization of primitive and committed peripheral blood progenitor cells in mice. Cancer Res 2002;62:6152–7.*
23. *Scalia R, Kochilas L, Campbell B, Lefer AM. Effects of defibrotide on leukocyte-endothelial cell interaction in the rat mesenteric vascular bed: role of P-selectin. Methods Find Exp Clin Pharmacol 1996;18:669–76.*
24. *Pellegatta F, Lu Y, Radaelli A, et al. Drug-induced in vitro inhibition of neutrophil-endothelial cell adhesion. Br J Pharmacol 1996;118:471–6.*
25. *Pellegatta F, Ferrero E, Marni A, Chierchia S, Forti D, Ferrero ME. The anti-ischemic drugs defibrotide and oligotide analogously inhibit leukocyte-endothelial cell adhesion in vitro. Transpl Int 1996;9 Suppl 1:S420–4.*
26. *Alberico P, Porta R, Pescador R, Ferro L. Is defibrotide's activity on leukocytes adenosine-receptor mediated? An "in vitro"-"ex vivo" appraisal. Thromb Res 1995;80:281–9.*
27. *Gupta D, Treon SP, Shima Y, et al. Adherence of multiple myeloma cells to bone marrow stromal cells upregulates vascular endothelial growth factor secretion: therapeutic applications. Leukemia 2001;15:1950–61.*
28. *Teicher BA, Crawford JM, Holden SA, et al. Glutathione monoethyl ester can selectively protect liver from high dose BCNU or cyclophosphamide. Cancer 1988;62:1275–81.*
29. *Ishiyama M, Tominaga H, Shiga M, Sasamoto K, Ohkura Y, Ueno K. A combined assay of cell viability and in vitro cytotoxicity with a highly water-soluble tetrazolium salt, neutral red and crystal violet. Biol Pharm Bull 1996;19:1518–20.*
30. *Koester SK, Roth P, Mikulka WR, Schlossman SF, Zhang C, Bolton WE. Monitoring early cellular responses in apoptosis is aided by the mitochondrial membrane protein-specific monoclonal antibody APO2.7. Cytometry 1997;29:306–12.*
31. *Mitsiades CS, Mitsiades NS, Bronson RT, et al. Fluorescence imaging of multiple myeloma cells in a clinically relevant SCID/NOD in vivo model: biologic and clinical implications. Cancer Res 2003;63:6689–96.*
32. *Kakeji Y, Maehara Y, Ikebe M, Teicher BA. Dynamics of tumor oxygenation, CD31 staining and transforming growth factor-β levels after treatment with radiation or cyclophosphamide in the rat 13762 mammary carcinoma. Int J Radiat Oncol Biol Phys 1997;37:1115–23.*
33. *Alvarez E, Westmore M, Galvin RJ, et al. Properties of bisphosphonates in the 13762 rat mammary carcinoma model of tumor-induced bone resorption. Clin Cancer Res 2003;9:5705–13.*

34. *Chauhan D, Uchiyama H, Akbarali Y, et al. Multiple myeloma cell adhesion-induced interleukin-6 expression in bone marrow stromal cells involves activation of NF-êB. Blood 1996;87:1104–12.*
35. *Chauhan D, Uchiyama H, Urashima M, Yamamoto K, Anderson KC. Regulation of interleukin 6 in multiple myeloma and bone marrow stromal cells. Stem Cells 1995;13 Suppl 2:35–9.*
36. *Fontelonga A, Kelly AJ, MacKintosh FR, et al. A novel high-dose chemotherapy protocol with autologous hematopoietic rescue in patients with metastatic breast cancer or recurrent non-Hodgkin's lymphoma. Bone Marrow Transplant 1997;19:983–8.*
37. *Mahtouk K, Hose D, Raynaud P, et al. Heparanase influences expression and shedding of syndecan-1, and its expression by the bone marrow environment is a bad prognostic factor in multiple myeloma. Blood 2007;109:4914–23.*
38. *Goldshmidt O, Zcharia E, Abramovitch R, et al. Cell surface expression and secretion of heparanase markedly promote tumor angiogenesis and metastasis. Proc Natl Acad Sci U S A 2002;99:10031–6.*
39. *Shafat I, Vlodavsky I, Ilan N. Characterization of mechanisms involved in secretion of active heparanase. J Biol Chem 2006;281:23804–11.*
40. *Yang YL, Lu MY, Jou ST, Lin KH, Lin DT. Hematopoietic stem cell transplantation in Taiwanese children with primary immunodeficiency. J Formos Med Assoc 2005;104:101–6.*
41. *Richardson P, Bearman SI. Prevention and treatment of hepatic venocclusive disease after high-dose cytoreductive therapy. Leuk Lymphoma 1998;31:267–77.*
42. *Wadleigh M, Ho V, Momtaz P, Richardson P. Hepatic veno-occlusive disease: pathogenesis, diagnosis and treatment. Curr Opin Hematol 2003;10:451–62.*
43. *Murry BP, Greiter-Wilke A, Paulsen DP, Hiatt KM, Beltrami CA, Marchetti D. Selective heparanase localization in malignant melanoma. Int J Oncol 2005;26:345–52.*
44. *Chen JQ, Zhan WH, He YL, et al. Expression of heparanase gene, CD44v6, MMP-7 and nm23 protein and their relationship with the invasion and metastasis of gastric carcinomas. World J Gastroenterol 2004;10:776–82.*
45. *El-Assal ON, Yamanoi A, Ono T, Kohno H, Nagasue N. The clinicopathological significance of heparanase and basic fibroblast growth factor expressions in hepatocellular carcinoma. Clin Cancer Res 2001;7:1299–305.*
46. *Rickles FR, Patierno S, Fernandez PM. Tissue factor, thrombin, and cancer. Chest 2003;124:58–68S.*
47. *Falanga A. Biological and clinical aspects of anticancer effects of antithrombotics. Pathophysiol Haemost Thromb 2003;33:389–92.*
48. *Bracht F, Schror K. Isolation and identification of aptamers from defibrotide that act as thrombin antagonists in vitro. Biochem Biophys Res Commun 1994;200:933–7.*
49. *Palumbo A, Rus C, Rossi D, et al. A multi-center phase I/II study of melphalan, prednisone, thalidomide and defibrotide in advanced multiple myeloma patients. Blood 2006;108:1016–7A.*
50. *Larocca A, Rossi D, Pregno P, et al. A multicenter phase I/II trial on combination of melphalan, prednisone, thalidomide and defibrotide in advanced stage multiple myeloma patients. Haematol Hematol J 2007;92:138–9.*