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Development of New Peptidomimetic NADPH Oxidase Inhibitors with Antithrombotic Properties

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The ability of synthetic peptides inhibitors of NOX1 to effectively block the production of ROS by the enzyme was studied with different methodologies. Specifically, taking advantage of our understanding of the active epitope of the regulatory NOX1 subunit NOXA1 as a potent inhibitor of NOX1-derived $O_2^{\bullet-}$ formation, a panel of peptidomimetic derivatives of this peptide were designed and synthesized with the aim of improving their activity and increasing their stability in plasma. The results highlighted that improved efficacy and potency was found for both the peptide-peptoid hybrid **GS2**, whereas stapled peptide **AC5** and its precursor showed higher stability despite lower biological potency. This study showed that minimal structural modifications of NOXA1 peptides are required to improve both their potency and stability to finally achieve best candidates as new potential anti-thrombotic drugs.

Introduction

Cardiovascular diseases are the main cause of death in the world with thrombosis among the most frequent. They affect arteries and veins in different organs, and the various risk factors are linked to lifestyle, but also to the presence of other pathologies, such as diabetes and hypertension. NADPH oxidase (NOX) enzymes are major sources of reactive oxygen species (ROS) and are involved in various physiological and pathological processes such as immunity, inflammation, atherosclerosis, diabetic nephropathy, and cancer.[1–4] Precisely, due to the involvement of NOX and ROS in the formation of thrombi, attempts have been made to find drugs capable of selectively inhibiting these enzymes.[5–8] Based on the homology of structure of NOXs and activation mechanisms within the NOX family, a sequence spanning amino acids 190–210 in the activating subunit of NOX1. NOXA1 was identified with homology for the active epitope of p67-phox, an activating subunit of NOX2.[9–10] A synthetic peptide corresponding to this domain of NOXA1 has been described as a selective inhibitor of NOX1.[11] Residues 195–205 of NOXA1 were found possessing a similar function to that of the activation domain of p67-phox, and in turn, it has been shown that this peptide serves as a competitive inhibitor of the NOXA1-NOX1 binding and corresponding enzymatic activation of the NOX1 complex.[11] As one of the main problems with peptides is that they are quickly degraded by peptidases, making them difficult to use as drugs in their original structure,[12] in this study we designed and tested peptidomimetics corresponding to $NOXA1_{195–205}$, which aimed to improve both peptide potency and stability in plasma. The newly synthesized peptidomimetics were subsequently evaluated with platelet aggregometry and then EPR (electron paramagnetic resonance) experiments to evaluate the effect of these compounds in inhibiting platelet aggregation and the consequent production of ROS, in particular $O_2^{\bullet-}$.

Results

Design and Synthesis of Peptidomimetics

Peptidomimetics were designed starting from the EPVDFLGKAKV sequence of the NOXA1 active epitope binding to NOX1 and introducing modifications to the sequence through different approaches, by introducing *N*-methyl amino acids (methyl-scanning), generating a peptide-peptoid hybrid, and synthesizing a stapled peptide in analogy to recent results by our group,[13] which was evaluated together with its non-cyclic precursor. Stapled peptides are defined as a peptide that has a covalent bond between two amino acid side chains, thus forming a peptide macrocycle.[14] This modification is generally carried out to increase the stability of the peptide and prevent it from being quickly degraded by plasma proteases.

All the peptides were synthesized using a microwave-assisted solid-phase synthesis protocol.[15,16] The peptide corresponding to the NOXA1 active epitope (aa 195–205) was

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synthesized as the reference compound and is indicated throughout the manuscript as peptide **AC1** (Table 1). Briefly, the peptides were synthesized using a ChemMatrix Rink Amide resin, with a 0.5 mmol/g loading capacity. DIC/Oxyma[17] was used as the activating mixture for coupling Fmoc-amino acids, and morpholine was used as a Fmoc deblocking agent. 95% TFA containing water and TIPS as scavengers, each in 2.5% amount was used as the cleaving mixture. Peptides were precipitated out of cold diethyl ether and purified by reverse-phase HPLC and characterized by analytical HPLC and ESI-MS.

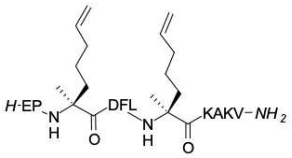
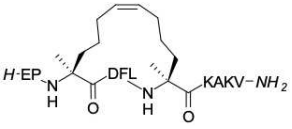
In view of studying the effect of local peptide modifications of the parent epitope EPVDFLGKAKV (NOXA1 peptide), we reasoned to focus our attention to valine and glycine residues to evaluate the effect of methylation on the potential of hydrogen-bonding interactions and of peptide stapling to stabilize a helical conformation between *i* and *i*+4 residues. In addition, the study of the central segment of the NOXA1 peptide epitope within the central phenylalanine-leucine dipeptide moiety (FL) was carried out to evaluate the functional profile and stability of an engineered peptide-peptoid hybrid capable to modulate hydrogen-bonding capability and conformational flexibility (**GS2**). Accordingly, compounds **AC2** and **AC3** were designed and synthesized to study the effect of *N*-methylation at different positions of the reference NOXA1 peptide. In particular, **AC2** possessed the *N*-methyl-glycine and **AC3** possessed both glycine and valine as *N*-methylated amino acids. The stapling between valine to glycine was achieved using ring-closing metathesis (RCM)[18] on solid-phase. To allow for peptide stapling via RCM, the two amino acids valine and glycine were replaced with the quaternary amino acid Fmoc-(*S*)-2-(4-pentenyl)Ala-OH Accordingly, **AC4** was synthesised as the reference linear precursor and **AC5** as the corresponding stapled peptide. The metathesis was carried out on the fully protected resin-bound peptide using 1st generation Grubbs catalyst (15 mg for 0.05 mmol resin-bound peptide) in 1,2-dichloroethane. After final Fmoc deprotection and peptide cleavage, subsequent purification by semi-preparative HPLC allowed to obtain **AC5** in 13% overall yield. The achievement of the ring closing metathesis was confirmed by ESI-MS and HPLC analysis (Supporting Information). Finally, the peptide-peptoid hybrid **GS2** was achieved on solid-phase using the submonomer approach for the peptoid fragment corresponding to pFpL (Table 1, entry 6).[19] Specifically, GKAKV was synthesized using standard SPPS (solid-phase peptide synthesis), then bromoacetic was coupled to free resin-bound *N*-terminal peptide fragment using DIC/Oxyma (2 M in DMF) as the activating mixture under microwave irradiation for 2 min at 40 °C. After resin washings, isobutylamine was used as 1 M DMF solution for the S_N2 reaction under microwave irradiation for 1'30'' at 80 °C to finalize the insertion of pL as the pseudo-Leu peptoid monomer. Similarly, bromoacetic acid and (*S*)-phenyl-ethyl-amine were used to install the pF peptoid monomer. Then, the compound was completed adding the EPVD sequence via standard SPPS.

Functional Inhibition of Platelets: Aggregation Assay

To evaluate how different peptidomimetics influence the ability of platelets to aggregate, the platelet aggregometry technique was used. To this end, using the aggregometer it was possible to monitor the progress of platelet aggregation for the different concentrations of each compound. DMSO (dimethyl sulfoxide) was used as a control at a concentration of 10 μM, while peptidomimetics were used at concentrations of 10, 3 and 1 μM.

Aggregation curves of the starting NOXA1 derived peptide **AC1** were obtained by aggregometer by measuring the OD signal (Figure S1, Supporting Information). The % aggregation of platelets with DMSO activated with collagen (10 μg/mL, Figure 1, left) and thrombin (0.1 U/mL, Figure 1, right) was determined with collagen and thrombin incubated with **AC1** at

Table 1. Structure and reaction data of NOXA1-derived peptidomimetics.

Entry	Sequence/structure	Overall yield, %	Purity, %
AC1	*H*-EPVDFLGKAKV-NH_2	27	90
AC2	*H*-EPVDFL(Me)GKAKV-NH_2 [a]	23	100
AC3	*H*-EP(Me)VDFL(Me)GKAKV-NH_2 [a,b]	23	100
AC4		14	98
AC5		13	100
GS2	*H*-EPVDpFpLGKAKV-NH_2 [c]	21	93

[a] (Me)G = N-methyl-Gly. [b] (Me)V = N-methyl-Val. [c] pL = *N*-isobutyl-Gly; pF = *N*-[(*S*)-1-phenylethyl]-Gly.

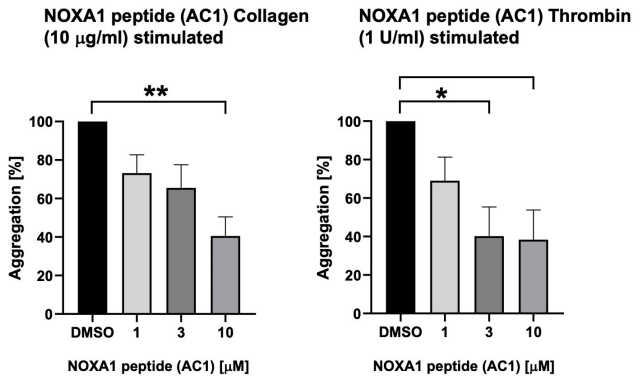


Figure 1. Left: Aggregation of reference NOXA1 peptide AC1 at three different concentrations after activation with collagen; right: aggregation of AC1 at three different concentrations after activation with thrombin. Each experiment was repeated three independent times (n = 3), with mean ± SEM shown in the graphs. The statistical significance was assessed by one-way ANOVA with Tukey post-test for pairwise comparisons (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

concentrations of 10, 3 and 1 μM, respectively. For all the peptidomimetics the activity was evaluated at 10, 3 and 1 μM under the same stimulatory concentrations of collagen and thrombin used for NoxA1ds. The reference NOXA1 derived peptide **AC1** proved to reduce the aggregation to 40% at 10 μM concentration on platelets activated with both collagen and thrombin (Figure 1). On platelets activated with collagen, **AC2** showed an effect of 50% for the highest concentration similarly to **AC1**, and upon decreasing the concentration, the activity of the compound was also gradually reduced (Figure S2). On the contrary, the effect on platelets activated with thrombin was still evinced although markedly reduced, suggesting an impaired role of the *N*-methylation at the glycine residue. **AC3** with collagen or thrombin did not show high activity, leading to a non-significant reduction in platelet aggregation for all concentrations (Figure S3), suggesting the importance of retaining the NH protons for both valine and glycine residues.

For **AC4** under collagen stimulation, the outcome was observed as with the previous compound, even a lower activity (Figure S4), while with thrombin, though still lower in activity than **AC1**, it showed a significant effect of 60% inhibition at 10 μM, which gradually decreased down to 1 μM where it still maintained an effect of approximately 30%. **AC5** with collagen showed similar efficacy to **AC2**, and lower than **AC1** for both stimuli, with a reduction between 30% and 50% but greater at a concentration of 1 μM and lower at 10 μM (Figure S5). **AC5** with thrombin stimulation presented a similar situation to **AC2** and **AC3**, with a greater reduction at a concentration of 1 μM (40%), intermediate at 10 μM (30%) and almost absent at 3 μM.

GS2 proved to be one of the most interesting compounds, with superior activity with respect to reference epitope **AC1**, which is why 0.3 μM and 0.1 μM concentrations were also tested for both collagen and thrombin. Under collagen stimulation, a reduction of 60–65% was highlighted for concentrations 10, 3 and 1 μM. Decreasing the concentration to 0.3 and 0.1 μM still resulted in inhibition but to an expectedly lesser degree (greater aggregation), approaching 100% of the platelet response, although not quite reaching it. Therefore, this result demonstrates that the compound has a high activity profile (Figure 2, left). With thrombin, at a concentration of 10 μM **GS2** showed an efficacy of 70%, which remained high even at a concentration of 1 μM (Figure 2, right).

Given the results obtained for **GS2**, we carried out a dose-response assay to establish the anti-aggregation potential quantitatively. Accordingly, **GS2** proved to be superior to **AC1** as the NOXA1 active epitope reference peptide, and resulted in a lower IC_{50} value (Table 2).

Inhibition of NOX-Dependent Superoxide Anion Production in Platelets

To evaluate the amount of ROS produced by platelets after their activation with collagen and thrombin, EPR spectroscopy was performed. The reference NOXA1 peptide **AC1** showed significant activity (> 50%) under the stimulus of collagen at 10 μM concentration (Figure 3). For **AC2**, under the stimulus of collagen or thrombin, an activity of 50% was obtained with the concentration of 10 μM (Figure S6). Under the stimulus of thrombin, this reduction was significant even at lower concentrations (presenting a slight anomaly at 3 μM which is a little lower than at 10 μM). With collagen the activity gradually decreased as the concentration decreased. These results indicated the improved effect of methylation at the glycine residue. Similarly, **AC4** showed a significant activity of 50% with 10 μM under thrombin stimulation and superior to **AC1** (Figure S7). This activity was maintained at a concentration of 3 μM and gradually decreased at a concentration lower than 1 μM.

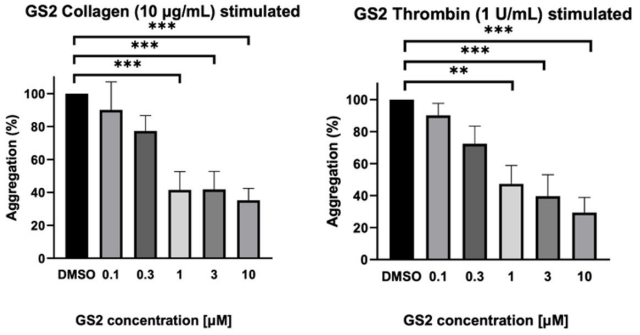


Figure 2. Stimulation with collagen (left) and thrombin (right) at five different concentrations of the GS2 peptidomimetic. Each experiment was repeated three independent times (n = 3), with mean ± SEM shown in the graphs. The statistical significance was assessed by one-way ANOVA with Tukey post-test for pairwise comparisons (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

Table 2. IC50 assay of platelet anti-aggregation potential of AC1 and GS2.

Entry	Collagen stimulated, μM	Thrombin stimulated, μM
AC1 (NOXA1 ref. peptide)	6.4 ± 2.1	2.4 ± 1.1
GS2	0.7 ± 0.5	0.9 ± 0.3

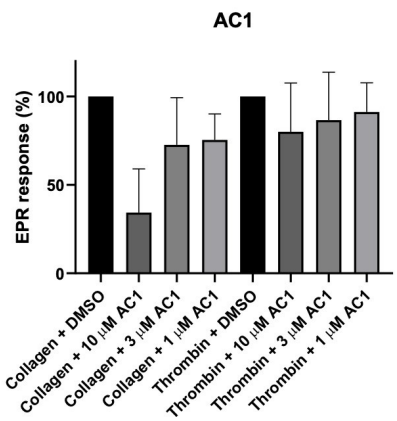


Figure 3. Percentage of EPR signal obtained from the CMH probe signal shows the amount of ROS produced by platelets incubated with AC1 after activation, either with collagen (10 μg/mL) or thrombin (0.1 U/mL). On the abscissa the control with DMSO and the three concentrations of AC1 (10, 3, 1 μM) with the two agonists are reported. Each experiment was repeated three independent times (n = 3), with mean ± SEM shown in the graphs. The statistical significance was assessed by one-way ANOVA with Tukey post-test for pairwise comparisons (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

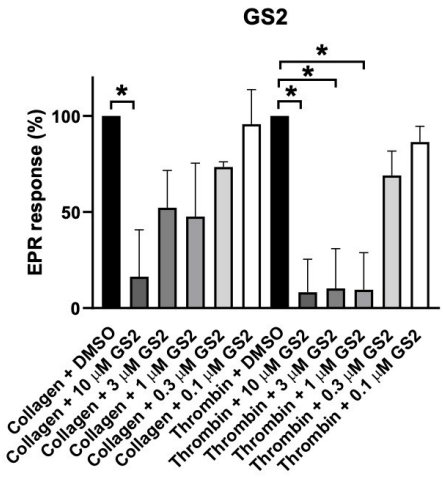


Figure 4. Percentage of EPR signal obtained from the CMH probe signal shows the amount of ROS produced by platelets incubated with GS2 after activation, either with collagen (10 μg/mL) or thrombin (0.1 U/mL). On the abscissa it is reported the control with DMSO and five concentrations of GS2 (10 to 0.1 μM) with the two agonists. Each experiment was repeated three independent times (n = 3), with mean ± SEM shown in the graphs. The statistical significance was assessed by one-way ANOVA with Tukey post-test for pairwise comparisons (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

Under collagen stimulation, however, there was no significant reduction in ROS production similarly to reference **AC1**, reaching almost 100% of the response at a concentration of 1 μM.

On the contrary, **AC3** did not show significant activity in reducing ROS production (about 20%), which remained constant for all concentrations, both for thrombin and collagen (Figure S7), and this was also the case for **AC5**, not experiencing significant effect in the reduction of ROS production (Figure S7). Thus, methylation of glycine and valine residues (**AC3**) proved to impair activity with respect to ROS production of platelets, and so as for peptide stapling (**AC5**) that proved to add conformational constraint to the peptide structure. Even in this case **GS2** turned out to be the best compound, with a significant activity of 80–90% at a concentration of 10 μM under the stimulus of thrombin, which remained high even at the lowest concentrations, suggesting the peptoid modification at Phe-Leu dipeptide moiety of the NOXA1 epitope facilitating such effect. Even under collagen stimulation, significant activity was highlighted at a concentration of 10 μM (Figure 4), thus resulting in superior activity with respect to the parent **AC1** peptide.

Plasma Stability Assay

Mass spectrometry was used to evaluate the stability of the compounds in the plasma before they are degraded. Spectra characterized by a series of peaks were obtained from which it was possible to identify the peak of interest and how long they remained intact in the plasma. The results showed **AC1** and **AC2** remaining in plasma for approximately 12 hours, but at 24 hours it was completely degraded (Table 3). **AC3** showed a similar trend, however at 24 hours it had not yet completely degraded, which instead happened at 48 hours. With respect to **AC4** and **AC5**, these were the most stable as they remained in the plasma for up to 96 h and 192 h, respectively. Finally, **GS2** was almost completely degraded after 12 hours incubation, and completely degraded after 24 hours.

Table 3. Mass spectrometry determination of % stability in plasma.

Entry	12 h	24 h	48 h	96 h
AC1	16	0	0	-
AC2	22	0	0	-
AC3	86	18	0	-
AC4	60	46	39	10
AC5[a]	63	38	30	27
GS2	9	3	-	-

[a] This compound showed 22% stability after 192 h of plasma incubation.

Discussion

In this work the potential inhibitory activity of some peptidomimetics towards the NOX1 enzyme was investigated. These peptidomimetics were synthesized starting from the $NoxA1_{195\text{-}205}$ peptide, whose inhibitory activity on NOX1 had already been confirmed.[10] The interest in blocking the activity of this enzyme (and of its family in general) arises from the fact that NADPH oxidases are involved in many pathological processes, including a series of cardiovascular diseases, including thrombosis. This pathology is associated with various causes, but a fundamental element for it to occur is the hyperactivation of platelets, resulting in the formation of aggregates which then lead to the formation of the thrombus. The platelet activation process is very complex and involves various factors, including ROS, the reactive oxygen species produced by NOX enzymes.

Therefore, starting from the peptide with known activity, modifications were made to make the enzyme inhibitors not only effective, but also more stable in plasma, with the final aim of being able to obtain drugs that can inhibit the formation of thrombi.

Through platelet aggregation and EPR spectroscopy experiments, it was evaluated whether and to what extent peptidomimetics inhibit aggregation and ROS production. Finally, the stability of the compounds in plasma was investigated using mass spectrometry, trying to understand how long it takes for them to be degraded.

We observed that compound **AC2**, which presents the substitution of glycine with an *N*-methyl-glycine as a structural modification, has a high activity on aggregation, both on platelets stimulated with thrombin and on those stimulated with collagen. Regarding the ROS production, **AC2** has an important effect on the reduction of radical production both after stimulation with thrombin and collagen. However, it does not remain in the plasma for long periods of time, as after 24 hours it is already completely degraded (probably due to the activity of various proteases). Therefore, when thinking about a hypothetical pharmacological therapy, a daily administration of the drug should be considered.

AC3, with two substitutions on *N*-methyl-valine and *N*-methyl-glycine, showed an efficacy regarding thrombin-induced aggregation, which however was not very high with collagen. This could suggest that the addition of the second methylated amino acid could somehow interfere with the NOX activation process induced by thrombin but not on that induced by collagen, and therefore still lead to aggregation after induction with the latter. Even with spectroscopy we did not highlight a very strong activity, only a low reduction in the EPR signal, supporting the idea that the second methylated amino acid can somehow reduce the affinity towards the enzyme. Compared to **AC2** however, **AC3** remains in the plasma for up to 24 hours, before being degraded within 48 hours.

AC5 and **AC4** corresponds to the stapled **AC1** peptidomimetic and its non-cyclic precursor, respectively. Stapled refers to a peptide that has a covalent bond between two amino acid side chains, thus forming a peptide macrocycle. This modification is usually carried out to increase the stability of the peptide and prevent it from being rapidly degraded by plasma proteases. Also, it generally stabilizes helical conformations of the peptide. The activity of **AC4** in aggregation was found being not particularly high under collagen stimulation, while it was strong at 10 μM under stimulation with thrombin. This behavior could be due, as for the second methylated amino acid in **AC3**, to the fact that the modification may interfere with the process induced by thrombin but not with that induced by collagen. In reducing the production of radical species, AC4 proved effective under both stimuli, proving anomalous as there is no inhibition in aggregation with collagen. **AC5**, possessing the peptide stapling moiety, showed an inverse situation compared to **AC4**, as a good response was highlighted in the inhibition of aggregation, while with EPR the inhibitory activity is practically zero, with a production of ROS practically equal to platelets without inhibitor. However, both remained stable in the plasma for the longest time, as expected given their structure and the modifications made. **AC4** was still present after 96 hours, and peaks related to **AC5** were found after 192 hours, without reaching total degradation in either case. Despite its poor plasma stability, **GS2** (a peptide-peptoid hybrid) was found to be an excellent compound in terms of activity, with a strong response under both collagen and thrombocyte stimulation, therefore being considered as an interesting candidate for further studies.

Conclusions

In conclusion, in the present work the ability of synthetic peptides inhibitors of NOX1 to effectively block the production of ROS by the enzyme was studied with different methodologies. The results obtained highlighted that **AC4** has a good activity in reducing the production of ROS under thrombin stimulation, which however is not reflected in aggregation; however, it is stable in plasma for up to 92 hours. Equally stable is **AC5** (the best in terms of stability) which however does not significantly reduce ROS production and aggregation. Improved efficiency, in terms of efficacy and potency, was highlighted for the peptide-peptoid hybrid **GS2**. However, these compounds do not remain stable in the plasma. Thus, to subsequently use them as new anti-thrombotic drugs, further modifications will need to be made to the best candidates to allow both remaining longer in the plasma without affecting their activity.

Experimental Section

Peptide Synthesis

Solid-phase peptide synthesis was carried out using an automated single-mode microwave synthesizer (Initiator Sixty, Biotage AB) using sealed reaction vessels and built-in internal pressure and temperature sensors. The peptides were synthesized on ChemMatrix Rink Amide resin, with a 0.5 mmol/g loading capacity and bead size of 100–200 mesh. Peptide couplings were carried out using 5 eq. the Fmoc-amino acids and 10 eq. DIC/Oxyma, as the activating mixture by heating at 90 °C for 3 min. 20% Morpholine was used as Fmoc deblocking agent by heating at 90 °C for 2 min (2x). Peptide cleavage was achieved using a mixture of 2.5% H_2O and 2.5% TIPS in TFA for 2 h followed by filtration. Cold diethyl ether was added to the filtrate, and peptide was precipitated. The mixture was centrifuged for 3 min at 2500 rpm, and the ether layer was separated. Then, the peptide was dried under vacuum overnight to give the crude peptide. Peptides were analyzed and purified using Dionex Ultimate 3000 system equipped with a reverse-phase analytical column Synergi 4 μm Fusion-RP 80 Å (150x4.6 mm) or semi-preparative column Synergi 10 μm Fusion-RP 80 Å (250x10.0 mm) and using acetonitrile (0.1% TFA) in H_2O (0.1% TFA). A flow rate of 1 and 5 mL/min were used for analytical and semi-preparative runs, respectively, and peak detection was achieved at 223 nm. All crude peptides were obtained in >90% purity. The molecular weight of all peptides was confirmed by electrospray mass spectrometry by direct inlet on a LCQ Fleet™ Ion Trap LC/MS system (Thermo Fisher Scientific) using electrospray (ES^+ or ES^-) ionization techniques.

Compound AC1

The reference NOXA1 peptide, **AC1**, was achieved in 27% yield using 100 mg of ChemMatrix Rink Amide resin and following the general procedure for peptide synthesis and HPLC purification. HPLC: 90% purity, rt = 9.01 min; ESI-MS: *m/z* 601 $[M+2H]^{2+}$, 1223 $[M+Na]^{+}$.

Compound AC2

The compound was achieved in 23% yield using 100 mg of ChemMatrix Rink Amide resin and *N*-Me-Gly in place of Gly as of **AC1**, following the general procedure for peptide synthesis and HPLC purification. HPLC: 100% purity, rt = 9.51 min; ESI-MS: *m/z* 608 $[M+2H]^{2+}$, 1216 $[M+H]^{+}$.

Compound AC3

The compound was achieved in 23% yield using 100 mg of ChemMatrix Rink Amide resin and *N*-Me-Gly and *N*-Me-Val in place of Gly and Val, respectively, following the general procedure for peptide synthesis and HPLC purification. HPLC: 100% purity, rt = 8.85 min; ESI-MS: *m/z* 615 $[M+2H]^{2+}$, 1229 $[M+H]^{+}$.

Compound AC4

The compound was achieved in 14% yield using 50 mg of ChemMatrix Rink Amide resin and following the general procedure for peptide synthesis and HPLC purification. Fmoc-(*S*)-2-(4-pentenyl)alanine was used in *i* and *i* + 4 relative positions in place of Val and Gly of the reference peptide **AC1**. HPLC: 98% purity, rt = 12.05 min; ESI-MS: *m/z* 663 $[M+2H]^{2+}$, 1325 $[M+H]^{+}$.

Compound AC5

The RCM-stapled compound was achieved in 13% yield using 50 mg of ChemMatrix Rink Amide resin and following the general procedure for peptide synthesis and HPLC purification. Fmoc-(*S*)-2-(4-pentenyl)alanine was used in *i* and *i* + 4 relative positions in place of Val and Gly of the reference peptide **AC1**. The cyclization was carried out on resin and before the deprotection of the last Fmoc-amino acid. Initially, the resin was washed 3 times with CH_2Cl_2. Then 5 mL (maximum capacity of the reactor used) of 1,2-dichloroethane (DCE) in the reactor and the solution was bubbled with nitrogen. After 15 min, 8 mg (amount for 0.025 mmol resin-bound peptide) of 1st-generation Grubbs catalyst was added and the solution was bubbled with nitrogen for 4 h. Then, the solution was filtered, and the resin was successively washed with MeOH (x2), CH_2Cl_2 (x2) and MeOH (x2). Final Fmoc deprotection and peptide cleavage from the resin were carried out as described in the general procedure for peptide synthesis. HPLC: 100% purity, rt = 9.99 min; ESI-MS: *m/z* 649 $[M+2H]^{2+}$, 1296 $[M+H]^{+}$.

Compound GS2

The compound was achieved in 14% yield using 100 mg of ChemMatrix Rink Amide resin and following the general procedure for peptide synthesis and a protocol for the insertion of the peptoid fragment. Specifically, GKAKV was synthesized using standard SPPS, then bromoacetic was coupled to free resin-bound *N*-terminal peptide fragment using DIC/Oxyma (2 M in DMF) as the activating mixture under microwave irradiation for 2 min at 40 °C. Then, after resin washings, isobutylamine was used as 1 M DMF solution for the S_N2 reaction under microwave irradiation for 1'30'' at 80 °C, to finalize the insertion of pL as the pseudo-Leu peptoid monomer. Similarly, bromoacetic acid and (*S*)-phenyl-ethylamine were used to install the pF peptoid monomer. Then, the compound was completed adding the EPVD sequence via standard SPPS. Final Fmoc deprotection and peptide cleavage from the resin were carried out as described in the general procedure for peptide synthesis. HPLC: 98% purity, rt = 12.05 min; ESI-MS: *m/z* 610 $[M+2H]^{2+}$, 1218 $[M+H]^{+}$.

Preparation of Platelets

Procedures utilizing human blood conformed to the principles outlined in the Declaration of Helsinki and were performed under local ethics approval (Ethikkommission der Ärztekammer Hamburg, Bearb.-Nr. #2322). Human blood was collected from consenting donors who were not under aspirin or other antiplatelet drug treatment and were nor diagnosed with a cardiovascular disease. Sodium citrate was used as an anticoagulant (3.2% w/v). The collected blood was subjected to centrifugation at 200xg at 37 °C for 15 minutes with gentle deceleration. Subsequently, PRP (platelet rich plasma) was isolated and indomethacin (concentration 10 μM) and PGE1 (prostaglandin E1; concentration 100 ng/mL) were added, and centrifuged again at 500 xg, at 37 °C for 15 minutes. The PPP (platelet poor plasma) was then taken and from this the pellet was resuspended in Tyrode buffer (136 mM NaCl, 1 mM $MgCl_2$, 2.7 mM KCl, 10 mM HEPES) with 5 mM glucose, previously placed in a bath at 37 °C. Finally, the density of the platelets was adjusted to $4x10^8$/ml.

Platelet Aggregation Assay

Before the aggregation test it was necessary to have a platelet density of $4x10^8$/mL. Subsequently these are incubated at 37 °C with the peptidomimetic at the concentrations of interest and with DMSO for the control sample for 15 minutes. A four-channel CHRONO-LOG® Model 490 4 + 4 laboratory aggregometer was used to measure the changes in optical density during the aggregation process. The platelet suspension was continuously agitated with a magnetic stirrer (1,200 rpm) and maintained at 37 °C.

Determination of ROS Produced by Activated Platelets

The electron paramagnetic resonance (EPR) technique with the cyclic hydroxylamine spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH), was employed to determine the presence of reactive oxygen species, including the superoxide radical ($O_2^{\bullet-}$). To carry out this measurement, CMH (200 μM), diethyldithiocarbamate (DETC, 5 μM) and deferotonin (DF, 25 μM) were then added to the platelet suspension before starting platelet aggregation.

Plasma Stability Assay

The stability of peptidomimetics in plasma at different times was determined by mass spectrometry using the MALDI-TOF technique. To prepare the sample to be analyzed by mass spectrometry, PPP (platelet poor plasma) incubated at 37 °C with peptidomimetics at a concentration of 100 μM each was used. A triplicate was analyzed for each compound and each incubation time (0, 12 h, 24 h, 48 h, 96 h, 192 h).

Supporting Information Summary

Aggregation curves of NOXA1-derived peptide (**AC1**); graphs of stimulation with collagen and thrombin in presence of **AC2–AC5** and **GS2**; amount of ROS produced by platelets incubated with **AC2–AC5** and **GS2**; Plots of plasma stability of **AC2–AC5** and **GS2**; copies of HPLC chromatograms and ESI-MS spectra for compounds **AC1–AC5** and **GS2**.

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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