

Targeting Intestinal Permeability for Graft-versus-Host Disease Treatment: A Therapeutic Perspective with Defibrotide

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Purpose: Acute graft-versus-host disease (aGVHD) is a significant cause of death in recipients of allogeneic hematopoietic stem cell transplantation. In this type of graft, the intestine is particularly affected, with the loss of intestinal barrier integrity playing a key role in its onset. In this scenario, the aim of the present research was to evaluate defibrotide, a heparin-like compound, marked for severe veno-occlusive disease, as an innovative therapeutic approach for restoring intestinal barrier integrity using an in vitro model and analyzing aGVHD patients' sera and clinical data.

Patients and Methods: Using an in vitro model of colon epithelium, we evaluated the modulation of tight junction proteins after defibrotide treatment, in basal condition or in presence of an inflammatory stimulus, by immunocytochemical and Western blotting analysis. Moreover, the study involved two patients with grade IV acute multisystem GVHD with great gastrointestinal compromise. Patients' sera were collected during the acute phase and remission of intestinal aGVHD and employed for the evaluation of a panel of 27 inflammatory cytokines using a Multiplex approach.

Results: Defibrotide treatment significantly increased the protein expression of Zonulin-1 and Occludin (untreated vs treated with 200 µg/mL, $p < 0.01$). In culture conditions mimicking inflammation, defibrotide countered the reduction of Occludin and Claudin-3 while preserving Zonulin-1 levels. Serum cytokine analysis of two patients receiving defibrotide for aGVHD showed significantly higher cytokine levels (IL-7, MIP-1β, IP-10, G-CSF, Eotaxin, IL-6) during the acute phase compared to remission after defibrotide treatment.

Conclusion: These findings suggest a potential therapeutic role for defibrotide in managing intestinal aGVHD by improving epithelial barrier integrity and reducing inflammation-related damage.

Keywords: defibrotide, tight junctions, intestinal inflammation, cytokines

Introduction

Hematopoietic stem cell transplantation (HSCT) has been demonstrated to be an effective treatment for several hematological malignant and non-malignant diseases. Despite its proven efficacy and the use of immunosuppressive prophylaxis, it is associated with early and late complications, among which there is the graft-versus-host disease (GVHD; OMIM#614395; ORPHA:39812). Acute GVHD (aGVHD) is the second most common cause of death in allogeneic HSCT recipients after the primary disease recurrence.^{1,2} Understanding the mechanisms responsible for the initiation and progression of this complication is fundamental to developing effective prevention and treatment strategies.^{3,4}

The aGVHD involves a cascade of events, including early inflammation and tissue injury, dysregulated immunity, until aberrant tissue repair with fibrosis that leads to irreversible tissue damage.^{5,6} The intestine is one of the organs most affected by aGVHD.^{7,8} Epithelial barrier loss can occur due to direct epithelial cells damage or through more subtle

changes in paracellular tight junction permeability.⁹ When dysregulated, these forms of intestinal barrier loss are thought to contribute to the initiation and propagation of the inflammation and damage progression, and it is considered a driving mechanism in aGVHD.^{10,11}

Despite the remarkable progress achieved in developing new and effective therapeutic strategies for treating severe aGVHD, no unique and safer treatment options are available nowadays. Steroid therapy is the first-line therapy, as well as the only one universally recommended. About 35–50% of patients with aGVHD develop refractory to systemic steroid therapy, and only 1–2% of patients with grade IV aGVHD survive more than two years.¹²

Defibrotide is a mixture of phosphodiester oligonucleotides (90% single-stranded and 10% double-stranded), obtained from controlled depolymerization of porcine intestinal mucosal DNA,¹³ indicated for treating severe hepatic veno-occlusive disease/sinusoidal obstruction syndrome (VOD/SOS) following allogeneic-Hematopoietic cell transplantation (allo-HCT).^{14–16}

It has been demonstrated that defibrotide exerts a protective role on activated endothelial cells^{13,17} mainly decreasing leukocyte extravasation and downregulating the expression of endothelial surface proteins involved in leukocyte recruitment.¹⁸

Defibrotide also has anti-inflammatory, anti-thrombotic and pro-fibrinolytic activities.^{13,19–21}

Even though clinical and experimental studies demonstrate that defects in the intestinal tight junction barrier and increased permeability are observed in various intestinal acute and chronic diseases, and systemic disorders, currently, the best therapy for barrier loss should target the disease itself.²² In this context, early reports suggest that restoring tight junction barrier function may have therapeutic benefits.^{23,24} Therapies targeted to restore the barrier function precisely may provide a substitute or supplement to immunologic-based treatments. However, the mechanisms of tight junction regulation will have to be defined in greater detail to make them viable as pharmacological targets.²⁵

The primary objective of this study was to evaluate *in vitro* on cells of the intestinal mucosa the effect of defibrotide, that, in a recent study,²⁶ has shown the ability to reduce the cytokine levels in a murine model of GVHD. In this work we have demonstrated for the first time that defibrotide can act *in vitro* towards damaged intestinal tight junctions leading to their rapid restoration supporting the repositioning of this drug for complete remission in patients with aGVHD of grade IV after failure of advanced-line therapies, including total lymphoablation with antithymocyte globulins.

Materials and Methods

Drug and Chemicals

Defibrotide (Defitelio[®] 80 mg/mL, Gentium Srl, Villa Guardia, Italy) derives from a kind concession of soon-to-expire waste lots by the Jazz Pharmaceuticals, for preclinical research purposes only. It was stored at room temperature according to the manufacturer's instructions.

MDP (N-Acetylmuramyl-L-alanyl-D-isoglutamine hydrate, Sigma-Aldrich, Saint Louis, MO, USA) was dissolved in saline solution, according to manufacturer instructions. MDP (10 μ M) was added to cell culture to mimic the inflammatory condition.²⁷

Cell Culture

HCT116 cells (human colon carcinoma cell line) were obtained from ATCC (Manassas, VA, USA) and cultured in Dulbecco's modified eagle medium (DMEM; Corning, New York, NY, USA) supplemented with 10% fetal bovine serum and with 2 mM L-glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin (all from GIBCO, Grand Island, NY, USA). Cells were left untreated or treated with 100 or 200 μ g/mL defibrotide, alone or in combination with 10 μ M MDP, for 24 hours. Where specified, the defibrotide was added both at the beginning of the experiments and after 8 hours.

Immunocytochemical Analysis

For immunocytochemical analysis, HCT116 cells were grown on coverslips in complete medium, treated for 24 hours, as described above, then fixed with freshly prepared 4% paraformaldehyde (for 10 minutes at room temperature) and washed in PBS 1X.

The cells on coverslips were then incubated with a Net Gel solution (150 mM NaCl, 5 mM EDTA, 50 mM TRIS-HCl pH 7.4, 0.05% NP40, 0.25% Carrageenan Lambda gelatin, and 0.02% Na azide) for 1 hour at room temperature to block non-specific binding.²⁸ Then, the cells were incubated with anti-zonulin-1 (ZO-1) polyclonal antibody, anti-Occludin monoclonal antibody (OC-3F10), both from ThermoFisher Scientific (Waltham, MA, USA) for 3 hours in Net Gel at room temperature. Samples were subsequently incubated with the specific secondary FITC and TRITC-conjugated antibodies in Net Gel for 45 min at room temperature. After two washes with NET gel and PBS, nuclei were counter-stained with DAPI (0.5 µg/mL) and coverslips were dried with ethanol and mounted in glycerol containing 1,4-diazabicyclo [2.2.2] octane (DABCO). The slides were analyzed with a Nikon Eclipse TE2000-E microscope (Carl Zeiss, Oberkochen, Germany).

Western Blot Analysis

HCT116 cells, cultured and treated as reported above, were lysed in ice-cold RIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 0.25% sodium deoxycholate) supplemented with Pierce Protease and Phosphatase Inhibitor mini tablets (Thermo Scientific, Rockford, IL, USA) on ice for 45 minutes. Protein determination was performed by using the BCA Protein Assay (Thermo Scientific, Rockford, IL, USA), according to the manufacturer's instructions. Samples were supplemented with the loading buffer (250 mM Tris pH 6.8, 2% SDS, 40% Glycerin, 20% b-mercaptoethanol) and boiled for 2 minutes. Equal amounts of proteins (50 µg) for each sample were migrated in acrylamide gels and blotted onto nitrocellulose filters. Western blot analysis was performed according to standard procedures using the following primary antibodies: anti-ZO-1 polyclonal antibody, anti-Occludin monoclonal antibody (OC-3F10), anti-Claudin 3 polyclonal antibody, anti-Claudin 4 monoclonal antibody (3E2C1), all from ThermoFisher Scientific, and anti-Tubulin monoclonal antibody from Sigma-Aldrich (St. Louis, MO, USA). After incubation with secondary antibodies (anti-mouse or -rabbit IgG HRP-conjugated; Sigma-Aldrich), a specific band detection was performed with the WesternBright Quantum kit (Advansta, Menlo Park, CA, USA). Image acquisitions were performed using the ImageQuant™ LAS 4000 imager and TL software (GE Healthcare, Buckinghamshire, UK). Densitometry of the Western blotting bands were analyzed with the Image J software (NIH). Western blotting was repeated at least three times with similar results and bands of interest were quantified with ImageJ software (NHI, USA), after normalizing with tubulin.

In-vivo Defibrotide Treatment: Therapeutic Approach and Ethical Approval

Two patients who underwent allogeneic HSCT after standard myeloablative conditioning were included in the study. Both the patients selected for the study had grade IV multisystem aGVHD with predominant involvement of the gastrointestinal tract. Defibrotide (Defitelio® 80 mg/mL, Gentium Srl, Villa Guardia, Italy) was administered as two-hour intravenous infusions of 6.25 mg/kg (25 mg/kg/day) every six-hour. The protocol followed the guidelines approved for VOD treatment, in accordance with the recommendations of the Haemato-oncology subgroup of the British Committee for Standards in Hematology (BCSH) and the British Society for Blood and Marrow Transplantation (BSBMT).

The transplant procedures and defibrotide treatment were performed at the Pediatric Bone Marrow Transplant Center (IRCCS Burlo Garofolo in Trieste), while all experiments with cell cultures were conducted at University of Ferrara. The Ethical Committee of the Institute for Research in Maternal and Child Health Burlo Garofolo of Trieste approved the study (reference no. 1105/2015). All laboratory experiments were carried out of the clinical study DF VOD-2013-03-REG, which investigates the efficacy of defibrotide to prevent conditioning-related organ injury in the course of allogeneic myeloablative HSCT.

The patient's parents provided informed written consent for the off-label defibrotide use and for the anonymous publication of clinical data and images.

All procedures were performed in accordance with the requirements of the Declaration of Helsinki.

Clinical Recovery of GVHD Patients Refractory to Conventional Treatments

Both patients were prednisone-resistant and had failed to respond to numerous treatments including ruxolitinib, tacrolimus, mycophenolate mofetil, infliximab, as well as rescue treatment with fludarabine and rabbit anti-thymocyte globulin (Thymoglobulin). During the third-line treatment, the first patient developed diffuse intestinal pneumatosis

involving massively the ileum and entire large bowel, the sigma, and the rectum up to the rectal ampulla included, with the presence of free air under the diaphragm, in the retroperitoneum, and mesenteric fat, bringing her to discouraging clinical conditions. The second patient continued to deteriorate despite several lines of aggressive immunosuppressive treatment after ten days of continuous severe bleeding, which required exceptional transfusional support. Additionally, worsening of liver function occurred. In the absence of valid therapeutic alternatives, we decided to try off-label treatment with defibrotide to preserve liver function at least. Both patients made full gut and liver recoveries within two weeks of continuous defibrotide administration associated with morphine infusion only.

Collection of Serum Samples

Peripheral blood samples were collected during the acute phase and remission of intestinal aGVHD, as part of diagnostic procedures, and used for research purposes only when clinical procedures had been completed. Patients' samples (~3–5 mL) were collected in sterile, serum-separator tubes and allowed to clot at room temperature for 30 minutes. Following clotting, the samples were centrifuged at $1500 \times g$ for 10 minutes at room temperature, to separate the serum from the cellular components. The resulting serum was carefully aspirated and transferred into sterile cryovials. Each vial was labelled with the patient identification number and stored at -80°C for long-term preservation until further analysis.

Cytokine Profile Evaluation

Patients' sera were tested for the evaluation of the following cytokines/chemokines (expressed in pg/mL): Interleukin (IL)1 β , IL2, IL4, IL5, IL6, IL7, IL8, IL10, IL12 (p70), IL13, IL17, granulocyte-colony stimulating factor (G-CSF), granulocyte/macrophage-colony stimulating factor (GM-CSF), Interferon (IFN)- γ , Monocyte chemotactic and activating factor (MCP1; MCAF), Macrophage Inflammatory Protein (MIP)1 β and Tumor Necrosis Factor (TNF)- α , using the human cytokine BioPlex assay (BioRad Laboratories, Milan, Italy), a magnetic bead-based multiplex kit. Samples used for the immunoassay test were frozen and thawed only once. Cytokine evaluation was performed according to the manufacturer's instructions on a Bio-Plex 200 instrument equipped with the Bio-Plex Manager software, using a five-parameters not-linear regression formula to compute sample concentrations from the standard curves.

For the patients' sera analysis, the control donors, for ethical reasons, were limited to infants and young children who had to undergo a medically indicated peripheral venous blood sampling before elective surgical interventions or with the scope of diagnostic procedures. Moreover, we excluded subjects affected by an acute or chronic infectious disease.

Statistical Analysis

All results are expressed as the mean \pm standard deviation (SD). Statistical analysis of bands densitometry was carried out using one-way analysis of variance (ANOVA), followed by Bonferroni multiple comparison test. We used, also, *t*-test to compare the cytokine levels of the two independent groups (remission and control groups) to determine if there was a statistically significant difference between them. Statistical analyses were performed using GraphPad Prism (version 5.0; GraphPad Software Inc., La Jolla, CA, USA).

Results

Effect of Defibrotide on Tight Junction Proteins in HCT116 Cells

To evaluate the potential efficacy of defibrotide in modulating intestinal permeability, we conducted in vitro experiments on a colorectal carcinoma cell line (HCT116 cells) used as an epithelium model of the large intestine. In a first step of experiments, we analyzed the expression of tight junction proteins in untreated and defibrotide-treated cells.

As shown in [Figure 1](#), immunoblot results revealed that the protein expressions of ZO-1 and Occludin were increased in cells treated with defibrotide compared to untreated cells. ZO-1 levels were significantly increased with 100 $\mu\text{g}/\text{mL}$ of defibrotide treatment ($p < 0.05$, [Figure 1A](#)), while Occludin expression was significantly induced when defibrotide was added at a concentration of 200 $\mu\text{g}/\text{mL}$ ($p < 0.01$, [Figure 1B](#)). In line with these results, immunohistochemical analysis also showed an increase in the expression levels of ZO-1 and Occludin in HCT116 cells treated with defibrotide ([Figure 2](#)).

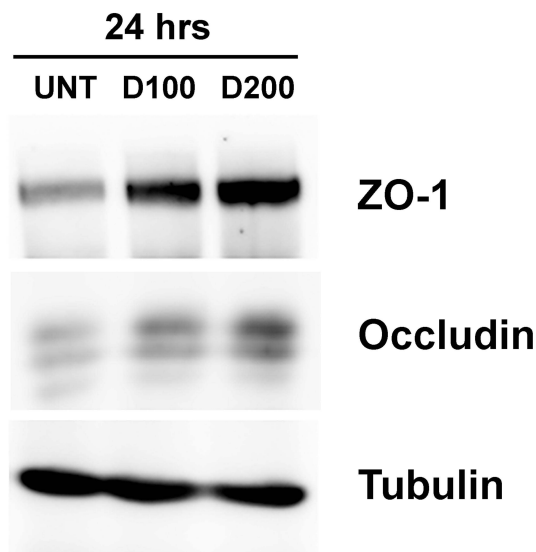
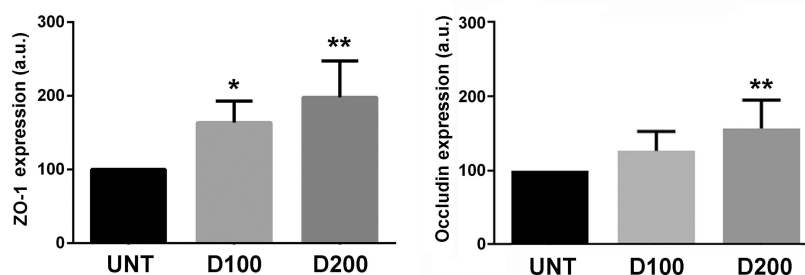
A**B**

Figure 1 Effects of defibrotide on the expression of tight junctions' proteins in HCT116 cells. **(A)** representative Western blotting images of ZO-1 and Occludin are shown. Immunoblotting was performed using 50 μ g of cell lysate. Tubulin staining is used as loading control. **(B)** The densitometric analyses of ZO-1 and Occludin are shown. Protein levels were normalized to tubulin and expressed as arbitrary units (a.u.). The experiments were performed at least in triplicate. UNT=untreated; D100=defibrotide 100 μ g/mL; D200=defibrotide 200 μ g/mL. Statistically significant p-values are shown (* p <0.05 vs UNT; ** p <0.01 vs UNT).

Effect of Defibrotide on Tight Junction Proteins Under Inflammatory Conditions

To assess the activity of defibrotide in counteracting the damage induced by inflammation, HCT116 cells were treated with 10 μ M MDP alone to mimic inflammation, or in combination with defibrotide (100–200 μ g/mL). After 24 hours of treatment, the cells were harvested and the protein levels of ZO-1, Occludin, Claudin 3 and Claudin 4 were analyzed by Western blot (Figure 3).

As previously described, defibrotide induced an increase in ZO-1 and Occludin levels, as well as Claudin 3 and Claudin 4, compared to untreated cells (Figure 3). Notably, except for ZO-1, the increase was more evident when defibrotide was added twice, at the beginning of the experiment and after 8 hours. Conversely, treatment with MDP induced a significant decrease in Occludin and Claudin 3 protein levels, while it seemed to minimally affect Claudin 4. As shown in Figure 3, the addition of defibrotide counteracted the effect of MDP, restoring the protein levels when added twice. Importantly, ZO-1 was not decreased by MDP treatment, and defibrotide was able to increase the levels of this protein at both concentrations, confirming the results previously described.

Clinical Efficacy of Defibrotide in GVHD Patients

Sera of two patients were collected during the acute phase and remission to analyse changes in cytokine and chemokine levels.

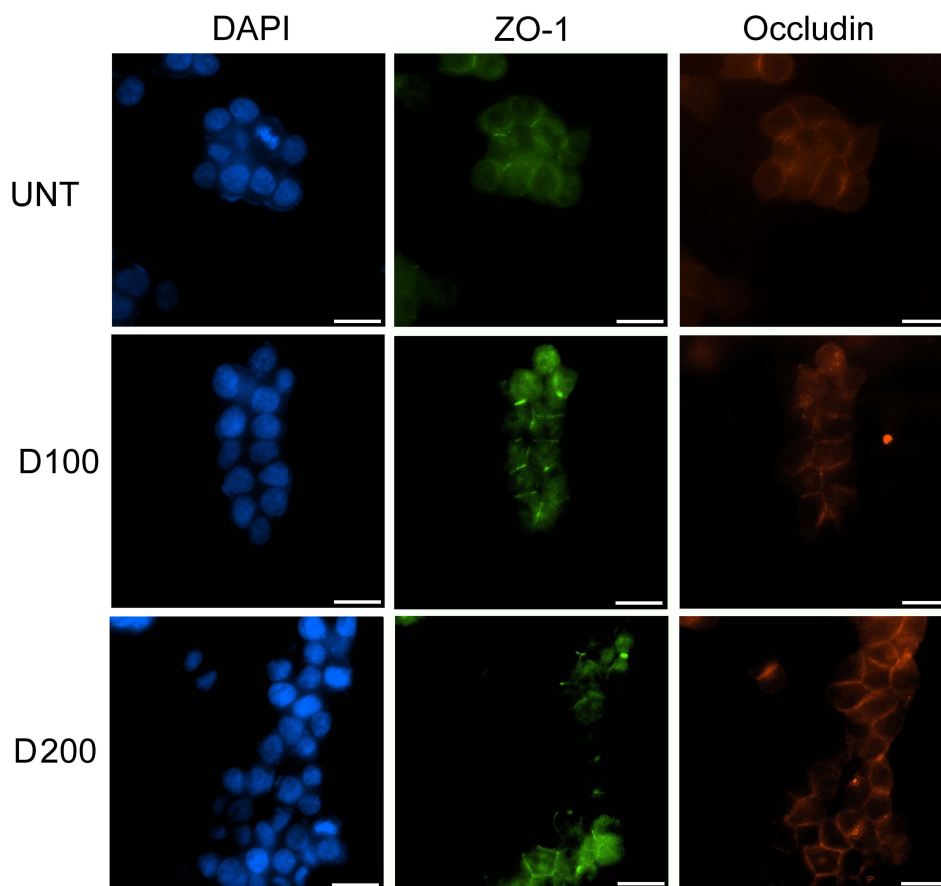


Figure 2 Immunofluorescence analysis of ZO-1 and Occludin in HCT116 cells. Representative images of HCT116 cells expressing ZO-1 (green) and Occludin (red). DAPI staining (blue) indicates nuclei. Magnification=40X/0.95. UNT=untreated; D100=defibrotide 100 $\mu\text{g}/\text{mL}$; D200=defibrotide 200 $\mu\text{g}/\text{mL}$, scale bar = 50 μm .

In **Figure 4**, cytokines and chemokines that present significantly different levels in the acute phase compared to remission are shown (the first patient is represented in red on the graphs, and the second patient in blue).

As represented in **Figure 4**, IL-7, MIP-1 β , IP-10, G-CSF, Eotaxin, and IL-6 had significantly different levels in the acute phase of the disease compared to the remission phase in at least one of the two patients. The results show that IL-7 levels were significantly higher in both patients than in the controls during the acute phase of the disease. Defibrotide treatment was successful, as cytokine levels significantly decreased in both patients, with a lower decrease in the first patient compared to the second, where cytokine levels had fallen below control (acute phase vs remission: Pt#1, $p < 0.05$; Pt#2, $p < 0.001$).

In our analyses, MIP-1 β levels were elevated in the acute phase of both patients and then significantly decreased after treatment with defibrotide. In the first patient, there was a more significant decrease of this chemokine, so that after therapy, MIP-1 β levels fell below the controls. The second patient, on the other hand, showed a less significant decrease in MIP-1 β , but still remained above the level of controls (acute phase vs remission: Pt#1, $p < 0.001$; Pt#2, $p < 0.05$).

A similar trend was observed for IP-10. Indeed, serum levels of this chemokine had increased in all patients during the acute phase and significantly decreased after resolution of the disease. Moreover, defibrotide treatment was able to decrease G-CSF levels, with significance in the first patient (acute phase vs remission: Pt#1, $p < 0.01$), where the glycoprotein levels during remission were like control.

Eotaxin and IL-6 were elevated in the serum of the first patient during the acute phase, but they were significantly reduced after defibrotide treatment (acute phase vs remission: Pt#1, $p < 0.001$). The second patient, on the other hand, showed a reverse trend, with IL-6 and eotaxin levels below the controls both during the acute and remission phases.

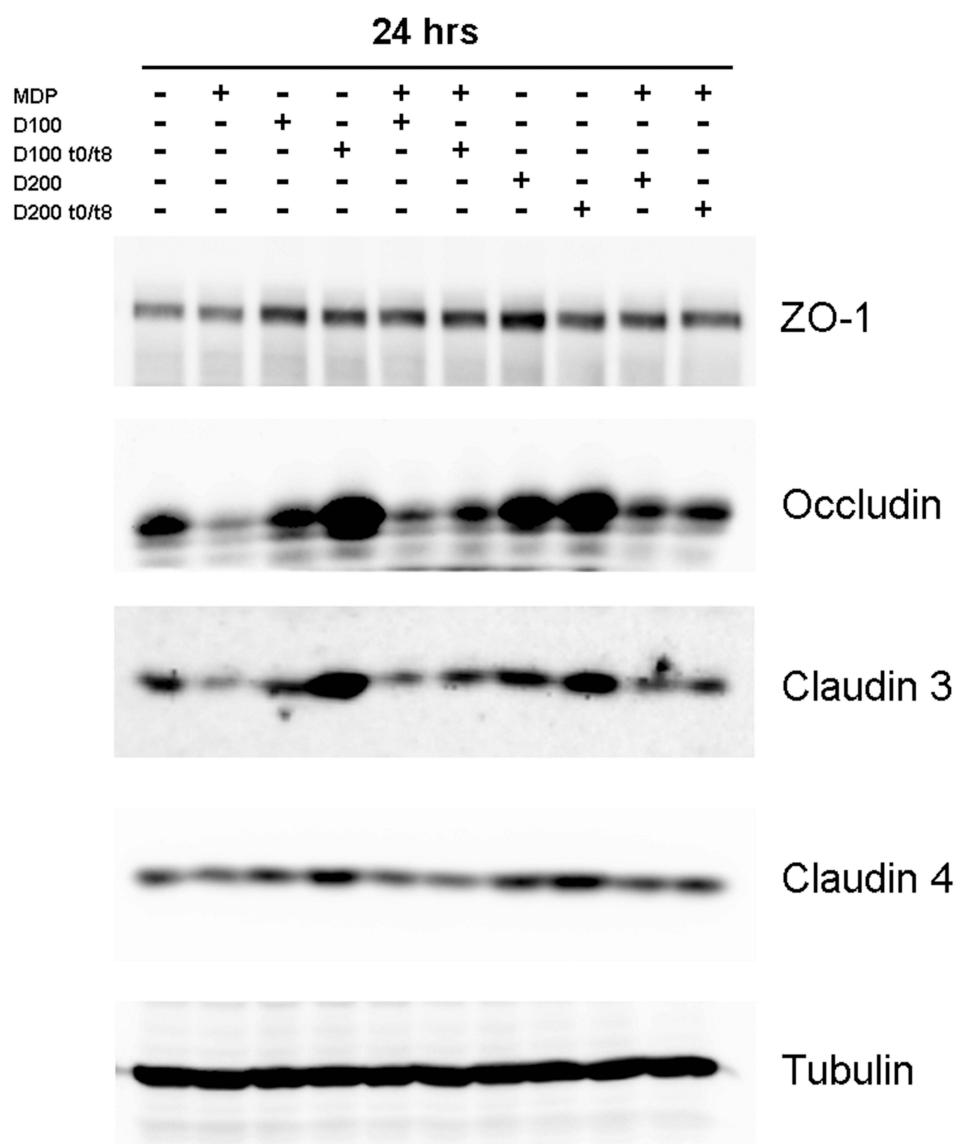


Figure 3 Effects of defibrotide on the expression of tight junctions' proteins in conditions of inflammation. Representative Western blotting images of ZO-1, Occludin, Claudin 3 and Claudin 4 are shown. Immunoblotting was performed using 50 μg of HCT116 cell lysate. Tubulin staining is used as loading control. MDP= 10 μM (inflammatory condition); D100=defibrotide 100 $\mu\text{g}/\text{mL}$; D100 t0/t8=defibrotide 100 $\mu\text{g}/\text{mL}$ added at time 0 and after 8 hours; D200=defibrotide 200 $\mu\text{g}/\text{mL}$; D200 t0/t8=defibrotide 200 $\mu\text{g}/\text{mL}$ added at time 0 and after 8 hours. The experiments were performed at least in triplicate.

Discussion

The integrity of the intestinal barrier is crucial for maintaining overall gut health and preventing the translocation of harmful pathogens and toxins into the bloodstream.²⁹ In our study, we analyzed the effects of defibrotide on colon physical barrier in experimental conditions mimicking the inflammatory state typical of bowel diseases, to evaluate the anti-inflammatory efficacy of this drug.

First, we analyzed the effect of defibrotide on the tight junctions (TJ), which consist of integral transmembrane proteins such as claudins, Occludin, and junctional adhesion molecules (JAMs), as well as zonula occludens (ZOs) cytoplasmic proteins, like ZO-1, ZO-2, and ZO-3, that connect transmembrane proteins to the actin cytoskeleton.^{30,31}

The results of our study demonstrate that defibrotide significantly enhances the expression of tight junction proteins, such as ZO-1 and Occludin, in HCT116 cells. This effect was observed both under normal conditions and in an inflammatory environment, suggesting that defibrotide may play a crucial role in maintaining and restoring intestinal barrier integrity.

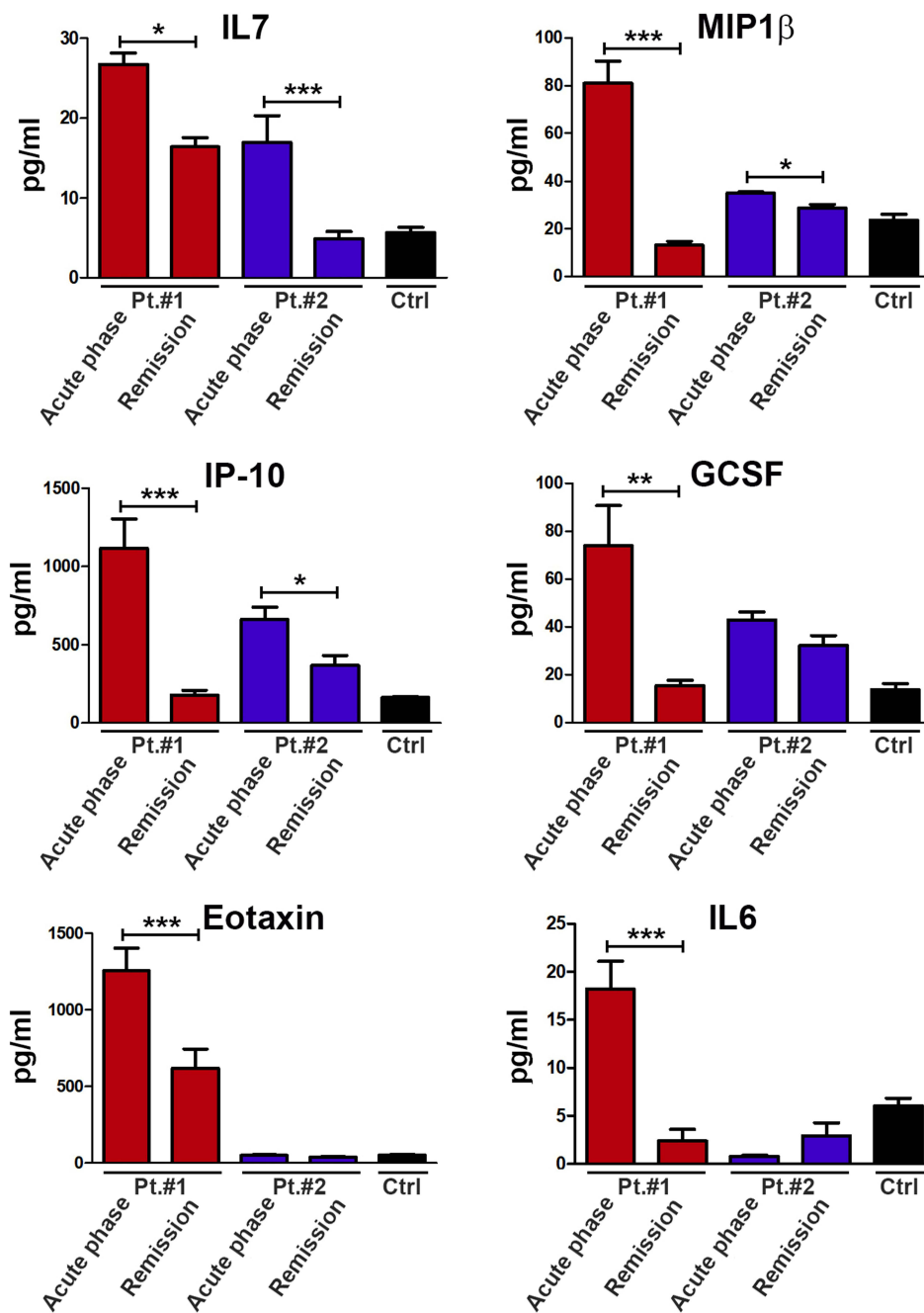


Figure 4 Levels of IL-7, MIP-1 β , IP-10, G-CSF, Eotaxin and IL-6 measured in acute phase, remission and control groups. Cytokines downregulated in the remission group in comparison to the acute phase group were measured in serum samples by multiplex immunoassays. Statistically significant p-values are shown in all comparisons (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

In particular, ZO-1 is a cytoplasmic peripheral membrane isoform that forms a scaffold anchoring the actin cytoskeleton and transmembrane proteins of the tight junctions.^{32,33} A recent study demonstrated, in a mouse model with intestinal epithelial-specific ZO-1 knockout, that ZO-1 is not required for epithelial barrier function but is crucial for the repair process of the mucosal epithelium.³⁴ In this context, the ability of defibrotide to increase the basal levels of ZO-1 could be important in hypothesizing a role for this compound in restoring the intestinal barrier.

TJ permeability is determined by the combination of different components, including barrier-forming junctional proteins Occludin, Claudins-1, -3, -4, and -8.³⁵ Claudins are a family of proteins distributed with distinct expression patterns in many organs and segments, such as the gastrointestinal tract. Claudins-3 and -4, predominantly expressed in

the distal regions of the intestine,^{36,37} are sealing claudins³⁸ that prevent the passage of molecules through the TJ and, like Occludin, are downregulated in diseases affecting the small and large intestine.³⁷ In line with these studies, Occludin, Claudins-3 and -4 were downregulated in our *in vitro* model mimicking intestinal inflammation, and this effect was counteracted by treatment with defibrotide.

We have also verified the efficacy of the systemic administration of defibrotide in clinical settings, evaluating the cytokine spectrum of two patients, who were successfully treated with defibrotide, as it has been demonstrated that the release of these molecules play an important role in the onset of GVHD.^{39,40}

In our study we identified different cytokines upregulated during the acute phase, with respect to controls, and downmodulated after the treatment with defibrotide: IL-7, MIP-1 β , IP-10, G-CSF, Eotaxin and IL-6.

IL-7 is a cytokine involved in T cell lymphopoiesis and in the homeostatic and extrathymic expansion of T cells in lymphopenic hosts.^{41,42} The immune benefits of this cytokine are, however, counterbalanced by the evidence that elevated plasma levels of IL-7, after allogenic-HSCT, are predictive of increased risk of aGVHD and mortality.^{43,44} IL-7 may promote the expansion of alloreactive T cells mediating GVHD.⁴⁵ In line with these studies, both patients present high levels of this cytokine during the acute phase, decreasing at the remission after the treatment with defibrotide.

MIP-1 β is a pro-inflammatory chemokine that increases the release of cytokines, such as IL-6, from fibroblasts and macrophages, as well as chemotaxis and trans-epithelial migration, mechanisms that contribute to the onset of inflammation.⁴⁶ For this chemokine, the levels before and after treatment of the first patient were significantly reduced, while in the second patient there was always a decrease, although less marked. A similar trend was also identified for IP-10, a CXC chemokine released by antigen presenting cells (APC), epithelial cells as well as endothelial and stromal cells.⁴⁷ It has been suggested that the onset of GVHD is triggered by activation of APCs by damage-associated molecular patterns and pathogen-associated molecular patterns, leading to the production of inflammatory cytokines, such as IFNs, which in turn can induce the production of chemokines, such as IP-10.⁴⁸

An effect similar to that shown for IL-17, MIP-1 β , IP-10 and G-CSF can also be observed in the first patient for eotaxin, that plays a role in promoting organ-specific migration of inflammatory cells in GVHD pathophysiology.⁴⁹ Our results support this hypothesis: indeed, the first patient showed significantly higher levels in the acute period of the disease than in the control. After treatment with defibrotide, the levels of chemokine decreased significantly in conjunction with the remission phase, where a reduction in inflammation is observed. In contrast, the second patient shows extremely low levels, comparable to control, already during the acute phase. This may be due to the different onset and severity of GVHD in the two patients.

IL-6 is a cytokine involved in multiple mechanisms such as inflammation, cancer and immunity.^{50,51} IL-6 is a pleiotropic cytokine that plays a key role in inflammatory diseases and the onset of GVHD.⁵² In line with the results obtained by Palaniyandi et al,²⁶ the first patient showed a significant reduction in IL-6 levels after treatment with defibrotide, supporting the hypothesis that the drug has an anti-inflammatory activity.

The results obtained in our study are in line with literature, as the levels of pro-inflammatory cytokines and chemokines during the acute phase of GVHD were higher than those found in the controls. Furthermore, from the results obtained, even if preliminary for the limited number of patients analyzed, it is possible to assume that defibrotide may lead to a reduction of the pro-inflammatory cytokine and chemokine profile, leading to an improvement in symptoms of GVHD and allowing its remission.

Conclusion

In conclusion, defibrotide was effective in lowering levels of proinflammatory cytokines to a baseline similar to the controls and also in restoring the expression of structural TJ's proteins. From the data obtained, it can therefore be assumed that this drug could be used in cases of aGVHD resistant to first- and second-line drugs and could resolve the condition of leaky gut. Consequently, defibrotide could become a repositioned drug, which would have the advantage of reducing development costs and time, since toxicological, pharmacokinetic and safety data had already been collected earlier.

The results presented in the study lay the basis for a more complex clinical investigation involving a broader range of cases, and further research will be necessary to validate our findings and to analyze the molecular mechanisms involved in defibrotide interaction with intestinal TJ.

Data Sharing Statement

The data underlying this study will be shared on request to the corresponding author.

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Disclosure

Erika Rimondi and Elisabetta Melloni are co-first authors for this study. Natalia Maximova and Annalisa Marcuzzi are co-last authors for this study. The authors report no conflicts of interest in this work.

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