Novel Tubulin Antagonist Pretubulysin Displays Antivascular Properties In Vitro and In Vivo

Verena K. Kretzschmann,* Donata Gellrich,* Angelika Ullrich, Stefan Zahler, Angelika M. Vollmar, Uli Kazmaier, Robert Fürst

Objective—Pretubulysin (PT) is a novel, synthetically accessible myxobacterial compound that acts as a tubulin-depolymerizing agent and inhibits cancer cell growth in vitro and in vivo. Moreover, PT was found to attenuate tumor angiogenesis. Here, we hypothesized that PT could exert antivascular activities on existing tumor vessels.

Approach and Results—We aimed to characterize the antivascular effects of PT and to elucidate the underlying mechanisms in endothelial cells. In vitro, PT rapidly induced endothelial hyperpermeability and a concentration-dependent disassembly of established endothelial tubes on Matrigel and in an ex vivo aortic ring model. It disrupted endothelial cell junctions and triggered F-actin stress fiber formation and cell contraction by the RhoA/Rho-associated protein kinase pathway without causing cell death. In vivo, using a hamster dorsal skinfold chamber preparation, PT significantly decreased blood flow and vessel diameter in hamster A-Mel-3 amelanotic melanoma tumors but not in the neighboring healthy tissue. In a second tumor model using mice with subcutaneous murine B16 melanoma tumors, a single dose of PT (10 mg/kg) caused a shut down of tumor blood flow and a strong central tumor cell necrosis within 24 hours. Repeated PT administration significantly decelerates tumor growth and seems to be well tolerated.

Conclusions—In summary, we could show for the first time that the antitumor effect of PT is, at least in part, mediated via its antivascular activities on existing tumor vessels. (Arterioscler Thromb Vasc Biol. 2014;34:294-303.)

Key Words: drug research □ endothelial cells □ neoplasms □ pharmacology

Tumor growth and metastasis depend on a functional vascular network, which supplies the tumor with nutrients and oxygen. Therefore, targeting the tumor vasculature offers an interesting antitumor strategy, which has intensively been explored in recent years. Two different pharmacological approaches exist in this field: (1) the angiogenesis inhibitors, which are already clinically established, such as bevacizumab or sorafenib. They act on the periphery of small tumors and prevent neovascularization from existing blood vessels. (2) The vascular disrupting agents (VDAs), which have recently gained importance in experimental antitumor treatment, are effective well below their maximum tolerated dose and lack the classic cytotoxic side effects of chemotherapeutics. They way of action completely differs from that of angiogenesis inhibitors: VDAs target the already established tumor blood vessels of larger solid tumors resulting either in apoptotic death of endothelial cells (ECs) or in cytoskeletal rearrangements, which alter the EC shape. These events emerge especially in the central part of the tumor and cause within a few hours after treatment strong hyperpermeability, vessel occlusion, shut down of tumor blood flow, ischemia, and enormous central necrosis, which can extend to as much as 95% of the tumor. Thereby, a peripheral viable rim of tumor cells remains, which is responsible for the rapid regrowth after single-dose treatment. To overcome this obstacle, VDAs are commonly applied in combination with chemotherapy, radiation, or angiogenesis inhibitors, which promote suppression of regrowth. Several VDAs are currently in clinical trials under intensive investigation, for example, combretastatin A-4 3-O-phosphate (CA4P) as well as its analogues OXi4503 and AVE8062. These are used as single compounds or in combination with other antivascular agents.

Pretubulysin (PT) represents a natural compound of myxobacterial origin, which was first isolated from Angiococcus disciformis An d48 in small amounts. It is a biosynthetic precursor of the microtubule-depolymerizing peptide tubulysin, which has been described recently to be highly active against different tumor cell lines. Structurally, PT is a linear tetrapeptide consisting of isoleucine and 3 unnatural amino acids. In contrast to tubulysin, PT is synthetically more easily accessible; in 2009, PT was first synthesized in the gram scale by Ullrich et al. Although the structure of PT is less complex, it shows nearly the same microtubule-depolymerizing potency as tubulysin and is also able to inhibit tumor cell...
growth. PT acts, like other tubulin-binding agents, through binding to the β-subunit of tubulin. Besides inhibition of cancer cell growth, PT has been shown recently to attenuate tumor angiogenesis. Here, we hypothesized that PT could also exert antivascular actions on already existing tumor vessels. Thus, we aimed to characterize the effects of PT in vitro in ECs and in vivo in different tumor models. Moreover, we investigated the underlying mechanisms of action.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results
PT Triggers the Depolymerization of Microtubules, Actin Stress Fiber Formation, Disassembly of Cell–Cell Connections, Endothelial Hyperpermeability, and the Destruction of Endothelial Tubes
PT treatment (30 and 300 nmol/L, 1 hour) induced the depolymerization of microtubules in primary EC (Figure 1A, i–iii). Noteworthy, PT showed an extent of depolymerization comparable with CA4P (Figure 1A, iv–vi). As shown in Figure 1B, PT (1 hour, 300 nmol/L) triggered the formation of thick, cell-spanning F-actin stress fibers (Figure 1B, i and ii) and the disruption of adherens junctions (vascular endothelial cadherin; Figure 1B, iii and iv) and tight junctions (claudin 5; Figure 1B, v and vi), thereby forming intercellular openings (marked by arrowheads). Focal adhesions showed a strong redistribution to pronounced clustered structures (Figure 1B, vii and viii). These changes are accompanied by an increase in endothelial permeability as assessed by 2 different assays; a time- and concentration-dependent decrease in electric impedance (ie, increase of permeability) was observed already on a few minutes of PT treatment reaching its minimum within 1.5 hours (100 nmol/L PT) or within 1 hour (300 nmol/L; Figure I in the online-only Data Supplement). The statistical evaluation of all tested concentrations at time point 1 hour is depicted in Figure 1C, i. A half maximal inhibitory concentration value of 71 nmol/L was calculated. In a second approach, macromolecular permeability (tracer: fluorescein isothiocyanate [FITC]-dextran, 40 kDa) was determined. Again, PT evoked a time- and concentration-dependent increase in permeability (Figure 1C, ii and iii). Aiming to investigate the ability of PT to destroy established endothelial tubes, we observed a disassembly of PT-treated tubes starting at 1.5 hours after PT addition (data not shown), leading to an almost complete disruption within 6 hours at 100 nmol/L PT (Figure 1D, i–vi). The total tube lengths, as well as the total number of tubes and of branching points, were strongly reduced by PT after 6 hours (Figure 1D, vii–xi). Moreover, established endothelial tubes that had sprouted from explanted mouse aortic rings were also significantly disrupted by PT (300 nmol/L) after 3 and 6 hours (Figure 1E, marked by arrowheads).

Short-Term Treatment With PT Is Not Toxic for ECs and Long-Term Treatment Exhibits Slight, But Fully Reversible Cytotoxic Stress
PT treatment did only marginally affect metabolic activity within 4 hours (Figure 2A) and led to a reduction of ≈20% within 26 hours (Figure 2B). The basal apoptosis rate of untreated cells (≈6%) was slightly increased by PT (24 hours) to ≈10% (Figure 2C), and also the number of membrane-damaged PI-positive cells rose from 5% to ≈15% (Figure 2D). Interestingly, removal of PT after 1 and 24 hours of treatment led to a full reestablishment of the normal EC morphology within 24 hours (Figure 2E). We conclude that short-term PT treatment is not toxic and that the slight cytotoxicity induced on 24 hours is fully reversible.

PT Triggers Its Effects on Endothelial Actomyosin via the RhoA/Rho-Associated Protein Kinase/Myosin Light Chain Pathway
PT treatment significantly increases the amount of activated RhoA (pull-down assay) within 30 minutes (Figure 3A; Figure III in the online-only Data Supplement). Also, the RhoA downstream target myosin light chain (MLC) was activated by PT within 30 minutes, as shown both by immunofluorescence stainings (Figure 3B) and by Western blot analysis (Figure 3C) of the MLC (Thr18/Ser19) phosphorylation status. This status is regulated by activation of the MLC kinase (MLCK) and by inactivation of the MLC phosphatase (MLCP). Interestingly, the MLCK inhibitor ML-7 did not alter the PT-evoked phosphorylation of MLC (Figure 3D). Further, the MLCP activation status was not affected by PT, as judged by analyzing the phosphorylation of the MLCP subunit myosin phosphatase targeting protein 1 (Figure 3E). This indicates that neither MLCK nor MLCP is involved in PT-induced signaling. However, on pretreatment of ECs with the Rho-associated protein kinase (ROCK) inhibitor Y27632, both the PI-stained MLC phosphorylation and stress fiber formation were completely prevented (Figure 3F). Moreover, we tested whether endothelial calcium levels were affected by PT treatment. Ratiometric calcium imaging in Fura 2–loaded ECs showed that cytosolic calcium concentrations were not influenced by PT (Figure 3G). Histamine was applied as positive control to confirm that ECs respond in a normal way to this calcium-augmenting autacoid. Besides Rho signaling, we also investigated whether the p38 mitogen-activated protein kinase (MAPK) pathway participates in stress fiber formation because we found a strong phosphorylation (activation) of this kinase and of its downstream target heat shock protein 27 within 30 minutes on PT treatment (Figure 3H). However, after applying the p38 MAPK inhibitor SB202180 (functionality was proven and is shown in Figure IV in the online-only Data Supplement.)
Figure 1. Pretubulysin (PT) triggers the depolymerization of microtubules, actin stress fiber formation, disassembly of cell–cell connections, endothelial hyperpermeability, and the destruction of endothelial tubes. 

A and B. Immunostaining and confocal microscopy of confluent endothelial cells. 

A. Human umbilical vein endothelial cells (HUVECs) were pretreated for 1 hour as indicated. Microtubules were stained with an anti-α-tubulin antibody (green) and nuclei were visualized by Hoechst 33342 (blue). One representative experiment of 4 is shown. Bar, 50 μm. 

B. HUVECs were treated with vehicle control or 300 nmol/L PT for 1 hour and were stained for actin (i and ii), for adherens junctions (iii and iv), for tight junctions (v and vi), and for focal adhesions (vii and viii). Arrowheads point at intercellular...
Figure 1 (Continued). open. One representative experiment of 3 is shown. Bars for B (i to vi), 50 μm and B (vii and viii), 20 μm. 
C. Changes in permeability on PT treatment were measured by impedance sensing (i) and by a macromolecular permeability assay (ii and iii). C. i. Human microvascular endothelial cells (HMECs) were grown to confluence on gold electrodes and treated as indicated. Impedance was measured every 10 seconds up to 12 hours. Quantitative analysis was performed at time point 1 hour after PT treatment (PT, mean±SEM, n=4). C. ii, Confluent HMEC monolayer grown on porous filter inserts were treated with vehicle control (○), 30 nmol/L PT (△), 100 nmol/L PT (■), and 300 nmol/L PT (●). Fluorescein isothiocyanate (FITC)-labeled dextran (40 kDa) was added to the upper compartment of the chamber. After 30, 60, 120, and 240 minutes the amount of FITC-dextran in the lower compartment of the chamber was determined. Quantitative analysis (iii) was performed at time point 4 hours. Control values were set as 1. Data are expressed as mean±SEM, n=4 (i), n=3 (ii and iii). One-way ANOVA followed by Tukey’s multiple comparison post hoc test was used. *P<0.05 vs control. D. HUVECs were seeded on Matrigel. After 16 hours, endothelial tubes were formed, which were then treated as indicated. Phase-contrast images before (i–iii) and after 6 hours of treatment (iv–vi) were captured. Magnification, ×10 for all panels. Tube disruption was quantified at time point 6 hours after PT treatment. Total tube length (vii), total tubes (viii), and total branching points (ix) were chosen for analysis. Control values were set as 100%. Bar graph data are expressed as mean±SEM and were obtained from 6 independent experiments. One-way ANOVA followed by Tukey’s multiple comparison post hoc test was used. *P<0.05 vs control. E. Fourteen aortic rings prepared from 6 different female C57BL/6 mice were placed on Matrigel. After 10 days established endothelial sprouts were treated with 300 nmol/L PT and phase-contrast images before (i), after 3 hours (ii), and after 6 hours (iii) of treatment were taken. Arrowheads indicate disrupted sprouts (ii and iii). One representative experiment of 14 is shown. Magnification, ×10. Quantification was performed using ImageJ 1.45s software. Normalized data (0 hours) are expressed as mean±SEM (n=14). One-way ANOVA followed by Tukey’s multiple comparison post hoc test was used. *P<0.05 vs control (0 hours). CA4P indicates combretastatin A4-phosphate; IC50, half maximal inhibitory concentration; and VE, vascular endothelial.

Data Supplement), PT was still able to exert its action on the actin cytoskeleton (Figure 3f). These data suggest that the effects of PT are mainly mediated by the RhoA/ROCK pathway, but not via calcium, MLCK, MLCP, or p38 MAPK.

PT Selectively Reduces Tumor Blood Flow in a Hamster Dorsal Skinfold Chamber Model

First, we determined the maximum tolerated dose of PT in a separate in vivo approach in mice. We revealed 10 mg/kg PT as the optimal dose (Figure V in the online-only Data Supplement). Then, the capability of PT to reduce tumor blood flow was assessed in a hamster dorsal skinfold chamber model. Blood flow in vessels of hamster A-Mel-3 amelanotic melanomas (Figure 4A, iii and iv) and of the surrounding healthy tissue (Figure 4A, i and ii) was analyzed. On intravenous injection of 10 mg/kg PT, we observed slight perfusion irregularities in tumor vessels already after 30 minutes (data not shown). After 2 hours (Figure 4A, iv, arrowheads), distinct criteria of microcirculatory impairment, that is, intraluminally stagnant FITC-dextran dye or discontinuity of fluorescence-contrasted...
Figure 3. Pretubulysin (PT) triggers its effects on endothelial actomyosin via the RhoA/Rho-associated protein kinase (ROCK)/myosin light chain (MLC) pathway. A, Confluent human umbilical vein endothelial cells (HUVECs) were pretreated as indicated. Active RhoA was isolated from cell lysates (pull-down assay) and was analyzed by Western blotting. One representative experiment of 4 is shown. B, Immunocytochemistry and confocal microscopy of di-(Thr18/Ser19) phosphorylated MLC2 in confluent HUVECs after 1-hour treatment with vehicle control (i) or with 300 nmol/L PT (ii). One representative experiment of 3 is shown. Bar, 50 μm. C, D, E, and H, Activation of MLC2
Figure 3 (Continued). (C), p38 mitogen-activated protein kinase (MAPK), and heat shock protein (HSP)-27 (H) and involvement of MLCK (MLC; D) and MLC phosphatase (E) were examined by Western blotting. Confluent HUVECs were pretreated as indicated (C, E, and H) or were pretreated with the MLCK inhibitor ML-7 (10 μmol/L, 30 minutes) followed by PT treatment (300 nmol/L, 1 hour; D). One representative blot of 4 (C) or 3 (D, E, and H) is shown. F and I, Immunocytochemistry and confocal microscopy of confluent HUVECs on treatment with vehicle control (1 hour; F, I, and ii), with the ROCK inhibitor Y27632 (10 μmol/L, 1 hour; F, iii and iv), with the p38 MAPK inhibitor SB203580 (20 μmol/L, 1 hour; I, i, ii), with PT (300 nmol/L, 1 hour; F, iv and vi; I, iii; iv) or in combination with Y27632 (30 minutes pretreatment) plus PT (300 nmol/L, 1 hour; F, vii and viii) and SB203580 (20 μmol/L, 30 minutes pretreatment) plus PT (300 nmol/L, 1 hour; I, iv). F-Actin stained with rhodamine phalloidine and MLC (Thr18/Ser19) are visualized. One representative experiment of 3 is shown. Bar, 50 μm. G, The change of intracellular Ca²⁺ [Ca²⁺]i was monitored using confluent HUVECs, which were incubated with Fura-2-AM for 30 minutes. Cells were then treated for 45 minutes with 300 nmol/L PT (gray line) or were left untreated (black line). At the end, histamine (100 μmol/L) was added as positive control. One representative figure of 3 is shown.

PT Diminishes Tumor Vessel Perfusion, Induces Central Necrosis, and Decelerates Tumor Growth in an Ectopic B16-F1 Mouse Melanoma Tumor Model

C57BL/6 mice bearing subcutaneous, highly vascularized B16-F1 mouse melanoma tumors were treated with PT (intra-venous injection, 10 mg/kg PT) as soon as the tumors had reached a size of ≈650 mm³. Twenty-four hours later, Hoechst 33342 was intravenously injected as perfusion marker and mice were euthanized after 1 minute. Both the tumor rim (Figure 5A) and the tumor center (Figure 5B) of PT- or vehicle-treated mice were analyzed. In control tumors, Hoechst 33342 (ie, perfusion) was detectable nearly in equal intensity at the rim as well as in the center, whereas PT-treated tumors showed a lesser Hoechst 33342 intensity at the rim and almost no staining in the center (Figure 5A and 5B). For quantification, the ratio of Hoechst 33342 fluorescence and total number of CD31-positive cells is given in Figure 5C. CD31-positive ECs were similarly distributed throughout the tumors, indicating that tumor vessel density was not altered by PT (Figure 5D). Hematoxylin and eosin staining of these tumors revealed that PT treatment increases necrosis, which spanned ≈30% of the whole tumor area (Figure 5E and 5F). In a second approach, PT treatment was started as soon as tumors reached a size of ≈100 mm³ and was applied repeatedly (on days 0, 3, and 6). Thus, we determined both the overall capacity of PT to affect tumor growth and also its tolerability. We found that the increase of tumor size was strongly attenuated by PT.
Figure 5. Pretubulysin (PT) diminishes tumor vessel perfusion, induces central necrosis, and decelerates tumor growth in an ectopic B16-F1 mouse melanoma tumor model. A to D, Subcutaneously growing B16-F1 mouse melanoma tumors were pretreated intravenously (IV) with dimethyl sulfoxide (DMSO) control (Co; 5%, 24 hours; upper row) or with PT (10 mg/kg, 24 hours; lower row). Hoechst 33342 (10 mg/kg) was injected IV 1 minute before tumor removal. Cryosections of 10 μm were prepared and stained with an anti-CD31 antibody to visualize endothelial cells. To obtain an overview about the tumor tissue, phase-contrast images were additionally taken. A and B, Representative images of PT- and Co-treated tumor rims and centers. Bar, 200 μm. C and D, For quantification, 13 pictures of the tumor rim and center of 2 different sections were analyzed. C, The mean fluorescence intensity (MFI) of Hoechst 33342 is divided by the number of CD31-positive cells. D, Number of CD31-positive cells. Data are expressed as mean±SEM (PT, n=8; DMSO, n=6). One-way ANOVA followed by Tukey’s multiple comparison post hoc test was used.
Taken together, our data demonstrate that a single dose of PT leads to a breakdown of tumor blood supply followed by excessive tumor necrosis. Moreover, repeated PT administration strongly decelerates tumor growth and is well tolerated.

**Discussion**

In this study, we present a preclinical characterization of the anti-vascular action of the myxobacterial tubulin-destabilizing compound PT by an in-depth investigation into the PT-induced effects on ECs in vitro and in different animal tumor models in vivo.

In vitro, we showed that the PT-triggered microtubule disassembly results in activation of RhoA, ROCK-mediated phosphorylation of MLC, actin stress fiber formation and activation of the contractile machinery, disassembly of interendothelial junctions, increase in permeability, and disruption of tube-like endothelial structures. Our study is in line with reports demonstrating that the disassembly of microtubules, for example, by CA4P or nocodazole, can activate the RhoA/ROCK/MLC pathway, which contributes to actin remodeling and hyperpermeability.23–26 Interestingly, it is still not clear how a disassembly of the tubulin network can activate RhoA, but it can be speculated that guanosine nucleotide exchange factors, which are linked to microtubules,27,28 could be involved. Recent studies showed that guanosine nucleotide exchange factor-H1, a Rho-specific guanosine nucleotide exchange factor, is inactive when it is bound to microtubules, whereas depolymerization results in its release and activation, accompanied by induction of stress fiber formation and MLC phosphorylation.29

Besides the RhoA/ROCK/MLC pathway, also p38 MAPK, a known important regulator of actin remodeling,23,26 was reported to be involved in the action of microtubule-disrupting compounds.25,26 In fact, we could show that p38 MAPK is strongly activated by PT. However, in contrast to these findings, activation of p38 MAPK is not needed to mediate the effects of PT on the F-actin cytoskeleton in our system. We speculate that the PT-induced activation of p38 MAPK might be associated with the process of early membrane blebbing, which was observed at higher concentrations of PT after long-term treatment (data not shown). Early membrane blebbing was intensively described by a study of Kanthou and Tozer,23 in which they could show that membrane blebbing in human umbilical vein ECs (HUVECs) occurred on the disruption of microtubules. This phenomenon is dependent on the activation of p38 MAPK, but was independent of MLCK-mediated MLC phosphorylation and, remarkably, of apoptotic blebbing.23

The PT-induced strong effects on cellular functions and morphology might at first suggest that PT is cytotoxic to the endothelium. However, cytotoxicity assays revealed that PT did neither strongly reduce metabolic activity nor heavily trigger apoptosis or necrosis after 24 hours. Furthermore, we could not detect any G2/M arrest (data not shown), which plays an important role in the onset of apoptosis-mediated cell death. Our findings are in contrast to data from previous studies, in which PT as well as CA4P was shown to induce G2/M
arrest and apoptosis within 24 to 48 hours.\textsuperscript{3,2,32} The reason for these different results may be a result of the fact that we worked with confluent, quiescent HUVECs, whereas the mentioned studies used proliferating ones, which are susceptible to the blockade of the spindle apparatus. This is supported by our own experiments showing that proliferating HUVECs pretreated with PT reveal a 4x higher induction of apoptosis (Figure II in the online-only Data Supplement) than confluent ones and a G2/M arrest (data not shown). Confluent HUVECs treated with CA4P also did not undergo changes in metabolic activity, G2/M arrest, apoptosis, or necrosis (data not shown). Moreover, HUVECs are even able to reassume their normal morphology after removing PT, which indicates that PT treatment exerts only minor cytotoxicity.

Using different in vivo tumor models, we showed that PT treatment induces a significant reduction in tumor blood perfusion, whereby perfusion of healthy tissue seems to be unaffected. We cannot exclude that normal blood vessels are not at all affected by PT, but we suggest that a mature endothelium covered by pericytes is more stable and, thus, resistant to PT treatment. This hypothesis is supported by a study showing that in vitro cocultures of ECs with smooth muscle cells are less affected by CA4P than ECs alone.\textsuperscript{33} In addition, differences between tumor and normal vasculature in regard to blood flow, permeability, interstitial fluid pressure, proliferation rate, or modifications of tubulin and actin could contribute to the observed selectivity.\textsuperscript{34,35} But are still a matter of debate. Reduced tumor vessel perfusion and, consequently, necrosis occurred mainly in tumor centers although vessel density was unchanged. This suggests that vessels in the tumor center are much less perfused, possibly because of constriction or local occlusion, which might occur through EC shape changes or early membrane blebbing.\textsuperscript{4} The reduced blood velocity caused by PT might also evoke an increase in blood viscosity, which would also promote vessel occlusion.\textsuperscript{26} Repeated applications of PT to tumor-bearing mice were intended to mimic a more therapeutic setting. Although this model does not allow to discriminate between effects of PT on cancer cells and on the tumor vasculature, the strong tumor growth-decelerating action of PT clearly demonstrates the overall anticancer potential of the compound. Importantly, the compound seems to be well tolerated because no obvious side effects were detected and the animals did not exhibit significant weight changes.

Our data might point toward a vascular disrupting activity of PT. However, it has to be mentioned that the in vivo data were gained at a dose of 10 mg/kg. As demonstrated, a 3-fold higher dose of PT is not tolerated anymore. Thus, 10 mg/kg seems to be (near) the maximum tolerated dose. VDAs are by definition active below their maximum tolerated dose. We did not establish the in vivo efficacy of lower doses of PT.

In summary, we characterized the antivascular action of PT on established tumor blood vessels, which is suggested as an important part of its overall anticancer effect. This natural compound of myxobacterial origin is synthetically accessible in large scales, which provides the opportunity for chemical modification. Thus, PT represents an interesting novel, tubulin-binding compound for further optimization into a vascular-targeting agent.

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### Disclosures

None.

### References

with antivascular properties. This compound represents an interesting and valuable new lead for further optimization as an anticancer agent in vivo without inducing severe cytotoxicity. Moreover, we gained insights into the underlying mechanisms of action. Because pretubulysin we report that pretubulysin, a novel microtubule-disassembling agent from myxobacteria, shows interesting antivascular features in vitro and and compounds that shut down the blood flow in existing tumor vessels are currently under intense preclinical and clinical evaluation. Here, antivascular tumor therapy has expanded our possibilities to fight solid tumors. Antiangiogenic compounds are already clinically available, structures and of simplified pretubulysin derivatives. Br J Pharmacol. 2012;167:1048–1061.


Significance

Antivascular tumor therapy has expanded our possibilities to fight solid tumors. Antiangiogenic compounds are already clinically available, and compounds that shut down the blood flow in existing tumor vessels are currently under intense preclinical and clinical evaluation. Here, we report that pretubulysin, a novel microtubule-disassembling agent from myxobacteria, shows interesting antivascular features in vitro and in vivo without inducing severe cytotoxicity. Moreover, we gained insights into the underlying mechanisms of action. Because pretubulysin is chemically fully accessible, this compound represents an interesting and valuable new lead for further optimization as an anticancer agent with antivascular properties.
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Materials and Methods

Compounds

Pretubulysin (PT) was synthesized as described previously\(^1\) and was kindly provided by Prof. Dr. U. Kazmaier (Saarland University, Saarbrücken, Germany). Combretastatin A-4 phosphate (CA-4-P) was a kind gift from OXiGENE (Waltham, MA, USA). The Rho-kinase inhibitor Y-27632 (# 10005583) was from Cayman Chemical (Ann Arbor, MI, USA), the MLCK inhibitor ML-7 (# E1-197) from Enzo Life Sciences (Lörrach, Germany), the p38 MAPK inhibitor SB203580 (# 559389) from Calbiochem (Nottingham, UK), histamine, TWEEN\(^\circ\) 20 and Triton X-100 from Sigma-Aldrich (Taufenkirchen, Germany).

Cell culture

Primary human umbilical vein endothelial cells (HUVECs) were prepared as previously described.\(^2\) Human Microvascular Endothelial Cells (HMEC-1)\(^3\) were kindly provided from the Center for Disease Control and Prevention (CDC, Atlanta, GA, USA) and were used until passage 11. B16-F1 mouse melanoma cells were from ATCC (Manassas, VA, USA) and were cultivated in DMEM (Sigma-Aldrich, Taufenkirchen, Germany) containing 10 % FCS. B16-F1 cells were used between passage 21 and 25.

Animals

All animal care and experimental procedures were in accordance with the local animal protection legislation (Government of Upper Bavaria). Experiments were carried out using male Syrian golden hamsters (6-8 weeks old, 60 ± 5 g body weight) and female C57BL/6 mice (6-12 weeks old, 20 ± 5 g body weight) both purchased from Charles River (Sulzbach, Germany). The animals were maintained in a specific pathogen-free environment, with food and water provided \textit{ad libitum}.

Immunoblotting

HUVECs were grown until confluence and were treated as indicated. Western blot analysis was performed as described previously.\(^4\) The following antibodies were used: anti-MYPT1 (H-130; sc-25618) rabbit polyclonal, anti-MLC (FL-172; sc-15370) rabbit polyclonal (Santa Cruz, Heidelberg, Germany), anti-phospho-MYPT1 (Thr696; ABS45) rabbit polyclonal and anti-actin (C4; MAB1501) mouse monoclonal (Millipore, Schwalbach, Germany), anti-HSP27 (2402) mouse monoclonal, anti-phospho-HSP27 (Ser82; 2401) rabbit polyclonal, anti-β-tubulin (2146) rabbit polyclonal, anti-p38 (9212) rabbit polyclonal, anti-phospho-p38 (Thr180/Tyr182; 9211) rabbit polyclonal, anti-phospho-MLC2 (Ser19; 3675) mouse monoclonal, anti-phospho-MLC2 (Thr18/Ser19; 3674) rabbit polyclonal (Cell Signaling/New England Biolabs, Frankfurt am Main, Germany), Alexa Fluor© 680 goat anti-mouse (A-21057; Invitrogen, Darmstadt, Germany), IRDye 800 goat anti-rabbit (611-132-122; Rockland, Gilbertsville, PA, USA), horseradish peroxidase-coupled goat anti-rabbit (111-095-144; Dianova, Hamburg, Germany) and horseradish peroxidase-coupled goat anti-mouse (BZL07046; Biozol, Eching, Germany). Antibodies were detected with the Odyssey infrared imaging system (LI-COR Bioscience, Lincoln, NE, USA) or with the Amersham ECL-detection system (GE-Healthcare, Munich, Germany).

Immunocytochemistry and confocal laser scanning fluorescence microscopy

HUVECs were cultured to confluence on 8-well μ-Slides (Ibidi GmbH, Martinsried, Germany) and treated as indicated. Cells were washed and fixed with 10 % paraformaldehyde (Sigma-Aldrich) at room temperature (ppMLC, vinculin and F-actin) or with ice-cold acetone at -20 °C (VE-cadherin, claudin-5 and F-actin). After permeabilization (0.2 % Triton X-100) and BSA blocking, cells were incubated with the following antibodies/reagents: anti-phospho-MLC2 (Thr18/Ser19) rabbit polyclonal (Cell Signaling), anti-vinculin (clone hVIN-1) mouse monoclonal (sc-25336; Santa-Cruz), anti-VE-cadherin (F-8) mouse monoclonal (sc-9989; Santa Cruz), anti-Claudin-5 rabbit polyclonal (34-1600; Invitrogen, Darmstadt, Germany),...
Alexa Fluor® 633 goat anti-mouse (A-21050), Alexa Fluor® 488 goat anti-rabbit (A-11008), Alexa Fluor® 488 goat anti-mouse (A-110011) and rhodamine-phalloidin (R-415; Invitrogen). Staining of polymerized microtubules was performed as described previously. Images were obtained with Zeiss LSM 510 META (Zeiss, Oberkochen, Germany) confocal microscope (40x or 63x oil immersions objectives).

Impedance measurements
Changes in endothelial permeability were measured by impedance sensing using the xCELLigence Real-time-cell-analyzer (RTCA) DP device (Roche, Penzberg, Germany) at 37 °C. According to the manufacturer, the cell index (CI), which represents a dimensionless value derived from the measured electrical impedance changes, was used to judge changes of endothelial barrier function. 4 x 10^4 HMECs per well were seeded on gold electrodes and grown to confluency. At the maximum CI, normalization was performed and HMECs were treated as indicated. Impedance was measured every 10 s up to 12 h. Quantitative analysis was performed at time-point 1 h after PT treatment.

Macromolecular permeability
The assay was performed as described previously. HMECs were treated as indicated and after 30, 60, 120 and 240 min, the amount of FITC-dextran (40 kDa) in the lower compartment was determined. Mean fluorescence of samples from untreated cells at t = 4 h was defined as 1. Data are expressed as percent increase of fluorescence versus control.

Cell viability and morphology analysis
Metabolic activity was measured by the CellTiter-Blue® assay (Promega Corp., Madison, WI, USA) as described previously. HUVECs were either simultaneously treated with PT and resazurin (4 h) or were pre-treated with PT for 22 h and then resazurin was added for 4 h. Fluorescence was detected at 560 nm. Apoptosis/necrosis: Quantification of apoptosis was carried out as described by Nicoletti et al. Cells undergoing necrosis were assessed by propidium iodide-staining (PI 10 µg/ml; Sigma Aldrich). 0.01% Triton served as positive control. Apoptotic and necrotic cells were analyzed by flow cytometry (FACSCalibur, BD Bioscience, Heidelberg, Germany). Morphology: After 1 h and 24 h images of HUVECs were taken using a Canon EOS 450 D digital camera and a Zeiss-Axiovert-25 inverted microscope (10x objective; Zeiss). Afterwards HUVECs were washed and fresh ECGM was added. 24 h later images of the same wells were taken again.

Active RhoA pull-down assay
Active RhoA isolation from HUVECs was performed according to the manufacturer’s protocol (Active Rho Pull-Down and Detection Kit; Thermo Scientific, Rockford, IL, USA). In brief: Active RhoA (RhoA-GTP) was separated from the whole cell lysate by using glutathione-S-transferase (GST)-Rhotekin RhoA binding domain (RBD)-fusion proteins immobilized on GST-agarose resin. After incubation of the whole cell lysate with the agarose-bound fusion protein, active RhoA was separated by spin columns and centrifugation. The part which does not contain Rho-GTP-loaded GST-RBD-agarose was denoted as “supernatant” and served as control. After Western blot analysis, quantification was carried out using the ImageJ 1.45s software.

Mouse aortic ring assay
14 aortae prepared from 6 different female C57BL/6 mice (animal #1 and #6: 3 rings, each; animal #2: 4 rings; animal #3 and #5: 1 ring, each; animal #4: 2 rings) were embedded into Matrigel® (Schubert & Weiss-OMNILAB, Munich, Germany). Once endothelial sprouts were established, treatment followed as indicated. Images were taken using a Zeiss Axiovert 200 inverted light microscope (10x objective; Zeiss) connected to an IMAGO-QE camera (TILL Photonics, Gräfelfing, Germany) and the appending software TILLvisION 4.0.1.2. Quantification was carried out using the ImageJ 1.45s software.
Tube disruption assay
1.2 x 10⁴ HUVECs in ECGM were seeded onto Matrigel® (Schubert & Weiss-OMNILAB)-coated µ-Slides Angiogenesis (Ibidi). Endothelial tubes were allowed to be build and were then treated as indicated. Images of each well were taken with the microscopic setting described under “mouse aortic ring assay” and analyzed with the tube formation module from WIMASIS (Munich, Germany) as described previously. Drug effects were assessed at time-point 6 h after treatment.

Cytosolic calcium measurement
Ratiometric calcium imaging was carried out with Fura-2-AM-loaded cells using a static system as described previously. HUVECs were treated with 300 nM PT and data was recorded for a total period of 55 min with images being acquired every 5 s. Each data point of the different graphs was calculated from a randomly chosen rectangle containing at least 20 adjacent cells, of which mean values are expressed. To obtain a positive calcium signal, histamine (10 µM) was added at time-point 45 min and data were recorded for further 10 min.

Determining the maximum tolerated dose (MTD) of PT
Different concentrations of PT (1, 10, 30 and 50 mg/kg) were injected intravenously into the tail vein of female C57BL/6 mice. Weight was measured every day up to 8 days.

Dorsal skinfold chamber preparation and in vivo fluorescence microscopy
The experiment was carried out as described previously. 10 d after tumor cell implantation, intravitral microscopy was performed. FITC-labeled dextran (500 kDa; 0.05-0.1 ml of a 5% solution in 0.9% NaCl; Sigma, Deisenhofen, Germany) was injected intravenously (IV) as plasma marker to visualize tumor microcirculation. A total of 5 regions of interest (ROIs) per animal were randomly selected. Intravitral microscopy images were recorded on digital videotape for 30 s for subsequent off-line analysis. Directly upon FITC-dextran injection and baseline image acquisition, 10 mg/kg PT or vehicle (5% DMSO) was administered intravenously. 30, 60 and 120 min following PT/DMSO injection, the capillary red blood cell velocity (\(V_{\text{RBC}}\) in mm/s), capillary diameter (D in µm) and functional vessel density (FVD in 1/mm) defined as the total length of perfused capillaries per area were determined in each of the five ROIs per animal, again.

B16-F1 mouse melanoma tumor model
C57BL/6 mice were injected s.c. into the left hind limb with 100 µl of a 1 x 10⁶ B16-F1 mouse melanoma cell suspension. The mice were entered into experiments once tumors reached a size of approximately 650 mm³ (assessed by \(\pi/6 \times \text{width} \times \text{height} \times \text{length}\). 10 mg/kg PT or 5% DMSO were injected IV into the tail vein. 24 h after treatment, tumors were removed and halved. One half was flash frozen, the other half was fixed in 4% paraformaldehyde and paraffin-embedded. One minute before B16-F1 tumor removal, Hoechst 33342 (bisbenzimide, 10 mg/kg; Sigma-Aldrich) was injected IV into the tail vein. Cryosections of 10 µm were prepared from the frozen tumors. Endothelial cells were detected using the monoclonal rat anti-mouse CD31 antibody (553370; BD). Hoechst 33342 and CD31 were visualized with a Zeiss LSM 510 META confocal microscope (40x objective). Thirteen random fields at the tumor periphery and the tumor center of two sections per tumor were analyzed. The fluorescence intensity of Hoechst 33342 was quantified using ImageJ 1.45s software according to Grosios et al. and vessel density was determined by counting CD31-positive vessel-like structures. The repeated treatment approach was started once tumors reached a size of about 100 mm³. PT or DMSO was injected every third day up to 3 times. Daily measurement of mouse weight served as health control. Sections of 5 µm were prepared from the paraffin-embedded tumors and stained with hematoxylin and eosin (Sigma-Aldrich). Tumor histology of two different sections per tumor were analyzed under 4x magnification using the Olympus CellSens Entry software in combination with an Olympus DP25 camera, connected to an Olympus Bx41
stereomicroscope (Olympus, Munich, Germany). Quantification was evaluated by determining necrotic regions using ImageJ 1.45s software.

**Statistical analysis**
The number of independently performed experiments (n) is stated in the respective figure legend. Bar graph data are expressed as means ± SEM. Statistical analysis was performed with Prism software (version 5.04; GraphPad Software, San Diego, CA, USA). For comparison of two groups an unpaired t-test was performed. Three or more groups were compared by one-way analysis of variance (ANOVA) followed by a Tukey’s multiple comparison post-test. Statistical significance is assumed if $P \leq 0.05$. 
References


Supplemental Figure I

**Figure I. Impedance sensing.** (A,B) Changes in permeability upon PT treatment were measured using an xCELLigence™ real-time cell analyzer. HMECs were grown to confluence on gold electrodes and treated as indicated. Impedance was measured every 10 s up to 12 h. (A) One representative curve is shown. The vertical black line represents the time point of normalization. (B) Quantitative analysis was performed at time point 30 min after PT treatment. Data are expressed as mean ± SEM, n = 4. One-way ANOVA followed by Tukey's multiple comparison post-hoc test was used. *$P \leq 0.05$ vs. control (Co).
Figure II. Quantification of apoptosis. Proliferating (80 % confluent) HUVECs were treated as indicated for 24 h. After permeabilization, cells were stained with propidium iodide (PI) and analyzed by flow cytometry, thereby counting the events with subdiploid DNA content. Quantitative evaluation was performed at time point 24 h. Data are expressed as mean ± S.E.M., n = 5. One-way ANOVA followed by Tukey’s multiple comparison post-hoc test was used. *P ≤ 0.05 vs. control (Co).
Figure III. Quantification of RhoA pull-down assay. Quantitative evaluation of the RhoA pull-down assays (see Figure 3A) was performed by ImageJ 1.45s. Data are expressed as mean ± S.E.M., n = 4. One-way ANOVA followed by Tukey’s multiple comparison post-hoc test was used. *P ≤ 0.05 vs. control.
Supplemental Figure IV

Figure IV. The p38 MAPK inhibitor SB203580 inhibits the PT-induced activation of pHSP-27. Confluent HUVECs were either pre-treated with vehicle control, with SB203580 (20 µM, 1 h), with PT (30, 100, and 300 nM, 1 h) or in combination with SB203580 (30 min pre-treatment) and PT. Equal amounts of protein were analyzed by Western blotting for phospho-HSP27(Ser82) and total-HSP-27. β-tubulin was used as loading control. One representative blot out of three is shown.
Figure V. *In vivo evaluation of the MTD of PT*. Female C57BL/6 mice were treated with different concentrations of PT (1, 10, 30 and 50 mg/kg; IV injection into the tail vein). To monitor the state of health, weight was measured every day up to 8 days after PT injection. 1 and 10 mg/kg PT were well tolerated, whereas 30 and 50 mg/kg were not tolerated and the experiment had to be stopped early. Data are expressed as mean ± S.E.M (n = 4 for 1 mg/kg; n = 5 for vehicle control, 10, 30 and 50 mg/kg). The weight of control mice was normalized to 1.
Supplemental Figure VI

Figure VI. The vehicle control does not affect perfusion, neither in tumor nor in normal tissue. (A-C) DMSO (5 %) was applied as vehicle control. Quantitative analysis of five regions of interest per tumor and normal tissue were selected to investigate microcirculatory parameters over a time period of 2 h. (A) Red blood cell velocity (vRBC) [mm/s], (B) vessel diameter (D) [µm] and (C) functional vessel density (fvd) [cm/cm²]. Data are expressed as mean ± S.E.M (n = 3). One-way ANOVA followed by Tukey’s multiple comparison post-hoc test was used. *P ≤ 0.05 versus muscle tissue.