Bioorganic & Medicinal Chemistry Letters 26 (2016) 5267–5271

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Structure–activity relationship of cyclic pentapeptide malformins as fibrinolysis enhancers

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ARTICLE INFO

Article history:
Received 26 August 2016
Revised 14 September 2016
Accepted 16 September 2016
Available online 16 September 2016

Keywords:
Malformin
Disulfide bond
Cyclic peptide
Fibrinolysis
Solid-phase peptide synthesis
Cytotoxicity

ABSTRACT

The formation of blood clots in blood vessels causes severe ischemic diseases such as cerebral infarction and myocardial infarction. While searching for microbial products that increase fibrinolytic activity using an in vitro fibrin degradation assay, we found malformin A1, a disulfide form of *cyclo*(–D-Cys-D-Cys-L-Val-D-Leu-L-Ile–), as an active compound. In this study, we synthesized malformin derivatives using a solid-phase peptide synthesis method and evaluated their fibrinolytic activity and cytotoxicity. Reduction of the disulfide bond and linearization of the cyclic peptide frame decreased the pro-fibrinolytic activity. Substitution of a branched-chain amino acid with lysine resulted in loss of activity. However, protection of the amino group in the lysine derivatives by the *tert*-butoxycarbonyl (Boc) group rescued the inactivity. Furthermore, the phenylalanine derivatives also exhibited a similar pro-fibrinolytic effect compared to malformin A1. These results suggest that the disulfide bond, the cyclic peptide frame, and the bulky hydrophobic side chains play a crucial role in the pro-fibrinolytic activity of malformin. The effective dose of the active derivatives for the in vitro fibrin degradation showed similar ranges (1–5 μM), while the order of cytotoxic potency for the active derivatives was as follows: Phe-derivatives > BocLys-derivatives > malformin A1 > reduced form. These results showed no correlation between pro-fibrinolytic activity and cytotoxicity, suggesting the possibility of the synthesis for non-toxic malformin derivatives possessing the activity.

Irregular thrombus formation causes severe ischemic diseases such as stroke or heart attack. Antithrombotic therapies are categorized into three groups: anticoagulants, antiplatelet agents, and thrombolytics. A thrombolytic therapy induces the conversion of proenzyme plasminogen, a proenzyme, to plasmin, a fibrinolytic enzyme, which in turn leads to the degradation of insoluble fibrin, the main component of thrombus. Although thrombolytic enzymes, such as tissue-type plasminogen activator (tPA) or urokinase-type plasminogen activator (uPA), are available for the treatment of thrombotic disorders, a low molecular weight compound that can enhance fibrinolytic activity is an unmet medical requirement for thrombolytic therapies. In particular, oral drugs that can reduce the formation of fibrin clots would be beneficial in preventing thrombotic disorders.

While searching for natural products that could increase fibrinolytic activity, we found malformin A1 (MA1) **1**, a disulfide form of *cyclo*(–D-Cys-D-Cys-L-Val-D-Leu-L-Ile–), as an active compound (Fig. 1A).[1] **1** was originally identified as a fungal metabolite that induces curvature and malformations in bean plants, and curvatures in corn roots.[2–5] Thereafter, the various biological activities of **1**, such as anti-bacterial activity,[6] anti-tobacco mosaic virus activity,[7] anti-protozoan activity against *Plasmodium* and *Trypanosoma*,[8] anti-proliferative activity against cancer cell lines,[9,10] and suppression of interleukin 1β-induced tissue factor expression,[11] have been reported. Furthermore, several types of natural analogs of **1** in which D-Leu or L-Ile was substituted with another branched-chain amino acid are known.[12,13] Among them, malformin C, also termed malformin A3 or malformin B1b, in

Abbreviations: tPA, tissue type plasminogen activator; uPA, urokinase type plasminogen activator; DIPEA, *N*,*N*-diisopropylethylamine; DCM, dichloromethane; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DMF, *N*,*N*-dimethylformamide; PyBop, benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate; DIPCI, *N*,*N*′-diisopropylcarbodiimide; HOBt, hydroxybenzotriazole; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; HATU, *O*-(7-aza-1*H*-benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; TIPS, triisopropylsilane; DBF, dibenzofulvene; Fmoc, 9-fluorenylmethyloxycarbonyl; Trt, trityl; Boc, *tert*-butoxycarbonyl; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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http://dx.doi.org/10.1016/j.bmcl.2016.09.045
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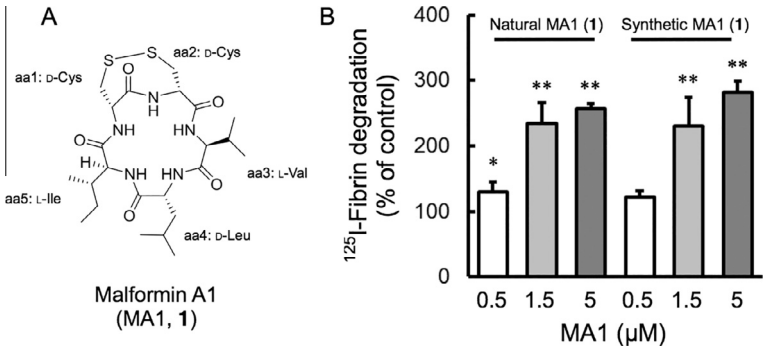
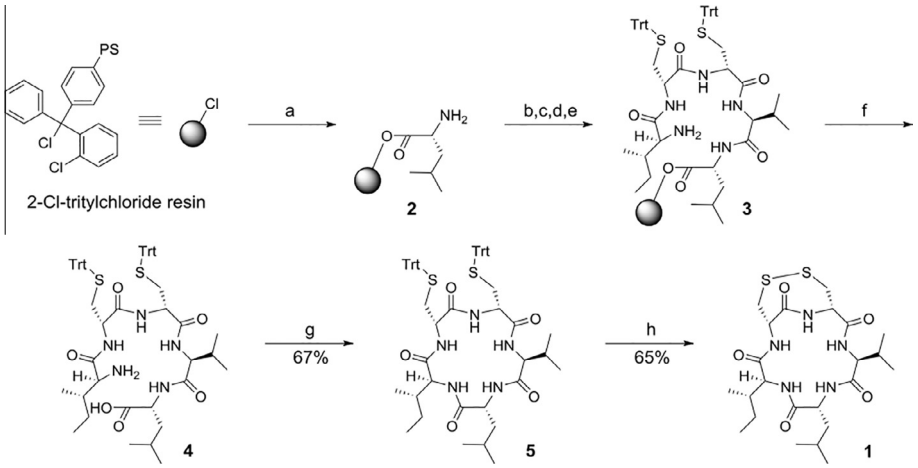


Figure 1. Malformin A1. (A) Structure of malformin A1 (**1**). (B) Effects of natural and synthetic malformin A1 (**1**) on an in vitro fibrin degradation assay. U937 cells in RPMI-1640 medium supplemented with human platelet-poor plasma were added to each well of the ^{125}I-fibrin-coated 96-well plate. After incubation for 3 h with malformin derivatives at the indicated concentration, ^{125}I-fibrin degradation products released into the supernatant were quantified using a γ-counter. Data are presented as means ± SD (n = 3). $^{*}p$ <0.05, $^{**}p$ <0.01, versus control.



Scheme 1. Solid-phase synthesis of malformin A1 (**1**). Reagents and conditions: (a) (i) Fmoc-D-Leu-OH, DIPEA, DCM, rt, 1 h; (ii) 2% DBU/DMF, rt, 5 min × 3; (b) (i) Fmoc-L-Val-OH, PyBOP, DIPEA, DCM/DMF (4:1), rt, 1 h; (ii) 2% DBU/DMF, rt, 5 min × 3; (c) (i) Fmoc-D-Cys(*S*-Trt)-OH, DIPCI, HOBt, DCM/DMF (4/1), rt, 1 h; (ii) 2% DBU/DMF, rt, 5 min × 3; (d) (i) Fmoc-D-Cys(*S*-Trt)-OH, DIPCI, HOBt, DCM/DMF (4:1), rt, 1 h; (ii) 2% DBU/DMF, rt, 5 min × 3; (e) (i) Fmoc-L-Ile-OH, PyBOP, DIPEA, DCM/DMF (4:1), rt, 1 h; (ii) 2% DBU/DMF, rt, 5 min × 3; (f) 20% HFIP/DMF, rt, 30 min; (g) HATU, DIPEA, DMF (0.01 M), rt, 3 h; (h) I_2, DMF, rt, 30 min. DIPEA = *N*,*N*-diisopropylethylamine, DCM = dichloromethane, DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene, DMF = *N*,*N*-dimethylformamide, PyBop = benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate, DIPCI = *N*,*N*-diisopropylcarbodiimide, HOBt = 1-hydroxy-1*H*-benzotriazole, HFIP = 1,1,1,3,3,3-hexafluoro-2-propanol, HATU = *O*-(7-aza-1*H*-benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate.

which the L-Ile in **1** has been substituted with L-Leu showed a number of biological activities.[8,14–18]

The mode of action of **1** in enhancing the fibrinolytic activity involves the plasminogen/uPA system, and depends on the coordinated action of vitronectin as a plasma component and an unknown mechanism in monocytoid cells.[1,19] In this study, for the purpose of increasing pro-fibrinolytic activity, decreasing cytotoxicity, and designing a chemical probe to identify the molecular target(s) of **1**, we synthesized malformin derivatives using a solid-phase peptide synthesis method and evaluated the efficacy of these derivatives on in vitro fibrin degradation and cytotoxicity.

Syntheses of malformin and its analogs have already been reported.[5,8,20] Ōmura's group reported an elegant route to malformin C synthesis via resin-bound macrolactamization and disulfide bond formation together with cleavage from the resin.[8] However, the yield of the cyclic peptide was relatively low, because of undesired interactions with the resin in the final oxidation step. Thus, our synthetic strategy involved a solid-phase peptide synthesis of a linear precursor, followed by macrolactamization, and an oxidative disulfide formation in liquid-phase synthesis. Synthesis of **1** is shown in Scheme 1. Fmoc-D-Leu-OH was anchored to a 2-chlorotrityl chloride resin and the Fmoc group was removed by treatment with 2% DBU/DMF to produce a resin-anchored amino acid **2**. The loading yield was estimated at 97% by determining the quantity of dibenzofulvene generated from the Fmoc group.[21] The remaining four amino acids were introduced to prepare a linear pentapeptide **3**. Coupling of Fmoc-L-Val-OH and Fmoc-L-Ile-OH were accomplished using PyBop and DIPEA. For the coupling of Fmoc-D-Cys(*S*-Trt)-OH, DIPCI and HOBt were used.[22] Cleavage from the 2-chlorotrityl resin with 20% HFIP/DCM released linear peptide **4**,[23] which was cyclized using HATU under high dilution conditions (0.01 M) to produce the cyclic peptide **5** in 67% yield. Finally, the cyclic peptide was subjected to an iodine-induced oxidative disulfide bond formation to produce **1** in 65% yield. The ^{1}H and ^{13}C NMR spectra, optical rotation, IR, and high-resolution MS were in complete accord with those of the natural product. New malformin derivatives substituted with various L- and D-amino acids were prepared by the same synthetic process (Table 1).

Evaluation of the effects of synthesized malformin derivatives on fibrinolytic activity was performed using an in vitro fibrin degradation assay system.[1,19] Synthetic **1** caused an enhanced fibrin degradation in the same dose-dependent manner as natural **1**, indicating that the synthesis of **1** has been successful (Fig. 1B).