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LP2, a stable lanthipeptide derived from cAng-(1-7), exerts myeloprotective action in mice

P. Namsolleck[1,2] | K. E. Rodgers[3] | R. Franklin[4] | G. N. Moll[1,5]

[1]Lanthio Pharma, Groningen, The Netherlands

[2]PCDA Pharma Consulting and Data Analytics, Nieuw-Roden, The Netherlands

[3]Department of Pharmacology, College of Medicine, Center for Innovation in Brain Science, University of Arizona, Tucson, Arizona, USA

[4]Constant Therapeutics LLC, C/O Casner & Edwards, Boston, Massachusetts, USA

[5]Department of Molecular Genetics, Groningen Biomolecular Sciences and Biotechnology, Institute, University of Groningen, Groningen, The Netherlands

Correspondence
G. N. Moll, Molecular Genetics, Linnaeusborg 7, 9725 AG Groningen, The Netherlands.
Email: g.n.moll@rug.nl

Abstract

Objectives: Linear unstable angiotensins stimulate hematopoiesis. Here we address: (1) Is cyclic angiotensin-(1-7) myeloprotective in mice? (2) Is cyclic angiotensin-(1-7) stable in rat? (3) Does LP2, a cyclic angiotensin-(1-7) with an N-terminal D-lysine, exert myeloprotective action in tumor-bearing mice?

Materials and Methods: Cyclic angiotensin-(1-7)'s capacity to restore levels of blood platelets and white blood cells was studied in gemcitabine-treated mice. The stability of cyclic angiotensin-(1-7) in rat was measured in blood samples taken after injection or infusion. The capacity of LP2 to restore total bone marrow cell levels in mice after treatment with 5-fluoruracil was measured. In addition, the capacity of LP2 to counter anemia in tumor-bearing mice treated with erlotinib was measured.

Results: Cyclic angiotensin-(1-7) dose-dependently restored blood platelet levels in gemcitabine-treated mice, whereas its capacity to restore levels of white blood cells was less. In vivo aminoterminal breakdown of cyclic angiotensin-(1-7) yielded cyclic angiotensin-(2-7) and cyclic angiotensin-(3-7). LP2 significantly ($p < .0001$ at 100 µg/kg/day) restored bone marrow cell counts in mice after treatment with 5-fluoruracil. LP2 also significantly ($p < .05$) countered anemia in tumor-bearing mice treated with erlotinib.

Conclusions: LP2 exerts myeloprotective action with perspectives for continuation of its clinical development.

KEYWORDS
5-fluoruracil, angiotensin, bone marrow, femur, lanthionine, stem cells

Novelty statements

What is the new aspect of your work?

- Lanthionine-stabilized angiotensins exert myeloprotective activity.

What is the central finding of your work?

- Antitumor peptide LP2 is myeloprotective and in vivo more stable than cAng-(1-7).

What is (or could be) the specific clinical relevance of your work?

- LP2 may enable prolonged chemotherapy cycles in cancer treatment.

1 | INTRODUCTION

Suppression or elimination of bone marrow progenitors may occur following chemotherapy or radiation and can result in anemia, thrombocytopenia, lymphopenia, and neutropenia.[1] These conditions often require the postponing of a chemotherapy cycle and/or the use of a reduced dose. For instance, thrombocytopenia poses an increased risk of bleedings. Postponing treatment and/or reduced dosing of cancer treatment may lower the chance of complete eradication of cancer. For the treatment of thrombocytopenia in adult patients with chronic liver disease or with primary chronic immune thrombocytopenia a thrombopoietin receptor agonist is approved, Avatrombopag.[2] For managing chemotherapy-induced myelosuppression in patients with extensive-stage small cell lung cancer, Trilaciclib is an FDA approved drug.[3] The current general modality to restore platelet levels in cancer are blood transfusions, which are costly and cumbersome.

A body of data has been obtained on the recovery of bone marrow induced by the natural angiotensin-(1-7) peptide, abbreviated: Ang-(1-7), a potent stimulator of progenitor cell proliferation. Ang-(1-7) acted synergistically with neupogen to increase the number of hematopoietic progenitors in the bone marrow.[4] In a phase II study in patients with breast cancer, Ang-(1-7) enhanced recovery of platelets, lymphocytes, leukocytes, and neutrophils.[5,6] Ang-(1-7) also has antitumor activity[7] suggesting a potentially beneficial combination of myeloprotection and antitumor activity by the same peptide.

Ang-(1-7) is broken down by many peptidases among which angiotensin converting enzyme (ACE). In 2009, a C-terminally lanthionine-constrained variant of Ang-(1-7) was reported whose ex vivo vasodilating activity was fully inhibited by Mas receptor antagonist D-Pro7-Ang-(1-7) and partially by D-Ala7-Ang-(1-7).[8] This cyclic Ang-(1-7), termed: cAng-(1-7), was fully resistant to ACE and had increased resistance to breakdown by peptidases from organ homogenates. This proteolytic resistance of cAng-(1-7) raised the hypothesis of lower doses and possibly less frequent administration and expansion of the routes of administration. cAng-(1-7), without formulation, could be delivered via the lungs and to some detectable extent orally.[9] Here we investigated whether cAng-(1-7) is also myeloprotective. Subsequently we assessed the stability of the N-terminus of cAng-(1-7) in vivo and thereafter continued myeloprotection studies with a cAng-(1-7) variant whose N-terminus is protected by an additional D-lysine.[10,11]

2 | MATERIALS AND METHODS

2.1 | Peptides

cAng-(1-7) was prepared by base-assisted sulfur extrusion of DRVdCIHC in which dC is a D-cysteine, as previously.[12] Reference peptides, cAng-(2-7) and cAng-(3-7), were obtained via N-terminal proteolytic degradation of cAng-(1-7) followed by purification by HPLC. LP2 has the sequence dKDRV[dAIHA]$_s$ in which dK is a D-lysine, dA a D-alanine and [dA···A]$_s$ a D-, L-lanthionine composed of two alanines with a thioether bridge (Figure S1B,C). LP2 was obtained via base-assisted sulfur extrusion of a D, L disulfide bridged precursor peptide, dKDRVdCIHC in which dK is a D-lysine and in which dC is a D-cysteine.[11]

2.2 | Evaluation of peptide-mediated myeloprotection after gemcitabine treatment

Female C57Bl/6 mice, 6–8 weeks old, $n = 7$ per treatment arm, received intravenous injection of 160 mg/kg gemcitabine and vehicle (saline) or 3 μg/kg/day cAng-(1-7), 10 μg/kg/day cAng-(1-7), 30 μg/kg/day cAng-(1-7) or 600 μg/kg/day of Ang-(1-7). On days −1, 3, 8, 15 and 21 after chemotherapy administration, blood was taken under anesthesia from the saphenous vein to assess platelets and white blood cell numbers. The mice were anesthetized by placing the nose of the animals in a 50 mL conical tube containing cotton ball soaked with isoflurane. Once anesthesia was accomplished, the animals were taken from the tube and held firmly. Approximately 50 μL of blood was obtained from the saphenous vein with a microfuge tube with a capillary end containing EDTA followed by mixing. The blood was held on ice until further processing and cell counting.

2.3 | Metabolism of cAng-(1-7) in rat

Two studies on the in vivo metabolism of cAng-(1-7) were performed by Pharmidex, UK. The first study, involving three Sprague Dawley rats was performed as follows. On the afternoon of the day prior to the start, rats were transferred to metabolism cages. Metabolic cages destined for rats >250 g were used wherein rats had free access to food and water. Cages were placed at $t = 0$ above dry ice for collection of urine samples. On the next morning, after a 30 min stabilization period with 2.5 mL/kg/h 0.9% NaCl, at $t = 0$, cAng-(1-7) in a 1 mg/mL solution was infused via the femoral vein at a rate of 2.5 mL/kg/h for 60 min, while taking blood samples at zero time, at 30 min and at 60 min, to give a total dose of 2.5 mg/kg. At time $t = 60$ min infusion was stopped but taking of serial blood samples (300 μL) was continued; subsequent samples were taken at 62, 65, 70 min and at 80 min. Blood samples at 0 min, and, post IV infusion initiation, at 30, 60 min, and, after arresting the infusion, at 62, 65, 70, 80 min were taken from the jugular vein cannula from each animal. Blood samples from the jugular vein cannulation of individual animals was delivered into labeled polypropylene tubes containing anticoagulant (heparin) and held on wet ice for a maximum of 30 min. The blood samples were centrifuged for plasma (4°C, 21 100 *g* for 5 min) and the resulting plasma transferred into labeled safe-lock Eppendorf 1.5 mL clear tubes. Urine samples were collected at $t = 24$ h after initiation of IV infusion. Number of urine samples: 4 including 1 from a control rat that is not infused. All plasma, urine and dosing solutions samples were maintained at −80°C until analysis by mass spec.

In the second experiment, which also involved 3 Sprague Dawley rats, instead of continuous infusion, a bolus of 0.8 mg cAng-(1-7)/rat

was subcutaneously administered. Blood samples were taken at the following time points: 0.03, 0.08, 0.17, 0.25, 0.50, 1.0, and 2.0 h. This second experiment was performed essentially as the first experiment. Urine samples were collected at $t = 24$ h after the s.c. bolus administration.

2.4 | Myeloprotection by LP2

Male C57BL/6 mice, 6–8 per group, were used. On day 0 either vehicle (saline) or 5-fluoruracil (5-FU) at a dose of 150 mg/kg was administered to mice, followed by subcutaneous injection of either G-CSF (5 μg/kg/day) or LP2 (10 μg/kg/day), or LP2 (30 μg/kg/day) or LP2 (100 μg/kg/day) until D14 at which day femur was isolated and total cell counts were made.

2.5 | Anemia in erlotinib-treated tumor-bearing mice

Studies with mice that bear patient-derived xenografts of colorectal carcinoma were done in accordance with the United Kingdom Coordinating Committee on Cancer Research regulations for the Welfare of Animals[13] and of the German Animal Protection Law and approved by the local responsible authorities, Berlin, Germany (Approval No. A 0010/19, LaGeSo Berlin, Germany). From arrival onwards, the animals were group-housed in individually ventilated cages (type GM 500) and placed in a TouchSLIMPlustm air handling unit (Techniplast S.p.A., Italy) providing HEPA filtered air. The animals were kept with a piece of gnaw wood and paper rolls (Enviro-dri) as environmental enrichment. Drinking water and autoclaved cereal-based commercial rodent VRF1 diet were provided ad libitum from the arrival of the mice until the end of the study. The NMRI *nu/nu* female mice at age 6–8 weeks were inoculated with PDX tumor subcutaneously and the tumor was grown to reach palpable tumor size before any treatment was initiated. Allocation of animals to the treatment arms allowed uniform distribution of initial tumor volume in both, placebo and verum groups. LP2 was dissolved in sterile PBS while PBS alone served as placebo (vehicle). Animals were treated with 50 mg/kg/day p.o. erlotinib or 0.2 μg/kg/day s.c. LP2 for maximally 28 days. Thereafter, blood samples were taken, and the hematocrit value and hemoglobin content were measured.

3 | RESULTS

The recovery of platelets was enhanced by Ang-(1-7) (Figure S2A,B). cAng-(1-7) clearly induced dose-dependent recovery of platelets with the dose of 30 μg/kg/day of cAng-(1-7) being nearly as effective as the dose of 600 μg/kg/day for Ang-(1-7). With respect to white blood cell recovery cAng-(1-7) did not appear to have any effect at the two lowest doses of 3 μg/kg/day and 10 μg/kg/day (Figure S3A,B). At 30 μg/kg/day, cAng-(1-7) appeared to induce white blood cell recovery but the effect was less and delayed compared to the effect of Ang-(1-7) at 600 μg/kg/day.

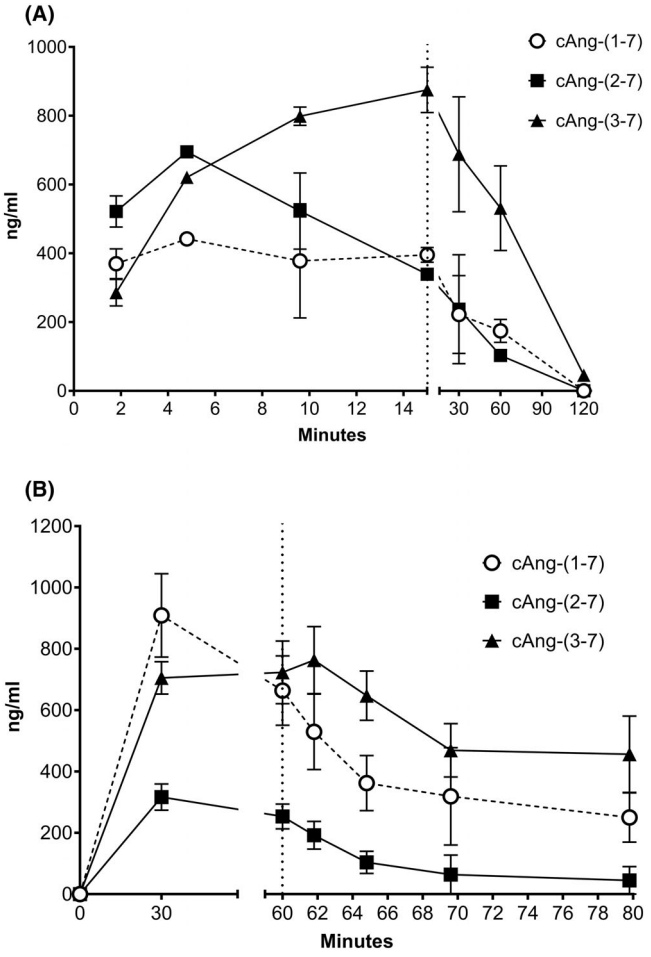

FIGURE 1 (A,B) Cyclic Ang-(1-7) is broken down in rat to cAng-(2-7) and cAng-(3-7). (A) Cyclic Ang-(1-7) is subcutaneously administered as a bolus at 0.8 mg/kg. At the indicated time points blood samples are taken and analyzed for the three different peptides. (B) Cyclic Ang-(1-7) at a dose of 2 mg/kg/h is continuously intravenously infused until $t = 60$ min. At the indicated time points blood samples are taken and analyzed for the three different peptides.

To investigate whether the above effects could be assigned to unmetabolized cAng-(1-7) itself or whether bioactive breakdown products of cAng-(1-7) should be considered, the in vivo fate of cAng-(1-7) was further studied in rats. cAng-(1-7) was subcutaneously administered as a bolus injection (Figure 1A). Alternatively, cAng-(1-7) was continuously intravenously infused in rats until $t = 60$ min (Figure 1B). In the latter case, infusion was stopped at $t = 60$ min while taking samples was continued until $t = 80$ min. Both experiments clearly demonstrated that already at the first time points, $t = 2$ min of the bolus experiment and $t = 30$ min for the infusion study, cAng-(2-7) and cAng-(3-7) were formed. At $t = 2$ min in the bolus study cAng-(2-7) was already present at higher level than cAng-(1-7). cAng-(3-7) was rapidly the predominant breakdown product. In urine after 24 h a low concentration of cAng-(1-7) was measured, cAng-(2-7) was below the limit of quantification, whereas cAng-(3-7)

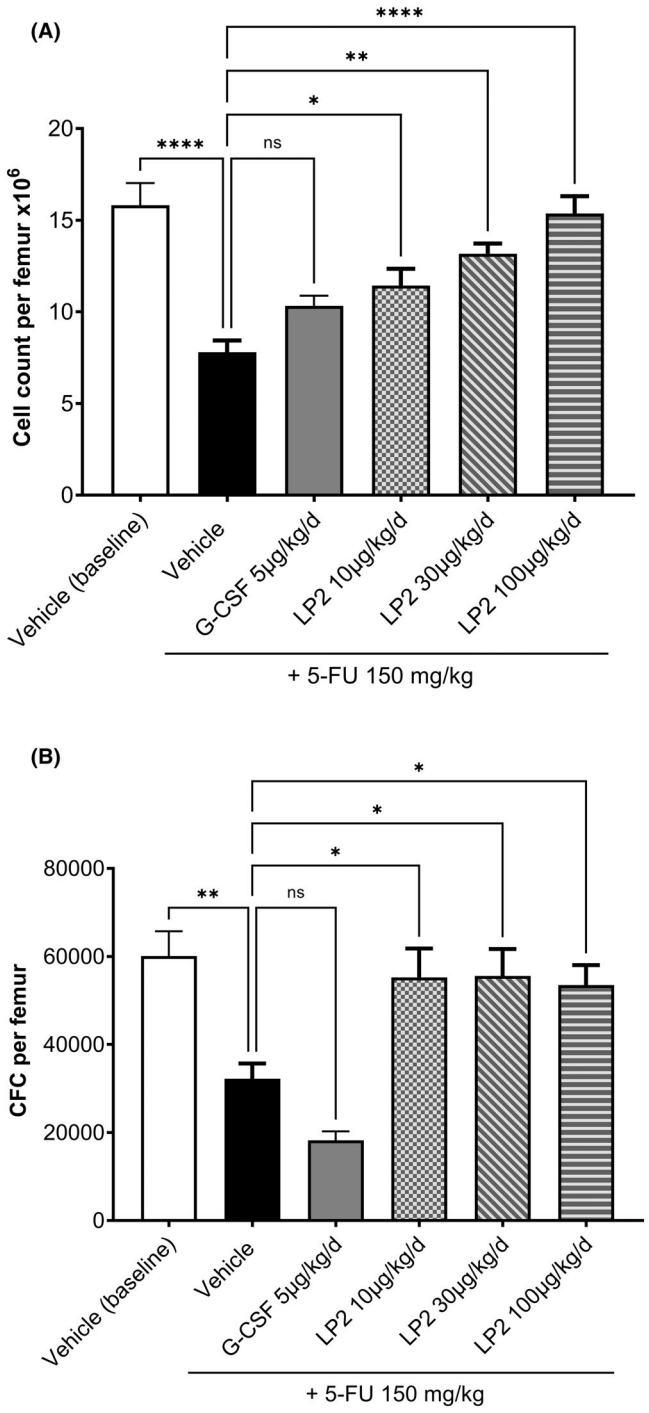

FIGURE 2 (A,B) Myeloprotection by LP2. Male C57BL/6 mice, 6–8 per group, received on day 0 either vehicle (saline) or 5-fluoruracil (5-FU) at a dose of 150 mg/kg, followed by subcutaneous injection of a low dose of G-CSF (5 µg/kg/day) or LP2 (10 µg/kg/day), or LP2 (30 µg/kg/day) or LP2 (100 µg/kg/day) until D14 at which day femur was isolated and cell counts were made. A: cell cunt per femur $\times$ 10^6; (B) colony forming units per femur. $n = 5–8$. One-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test; *$p < .05$; **$p < .01$; ***$p < .001$; ****$p < .0001$.

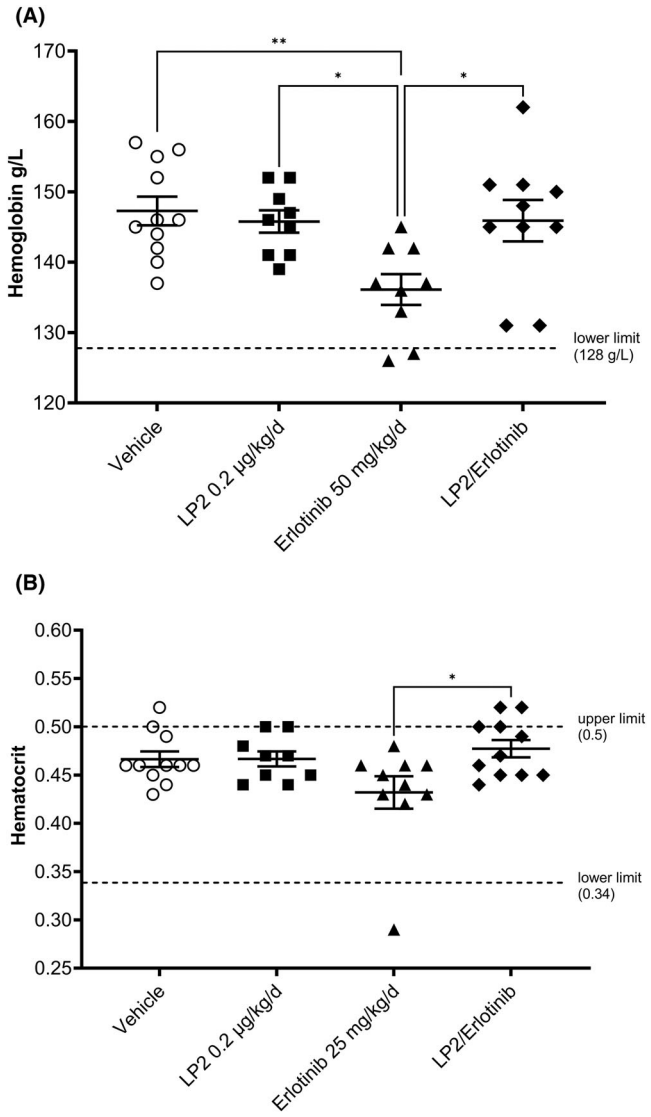

FIGURE 3 (A,B). LP2 protects against erlotinib-induced anemia. NMRI *nu/nu* female mice at age 6–8 weeks were inoculated with PDX of CRC tumor subcutaneously and the tumor was grown to reach palpable tumor size before any treatment was initiated. Erlotinib was administered at 50 mg/kg/day. Animals were treated once daily, either with erlotinib at 50 mg/kg/day, with LP2 at 0.2 µg/kg/day or their combination for maximal 28 days. Thereafter, blood samples were taken and the hemoglobin content (A), and hematocrit value (B) were measured. $N = 9–11$. One-way analysis of variance (ANOVA) with Tukey's multiple comparisons test; *$p < .05$; **$p < .01$.

had the highest concentration (Table S1). The data are consistent with (amino)peptidase-mediated stepwise formation of cAng-(3-7) via cAng-(2-7), although, in view of the absence of quantifiable cAng-(2-7) in urine, direct formation of cAng-(3-7) from cAng-(1-7) by trypsin-like activity cleaving after the arginine may also take place. Usually, a lanthionine ring protects the potential cleavage sites within the ring and one or two potential cleavage sites next to the lanthionine ring. However, at higher concentration also cleavage can occur directly next to a ring; for instance, trypsin at elevated dose can cleave off nisin's rings AB from rings CDE. Here, the lanthionine ring from position 4 to 7 in cAng-(1-7) protected cAng-(3-7) from further degradation. For native Ang-(1-7) conversion of Asp1 to Ala1 has been observed yielding a peptide termed alamandine.[14] No such conversion

of the amino acid at position 1 was observed for cAng-(1-7). In conclusion, cAng-(1-7) is rapidly degraded to cAng-(2-7) and cAng-(3-7), each with potential bioactivities. Consequently, the myeloprotective effects after administration of cAng-(1-7) cannot yet be assigned to any of (the combinations of) cAng-(1-7), cAng-(2-7) or cAng-(3-7).

In view of its stability, subsequent studies were performed with LP2, which is composed of cyclic angiotensin-(1-7) with an N-terminal D-lysine. D-Amino acids locally protect peptides against breakdown by peptidases. The D-lysine at the N-terminus thus protects the N-terminus of LP2, whereas the lanthionine ring protects the C-terminus. LP2 has a half-life in man of 2.1–2.6 h. After administration by subcutaneous injection, 66%–85% of the administered LP2 is found back intact in human urine.[11] This means that any effect induced by LP2 can be readily assigned to LP2. Furthermore, its stability may potentially allow lower doses, less frequent administration and facilitate other routes of administration.

In a next experiment myelosuppression was induced by 5-FU and protective effects on bone marrow cells by three doses of LP2 were measured. Figure 2A shows that LP2 dose-dependently protects against 5-FU-induced myelosuppression measured as cells per femur, whereas Figure 2B shows that at all doses LP2 protected the number of colony-forming cells (all cells) per femur.

Subsequently, protective effects of LP2 on anemia in erlotinib-treated, colorectal-tumor-bearing mice were investigated. Most tyrosine kinase inhibitors, like erlotinib, cause unwanted hematologic side effects like anemia, thrombopenia and neutropenia. Figure 3A shows that LP2 significantly protects the hemoglobin level in erlotinib-treated, tumor-bearing mice. Similarly, LP2 protects the hematocrit value in erlotinib-treated, tumor-bearing mice (Figure 3B).

4 | DISCUSSION

A vast amount of preclinical and clinical data convincingly demonstrates the multilineage myeloprotective effect of the labile endogenous angiotensin-(1-7). This study started with cyclic angiotensin-(1-7), an analog of angiotensin-(1-7) in which Tyr 4 and Pro7 have been replaced by a stabilizing lanthionine (dAla4-s-Ala7, in which dAla is a D-alanine). Previous studies showed that ex vivo vasodilating activity of cAng-(1-7) could be inhibited by Mas receptor antagonists D-Pro7-Ang-(1-7) and to a lesser extent D-Ala7-Ang-(1-7).[8] cAng-(1-7) did not act via AT_1R, AT_2R or MrgD (unpublished data Lanthio Pharma). Its lanthionine confers resistance to ACE and several other peptidases. A previous study on cAng-(1-7)-mediated therapeutic action in a rat model of cerebral stroke hypothesized that cAng-(1-7) could stimulate the formation and migration of stem cells to the injured site.[15] Here we found that cAng-(1-7) stimulated the formation of platelets and, consistent with its increased stability compared to native angiotensin-(1-7),[8] was effective at a lower dose than the native linear angiotensin-(1-7). cAng-(1-7) was, however, less effective than native angiotensin-(1-7) in stimulating the formation of white blood cells. As constrained peptides usually have enhanced receptor specificity it is tempting to speculate that the lower capacity of stimulating white blood cells, might result from enhanced receptor specificity of cAng-(1-7) compared to native Ang-(1-7). Alternatively, both peptides might interact with the same receptor but in a different way.

Previous studies demonstrated that cAng-(1-7) was resistant to ACE and more resistant, but not fully resistant, to breakdown by peptidases in peptidase-rich organ homogenates.[8,9] Here we found that cAng-(1-7) is rapidly broken down via N-terminal degradation yielding cAng-(2-7) and cAng-(3-7). Assessing the activity of each of cAng-(1-7) and cAng-(2-7) is complicated by the rapid conversion of cAng-(1-7) to cAng-(2-7) and by their conversions to cAng-(3-7).

In view of the above findings on N-terminal breakdown of cAng-(1-7), we continued with LP2, an AT_2R agonist[16] which is structurally equivalent to cAng-(1-7) extended with an N-terminal D-lysine which protects against N-terminal breakdown. Indeed, LP2 is highly stable in man and is even largely found back in an intact form in urine.[11] LP2 has a half-life in man of 2.1–2.6 h. After administration by subcutaneous injection, 66%–85% of the administered LP2 is found back intact in human urine. Preferential stimulation of the AT2 receptor would direct the hemangioblast toward the hematopoietic lineage.[17] Such mechanism would suggest that LP2 could be myeloprotective also in case of myelosuppression induced by other drugs than 5-fluoruracil.

In tumor-bearing mice treated with erlotinib, LP2 protected against anemia. Previous studies showed LP2-mediated inhibition of growth of patient-derived xenografts in mice of lung, and colorectal tumors and of cell-derived xenografts of glioblastoma.[18] The AT_2R agonist LP2, which is daily subcutaneously injected, differs in many ways from the myeloprotective drug Trilaciclib, a cyclin-dependent kinase 4/6 inhibitor, which is intravenously infused prior to chemotherapy.[19] Trilaciclib induces a transient, reversible G1 cell cycle arrest of proliferating hematopoietic stem and progenitor cells in bone marrow, thus protecting them from damage during chemotherapy. Its myeloprotection has been demonstrated in clinical studies for small cell lung cancer.[20–22] Trilaciclib effectively protects against neutropenia[21] and enhances the antitumor activity of chemotherapy and immune checkpoint inhibitor combinations.[23] LP2 shows synergistic antitumor effects with erlotinob and 5-fluoruracil.[24] Dosing levels are several orders of magnitude higher, in the mg/kg range, for Trilaciclib[23] than for LP2 which is dosed in the µg/kg range. Trilaciclib causes some side effects[3]; whereas—thus far—no side effects of LP2 have been observed. LP2 has shown excellent safety and pharmacokinetics in preclinical animal studies and in clinical phase Ia in healthy volunteers.[11] A next clinical trial with LP2 in cancer patients might allow the assessment of myeloprotective action of LP2 in man. The here demonstrated myeloprotective action of the antitumor peptide LP2 points to further clinical development of LP2.

AUTHOR CONTRIBUTIONS

All authors contributed to the design of experiments. P. Namsolleck and G. N. Moll wrote the manuscript.

CONFLICT OF INTEREST STATEMENT

PN, KER and RF have no conflict of interest. GNM is owner of Lanthio Holding.

DATA AVAILABILITY STATEMENT

The data from this study are readily available after reasonable request to the corresponding author.

ORCID

K. E. Rodgers https://orcid.org/0000-0002-4590-7728
G. N. Moll https://orcid.org/0000-0001-5762-5667

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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