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Thrombin-Inhibiting Anticoagulant Liposomes: Development and Characterization

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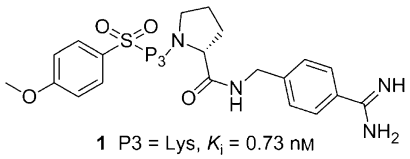
Many peptides and peptidomimetic drugs suffer from rapid clearance in vivo; this can be reduced by increasing their size through oligomerization or covalent conjugation with polymers. As proof of principle, an alternative strategy for drug oligomerization is described, in which peptidomimetic thrombin inhibitors are incorporated into the liposome surface. For this purpose, the inhibitor moieties were covalently coupled to a palmitic acid residue through a short bifunctionalized ethylene glycol spacer. These molecules were directly added to the lipid mixture used for liposome preparation. The obtained liposomes possess strong thrombin inhibitory potency in enzyme kinetic measurements and anticoagulant activity in plasma. Their strong potency and positive ζ potential indicate that large amounts of the benzamidine-derived inhibitors are located on the surface of the liposomes. This concept should be applicable to other drug molecules that suffer from rapid elimination and allow covalent modification with a suitable fatty acid residue.

Introduction

The therapeutic potential of peptidic drug candidates is often hampered by their limited proteolytic stability and short half-lives as a result of rapid renal clearance. Many unmodified peptides are eliminated from plasma within a few minutes after administration. Therefore, various strategies to extend the stability of peptides have been developed that also prolong their half-lives.[1] This includes N- and C-terminal modifications, incorporation of D-configured or N-methylated amino acids, replacement of peptide bonds and labile amino acids with unnatural residues, and numerous types of cyclizations.[2] Additional strategies are based on various types of conjugation to increase the molecular mass of the drug molecules, which results in reduced renal clearance. This includes PEGylation (PEG = polyethylene glycol), conjugation with polymers of *N*-acetylneuraminic acid (polysialic acids) and hydroxyethyl starch (HESylation), and conjugation with random polymers of proline, alanine, and serine (PASylation).[3,4] The half-lives of drug molecules can also be extended by conjugation with albumin mediated by a special endosomal recycling mechanism by using the high affinity of albumin to the neonatal Fc receptor (FcRn).[5] An additional strategy is based on multimerization; for instance, the plasma half-life of a dimeric erythropoietin, obtained by chemical conjugation, was enhanced to 24 h compared to 4 h for the monomeric form.[6]

Similar problems of rapid renal clearance and in some cases also hepatobiliary clearance were found by us for various benzamidine-derived peptidomimetic and substrate–analogue inhibitors of various trypsin-like serine proteases.[7–10] Therefore, we investigated the possibility to achieve suitable oligomerization of related protease inhibitors onto the surface of liposomes for potential half-life prolongation. For this purpose, we used previously described benzamidine-derived inhibitors **1** and **2** (Figure 1) that inhibit thrombin in the low nanomolar



1 P3 = Lys, K_i = 0.73 nM
2 P3 = Asp, K_i = 1.6 nM

Figure 1. Structures of used substrate analogue thrombin inhibitors.[11]

range and possess negligible potency against related proteases such as factor Xa, plasmin, urokinase-type plasminogen activator (uPA), plasma kallikrein, and matriptase.[11] Thrombin is a trypsin-like serine protease and a key enzyme of the blood coagulation cascade. Various direct thrombin inhibitors, such as oral dabigatran etexilate and parenteral r-hirudin, bivalirudin, and argatroban, have been approved and can be used as anticoagulants for various prophylactic and acute applications.[12] Moreover, thrombin is also an excellent tool for studying receptor–ligand interactions.[13–15] The protein data bank contains nearly 400 crystal structures in complex with various types of inhibitors; it is easily accessible and numerous test systems to determine the potency of new inhibitors exist.

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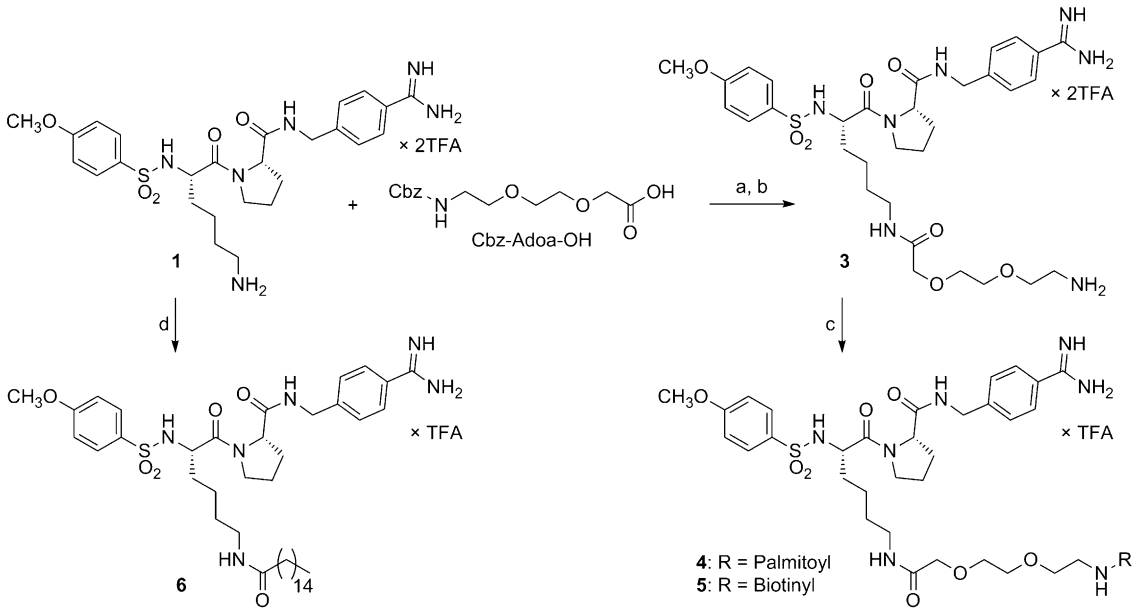
The used inhibitor type contains an L-configured trifunctional amino acid in the P3 position. X-ray structure analysis of a close analogue in complex with thrombin revealed that its P3 side chain is directed into the solvent and should tolerate further modification without losing potency.[11] In this manuscript, we describe the covalent coupling of these inhibitors with palmitic acid, which enables their incorporation into liposomes. The fatty acid residue was attached either directly to the P3 side chain or through a short bifunctional ethylene glycol spacer. The inhibitory potency of the inhibitors before and after incorporation into the liposomes was determined by enzyme kinetic studies and by measurement of their anticoagulant activities in plasma. The results will be described in this publication.

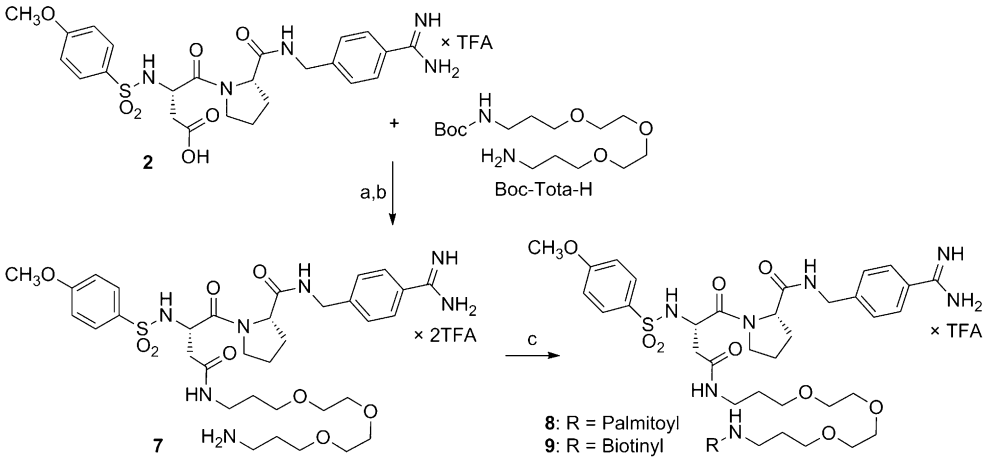
Results

Synthesis

The new Lys- and Asn-derived thrombin inhibitors were prepared as shown in Schemes 1 and 2, respectively. Inhibitor **1**[11] was coupled to 8-(benzyloxycarbonylamino)-3,6-dioxaoctanoic acid (Cbz-Adoa-OH), and this was followed by removal of the protecting group. This provided compound **3**, which was further coupled to palmitic acid or biotin to give inhibitor **4** or **5**, respectively. Furthermore, compound **1** was directly coupled to palmitic acid to give inhibitor **6** lacking the ethylene glycol spacer. Similarly, Asp compound **2** was treated with 1-(*tert*-butoxycarbonylamino)-4,7,10-trioxa-13-tridecanamine (Boc-Tota-



Scheme 1. Synthesis of Lys-containing inhibitors **3**–**6**. *Reagents and conditions*: a) BOP, DIPEA (2 equiv.) in DMF; b) 33% HBr in acetic acid, preparative HPLC; c) palmitic acid or biotin, BOP, DIPEA (2 equiv.) in DMF, preparative HPLC; d) palmitic acid, BOP, DIPEA (2 equiv.) in DMF, preparative HPLC.



Scheme 2. Synthesis of Asn-containing inhibitors **7**–**9**. *Reagents and conditions*: a) BOP, DIPEA (2 equiv.) in DMF; b) TFA, preparative HPLC; c) palmitic acid or biotin, BOP, DIPEA (2 equiv.) in DMF, preparative HPLC.

H). Cleavage of the Boc group provided inhibitor **7**, which was converted into palmitoyl and biotinyl derivatives **8** and **9**, respectively.

Moreover, Boc-Tota-H was directly coupled with biotin, which was followed by acidic deprotection. The obtained intermediate was treated with palmitic acid to provide compound **13** [Me-$(CH_2)_{14}$-CO-Tota-Biotin; for the synthesis, see Scheme S1 in the Supporting Information], which was used for the preparation of bifunctionalized liposomes (Figure 2).

Biological assays

Enzyme kinetic studies

The inhibitory potencies of the synthesized inhibitors against thrombin and a few related trypsin-like serine proteases are summarized in Tables 1 and 2. The results indicate that all of these compounds are highly selective thrombin inhibitors with relatively weak affinity ($K_i > 3\ \mu M$) against the other studied proteases. As expected, further elongation of the P3 side chain with the used ethylene glycol derivatives was well accepted and resulted in low nanomolar inhibitors in both series. A slightly decreased potency was only found for compound **6** obtained after direct coupling of the palmitoyl residue to the Lys side chain. In contrast, the coupling of the Tota moiety to the Asp side chain of inhibitor **2** provided Asn derivatives **7**–**9** with slightly enhanced thrombin affinity. This tendency confirms previous data, for which thrombin inhibition after incorporation of Asn in the P3 position was stronger than that of the Asp analogue.[11]

Preparation of anticoagulant liposomes

Owing to slightly better chemical accessibility, P3 Lys-containing palmitoylated inhibitors **4** and **6** were used for the preparation of liposomes made by the thin-film hydration method.[16,17] The liposomes were obtained from a mixture of

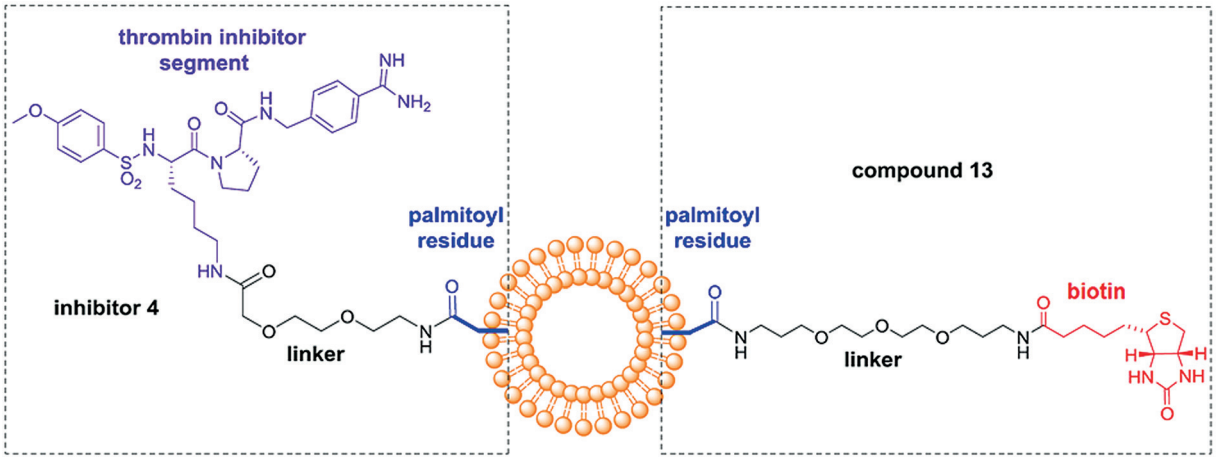
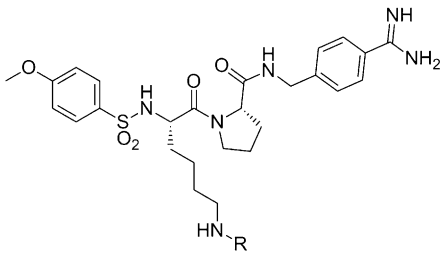


Figure 2. Schematic presentation of bifunctionalized liposomes containing inhibitor **4** and biotin derivative **13** on their surface.

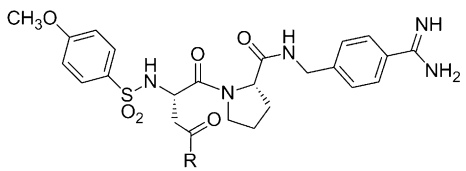
Table 1. Inhibitory potency of P3-Lys-derived inhibitors.



Compd	R	K_i [nM][a] Thrombin	FXa	K_i [μM][a] Plasmin	uPA	IC_{200} [μM] aPTT
1	H-	0.73	3.92 ± 0.21	3.74 ± 0.31	25.31 ± 4.28	0.10
3	Adoa-	0.82 ± 0.07	7.28 ± 0.93	3.12 ± 0.69	30.32 ± 5.37	0.12
4	Palmitoyl-Adoa-	1.39 ± 0.06	5.93 ± 0.49	4.16 ± 0.48	44.49 ± 3.18	0.32
5	Biotin-Adoa-	0.89 ± 0.04	3.52 ± 0.49	3.59 ± 0.57	31.36 ± 4.25	0.71
6	Palmitoyl-	2.36 ± 0.62	6.13 ± 0.99	7.41 ± 0.18	51.01 ± 6.53	5.29

[a] Data are the mean ± SD obtained for three measurements.

Table 2. Inhibitory potency of P3-Asp/Asn-derived inhibitors.



Compd	R	K_i [nM][a] Thrombin	FXa	K_i [μM][a] aPTT	uPA	IC_{200} [μM] aPTT
2	HO-	1.6	5.62 ± 0.25	17.92 ± 1.32	26.92 ± 1.43	–
7	Tota-	0.41 ± 0.02	6.02 ± 0.98	18.32 ± 1.52	26.42 ± 1.94	0.05
8	Palmitoyl-Tota-	0.87 ± 0.06	4.73 ± 0.20	17.99 ± 1.65	28.32 ± 1.26	0.09
9	Biotin-Tota-	0.35 ± 0.06	3.67 ± 0.29	23.64 ± 1.75	56.25 ± 1.20	–

[a] Data are the mean ± SD obtained for three measurements.

DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine), CH (cholesterol), and palmitoylated inhibitor (ratio 65:30:5 mol%).

Moreover, bifunctionalized liposomes containing a thrombin inhibitor and an additional biotin moiety on the surface were prepared by the same method (Figure 2). In this case, the same mixture of DPPC, CH, and palmitoylated inhibitor **4** or **6** was used, which contained additional compound **13** (Palmitoyl-Tota-Biotin, 10% relative to thrombin inhibitor **4** or **6**).

Analysis of the supernatants after liposome preparation revealed that the inhibitor was most likely fully incorporated into the liposomes. In the least, it was not possible to detect free inhibitor **4** or **6** by HPLC or MS analysis. Moreover, no thrombin inhibitory potency was observed upon analyzing the supernatants in enzyme kinetic assays.

Physicochemical characterization of the liposomes

The ζ potential is the overall charge of a particle in a particular medium, and it can be used to predict its interaction with other molecules. The determined particle sizes and ζ potentials are summarized in Table 3. For the unmodified DPPC/CH liposomes, a negative ζ potential was found owing to the presence of negatively charged phosphoester groups. In contrast, a positive potential was determined for all liposomes containing the positively charged thrombin inhibitors. Relative to that of the pure DPPC/CH formulation, all modified liposomes possess an increased particle size.

In addition to measurements by dynamic light scattering (DLS), atomic force microscopy (AFM) was conducted. AFM allows visualization of formulations, and it confirmed the particle size measurements performed by DLS. For size determination, all objects within a representative scan area were individually evaluated (Figure 3). For the DPPC/CH liposomes (Figure 3A), spherical liposomes with a size range of 78 ± 11 nm could be visualized and a monomodal size distribution was determined. The monofunctionalized liposomes in Figure 3B,C also show spherical shapes and sizes similar to those obtained from the DLS measurements (Table 3). For the liposomes containing 5 mol% inhibitor **4**, a size of 324 ± 65 nm was measured, whereas inhibitor **6** led to liposomes that were 335 ± 87 nm in size. On the AFM images of these formulations, aggregates of lipids are seen beside the liposomes. The addition of biotinylated compound **13** to the formulation resulted in a slight further increase in the liposome size (370 ± 60 nm for DPPC/CH/**4**/**13**, Figure 3D; 390 ± 70 nm for DPPC/CH/**6**/**13**, Figure 3E), and again, some lipid aggregates are visible, especially for the bifunctionalized liposomes containing inhibitor **6**.

Thrombin inhibition by the prepared liposomes

The inhibitory potency of the anticoagulant liposomes was determined by enzyme kinetic measurements by using the fluorogenic substrate Tos-Gly-Pro-Arg-AMC (Tos = tosyl, AMC = 7-amido-4-methylcoumarin) in the presence of thrombin (Table 3). The provided inhibitor concentration on the *x* axis (Figure 4) was calculated on the basis of the total inhibitor amount used for the preparation of the liposomes, assuming that it was completely incorporated and equally distributed on

Table 3. Thrombin inhibitory potency, anticoagulant activity, particle size, and ζ potential of the prepared liposomes.

Composition	ζ potential [mV][a]	Particle size [nm][a]	IC_{50} [nM][a,b]	IC_{200} [μM] aPTT
DPPC/CH	−8.60 ± 3.13	75.27 ± 15.96	–	–
DPPC/CH/**4**	+29.74 ± 10.05	353.27 ± 159.14	0.55 ± 0.03	1.61
DPPC/CH/**6**	+23.56 ± 4.79	330.35 ± 124.12	81 ± 8.09	7.26
DPPC/CH/**4**/**13**	+29.95 ± 11.43	392.78 ± 191.37	4.0 ± 0.24	2.16
DPPC/CH/**6**/**13**	+37.18 ± 16.19	343.23 ± 181.85	116 ± 14.4	7.97

[a] Data are the mean ± SD obtained for three measurements. [b] IC_{50} values were determined in enzyme kinetic measurements with a fluorogenic substrate.

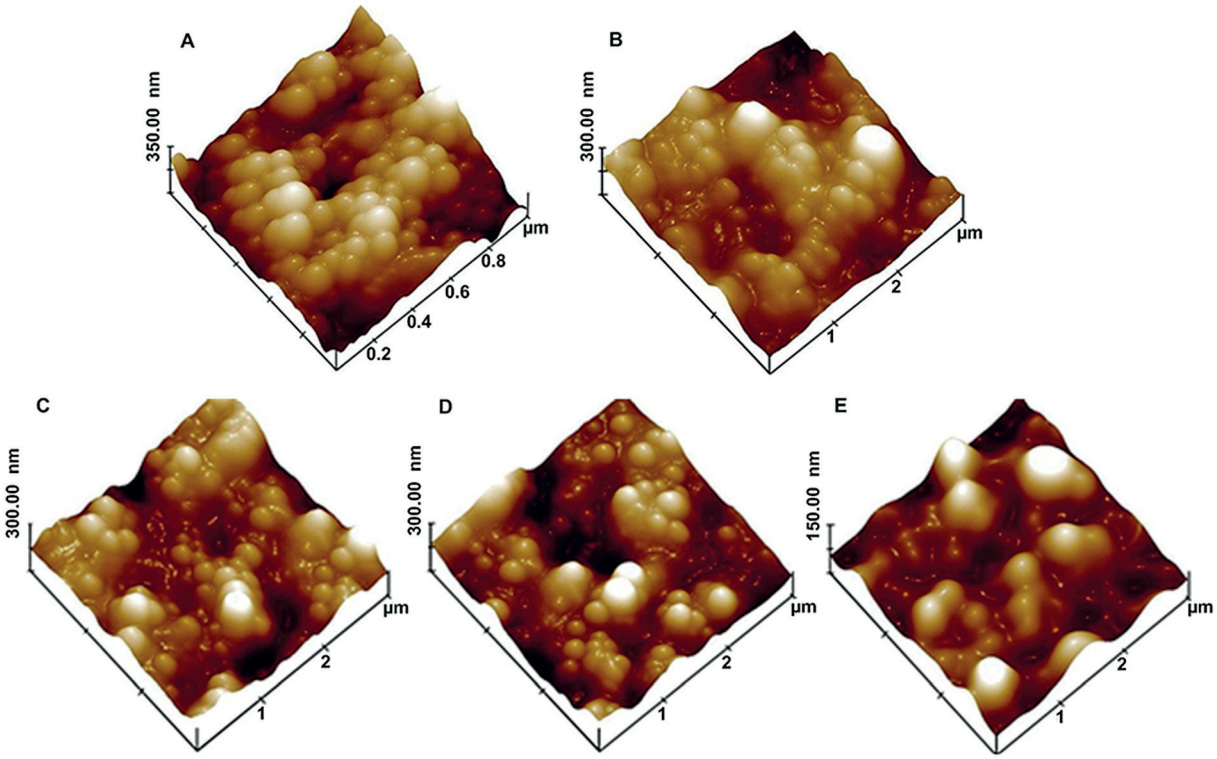


Figure 3. Visualization of the size distribution and morphology of the liposomes by using AFM: A) DPPC/CH, B) DPPC/CH/**4**, C) DPPC/CH/**6**, D) DPPC/CH/**4**/**13**, E) DPPC/CH/**6**/**13**.

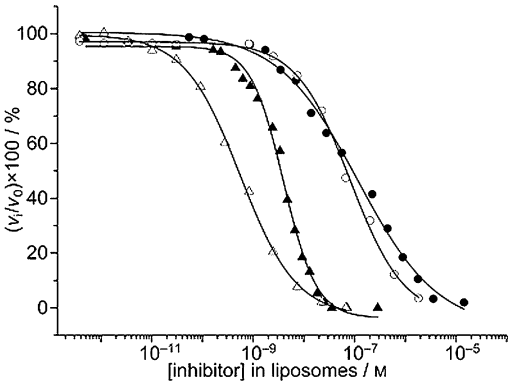


Figure 4. Inhibition of the thrombin-catalyzed cleavage of the fluorogenic substrate Tos-Gly-Pro-Arg-AMC (10.9 µM) by the prepared liposome formulations: DPPC/CH/**4** (△), DPPC/CH/**6** (○), DPPC/CH/**4**/**13** (▲), and DPPC/CH/**6**/**13** (●). The inhibitor concentrations in the liposomes were calculated on the basis of the total inhibitor amount used for their preparation, assuming that the inhibitors were completely incorporated and distributed on the surface.

the surface. However, it cannot be excluded that some amount of the inhibitor is incorporated in the inner liposome membrane.

The monofunctionalized liposomes prepared from inhibitor **4** containing the Adoa spacer possess excellent inhibitory potency (IC_{50} = 0.55 nM), whereas the liposomes containing compound **6** lacking the ethylene glycol linker have strongly reduced activity (IC_{50} = 81 nM). This indicates the importance of the flexible spacer between the inhibitor head and the surface of the liposomes to improve the accessibility for the target enzyme. Despite slightly decreased thrombin affinity relative to that of the monofunctionalized liposomes, a similar tendency was found for both bifunctionalized formulations. Particles prepared from inhibitor **4** and biotinylated compound **13** inhibit thrombin with IC_{50} = 4 nM, whereas those containing inhibitor **6** possess reduced activity (IC_{50} = 116 nM, Table 3). Moreover, measurements with inhibitor-free DPPC/CH or DPPC/CH/**13** liposomes show no inhibitory potency against thrombin. For these control measurements, the liposome formulations were diluted similar to that described for the more potent inhibitor-containing DPPC/CH/**4** liposomes.

Anticoagulant activity in plasma

The anticoagulant activities of the free Lys-derived inhibitors and the prepared liposomes were determined by the activated partial thromboplastin time (aPPT) clotting assay in human plasma and are expressed as their IC_{200} values, which correspond to the inhibitor concentrations required to double the clotting time relative to that of the control (Figure 5). As described above, the inhibitor concentration of the liposomes was calculated by assuming complete incorporation of compound **4** or **6**. Free inhibitors **1** and **3** possess excellent anticoagulant potency with IC_{200} values of 0.1 and 0.12 µM, respectively. A slightly reduced activity is found for palmitoyl-Adoa and biotin derivatives **4** and **5**, whereas analogue **6** lacking the Adoa spacer has relatively poor potency (Table 1). A similar tendency is observed for the mono- and bifunctional liposome

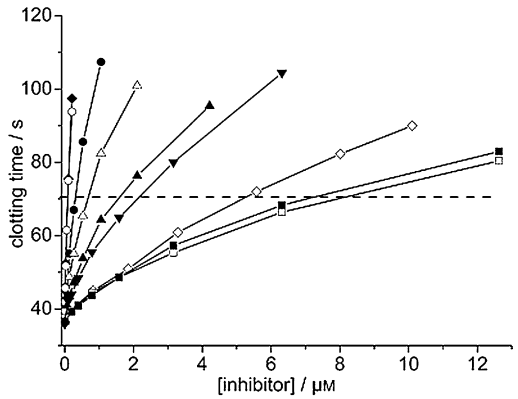


Figure 5. aPPT-assay of the Lys-derived inhibitors and liposome preparations in plasma [free inhibitors: **1** (◆), **3** (○), **5** (●), **4** (△), **6** (◇); monofunctionalized liposomes: DPPC/CH/**4** (▲), and DPPC/CH/**6** (■); bifunctionalized liposomes: DPPC/CH/**4**/**13** (▼), and DPPC/CH/**6**/**13** (□)]. The dotted line represents the doubling of the clotting time in the absence of inhibitor.

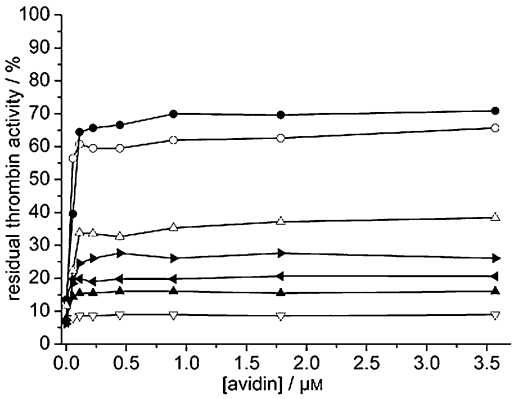


Figure 6. Avidin-mediated neutralization of thrombin inhibition. Different avidin concentrations were added to the free biotinylated inhibitors **5** (●) and **9** (○), to monofunctionalized DPPC/CH/**4** liposomes (▽), and to bifunctionalized DPPC/CH/**4**/**13** liposomes containing different concentrations of inhibitor **13** (▲: 10 mol%, ◀: 20 mol%, ▶: 50 mol%, △: 100 mol% relative to inhibitor **4**). Measurements were performed with 15 pM thrombin in the presence of 11 μM Tos-Gly-Pro-Arg-AMC.

preparations, whereas the particles containing compound **4** with the Ado spacer are much more potent than their analogues based on inhibitor **6** (Table 3).

Neutralization of bifunctionalized DPPC/CH/4/13 liposomes by avidin

In previous work, a chimeric anticoagulant that could be neutralized was prepared[18] by coupling the active site directed thrombin inhibitor CRC220[19] to an idraparinux-derived synthetic pentasaccharide[20] through a short spacer consisting of a short ethylene glycol unit attached to a Lys-containing dipeptide segment. The thrombin inhibitor segment enabled the inhibition of fluid phase and clot-bound thrombin, whereas the pentasaccharide led to antithrombin-mediated factor Xa inhibition and a prolonged half-life of approximately 3 h with more predictable pharmacokinetics. Moreover, the Lys side chain was further coupled to biotin, which enabled neutralization of these anticoagulants after injection of avidin.[21]

To adapt this antidote strategy, bifunctionalized liposomes were prepared by adding biotinylated compound **13** during their preparation (10% compound **13** relative to inhibitor **4** or **6**, Figure 2). The obtained liposomes (DPPC/CH/**4**/**13** and DPPC/CH/**6**/**13**) possess slightly reduced inhibitory potency against thrombin relative to the monofunctionalized formulation. Also in this case, the bifunctionalized liposomes containing inhibitor **4** are more potent than their analogues with inhibitor **6**.

To test the neutralization of the thrombin inhibitory potency of the free biotinylated inhibitors and selected liposome formulations, the inhibitor amount was adjusted to provide a residual thrombin activity of approximately 10% in the absence of avidin (Figure 6). Addition of avidin to the DPPC/CH/**4**/**13** liposomes had only a minor effect on the neutralization of their thrombin inhibitory potency. To enhance the efficacy of the antidote, additional liposomes with higher concentrations of biotinylated derivative **13** (20, 50, and 100% relative to inhibitor **4**) were prepared. However, despite a slight concentration-dependent effect, it was only possible to achieve partial neutralization of thrombin inhibitory potency by the addition of avidin (Figure 6). A stronger neutralizing effect was observed for free biotinylated inhibitors **5** and **9**, whereas no influence was found for the control DPPC/CH/**4** liposomes lacking biotinylated derivative **13**.

Discussion

Various groups have described the development of antithrombotic nanoparticles and micelles by using different antiplatelet and anticoagulant strategies. For instance, antiplatelet activity was reported for nanosilver particles that accumulate within platelets and reduce interplatelet proximity.[22] Anticoagulant micelles were obtained by mixing covalently coupled conjugates of DSPE-PEG2000-maleimide (DSPE = 1,2-distearoyl-sn-glycero-3-phosphoethanolamine) with a cysteine-containing derivative of the bivalent 20-mer thrombin inhibitor bivalirudin (hirulog) and with a clot-targeting pentapeptide sequence Cys-Arg-Glu-Lys-Ala.[23] Moreover, D-Phe-Pro-Arg-chloromethyl ketone (PPACK)-functionalized perfluorocarbon (PFC)-core nanoparticles were prepared and were shown to possess significant thrombin inhibitory potency in enzyme kinetic studies and to inhibit clotting in plasma.[24] However, chloromethyl ketone derived inhibitors have limited selectivity, and previous studies revealed that the irreversible thrombin inhibitor PPACK suffers from a very short half-life of approximately 2–3 min in vivo.[25,26] In a following study, these PFC-core nanoparticles were conjugated with bivalirudin covalently coupled to a DSPE-carboxy-PEG2000 derivative.[27] Although no specific information was provided for the quality and yield of this reaction, the described carbodiimide-mediated coupling of unprotected bivalirudin without any pre-activation of the DSPE-carboxy-PEG2000 derivative might be challenging because of several functional groups present in this relatively large peptidic thrombin inhibitor. The same group also reported the prepara-

tion of anticoagulant liposomes obtained by coupling PPACK to preformed carboxy-functionalized liposomes.[28] Compared with free PPACK, these liposomes possess reduced inhibitory potency against thrombin, as determined by using an enzyme kinetic assay. However, they still showed significant anticoagulant activity in an aPPT assay in plasma and decreased thrombus formation in vivo.

In our work, we investigated a different strategy for the preparation of anticoagulant liposomes. For this purpose, a highly potent and specific thrombin inhibitor was coupled through a short ethylene glycol spacer to a palmitic acid. This palmitoylated inhibitor is synthetically well accessible and can be easily purified and characterized. Moreover, in contrast to highly reactive chloromethyl ketones,[25] it is a chemically stable compound. To obtain anticoagulant liposomes, the 4-MeO-bs-Lys(Adoa-Palmitoyl)-Pro-4-Amba·TFA (**4**) conjugate was simply mixed with standard reagents such as 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and cholesterol (CH) during liposome preparation. This convenient strategy avoids chemical modification of preformed and functionalized liposomes, which can lead to side reactions on the inhibitor moiety and requires further purification steps, for example, removal of coupling reagents. On the basis of the IC_{50} values obtained from enzyme kinetic studies, we found that liposomes prepared from inhibitor **4** containing the flexible ethylene glycol spacer are more than 100-fold more potent than liposomes prepared from 4-MeO-bs-Lys(Palmitoyl)-Pro-4-Amba·TFA (**6**) without the linker. This indicates that the potency of the liposomes strongly depends on a sufficient distance between the phospholipid surface and the thrombin inhibitor head. We assume that the longer distance improves the accessibility of the inhibitor for thrombin and probably enables simultaneous binding of several thrombin molecules to a single particle. A similar tendency with improved anticoagulant potency of the liposomes containing inhibitor **4** was observed in the plasma clotting assay.

Bifunctionalized liposomes containing the inhibitor **4** and biotinylated Me-$(CH_2)_{14}$-CO-Tota-Biotin (**13**) on their surface were prepared to adopt an avidin-mediated neutralization strategy previously described for other types of thrombin inhibitors.[21] However, only a weak reduction of the inhibitory potency could be achieved for bifunctionalized liposomes, which contain 10% of compound **13** compared to inhibitor **4**. Although the efficacy of neutralization was slightly enhanced for liposomes containing increasing amounts of derivative **13**, only partial neutralization was possible. Surprisingly, neutralization of free biotinylated thrombin inhibitors 4-MeO-bs-Lys(Adoa-Biotinyl)-Pro-4-Amba·TFA (**5**) and 4-MeO-bs-Asp(Tota-Biotinyl)-Pro-4-Amba·TFA (**9**) by addition of avidin was more effective. At present, we have no explanation for the found discrepancies and relatively poor efficacy of this antidote strategy.

Conclusions

The described strategy for the preparation of anticoagulant liposomes can be considered as proof of principle for the convenient incorporation of protease inhibitors into lipid formulations. Their significant anticoagulant activity and strongly increased positive ζ potential indicate that a large amount of the inhibitor is present on the surface. However, we cannot exclude that some molecules are incorporated in an opposite direction, whereby the inhibitory portion is located inside the liposomes. The same strategy could be applied to other drug molecules that tolerate modification with a linker coupled to a suitable fatty acid. Although we did not perform any pharmacokinetic studies, we assume that incorporation of these inhibitors into liposomes should increase their half-lives in circulation relative to that of the unmodified peptidic drug molecules. Until now, we have only used one standard mixture of DPPC/CH (molar ratio ≈2:1) as the liposomal formulation. Certainly, their stability can be further improved, for example, by adding small amounts of PEG-containing lipids such as 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[maleimide(polyethylene glycol)-2000], which protect the liposomes from rapid phagocytotic elimination.[29]

Experimental Section

General methods

Analytical HPLC experiments were performed with a Shimadzu LC-10 A system (analytical HPLC column: Nucleodur C_{18}, 5 μm, 100 Å, 4.6×250 mm, Machery-Nagel, Düren, Germany) with UV detection at $\lambda = 220$ nm. A linear gradient (1% increase in solvent B per minute, flow rate 1 mL min^{-1}) of 0.1% trifluoroacetic acid (TFA) in acetonitrile (solvent B) in 0.1% TFA in water (solvent A) at different starting concentrations of solvent B was used. The final inhibitors were purified to more than 95% purity (based on detection at $\lambda =$ 220 nm) by preparative HPLC (pumps: Varian PrepStar Model 218 gradient system, detector: ProStar Model 320, fraction collector: Varian Model 701) equipped with either a C_{18} column (Nucleosil, 5 μm, 300 Å, 32 mm×250 mm, Macherey–Nagel, Düren, Germany) or a C_8 column (Nucleodur, 5 μm, 100 Å, 32×250 mm) by using the same solvents (gradient: 0.5% increase in solvent B per minute, flow rate 20 mL min^{-1}). All final compounds were obtained as lyophilized TFA salts after preparative HPLC. The molecular masses of the synthesized compounds were determined with a QTrap 2000 ESI spectrometer (Applied Biosystems, now Life Technologies, Carlsbad, CA). ^{1}H NMR and ^{13}C NMR spectra were recorded by using a Jeol-ECX500 (Jeol Inc., Peabody, MA) and were referenced to internal solvent signals. The reagents for synthesis, including the amino acid derivatives, coupling reagents, and solvents, were obtained from Orpegen, Bachem, Calbiochem, Alfa Aesar, Fluka, Acros, or Aldrich. 1-(*tert*-Butyloxycarbonylamino)-4,7,10-trioxa-13-tridecanamine (Boc-Tota-H) and 8-(Cbz-amino)-3,6-dioxaoctanoic acid (Cbz-Adoa-OH) were purchased from Iris Biotech GmbH (Marktredwitz, Germany).

Synthesis

Inhibitors 1 and 2: Synthesized as previously described.[11]

4-MeO-bs-Lys(Adoa)-Pro-4-Amba·2 TFA (3): A mixture of compound **1**[11] (100 mg, 0.13 mmol) and Cbz-Adoa-OH (39 mg, 0.13 mmol) in DMF (2 mL) was cooled to 0 °C and then treated with *N*,*N*-diisopropylethylamine (DIPEA; 44 μL, 0.26 mmol) followed by the stepwise addition of 1-benzotriazolyloxytris(dimethylamino)-phosphonium hexafluorophosphate (BOP; 57.5 mg, 0.13 mmol).

The mixture was stirred for 15 min at 0 °C and for 3 h at room temperature. The solvent was evaporated under reduced pressure, and the remaining residue was treated with 33% HBr in acetic acid (1 mL). The solvent was removed under reduced pressure, and the product was purified by preparative HPLC and lyophilized from water to afford a white solid (66.8 mg, 56%). ^{1}H NMR (500 MHz, [D$_6$]DMSO): δ = 9.22 (s, 2H), 9.18 (s, 2H), 8.36 (t, J = 6.1, 1H), 7.80 (s, 3H), 7.73–7.62 (m, 6H), 7.37 (d, J = 8.5, 2H), 7.05–7.01 (m, 2H), 4.31 (dd, J = 16.3, 6.0, 1H), 4.25 (dd, J = 16.3, 6.1, 1H), 3.89–3.83 (m, 4H), 3.80 (s, 3H), 3.60–3.54 (m, 6H), 3.48–3.42 (m, 1H), 3.39–3.32 (m, 1H), 3.01–2.91 (m, 4H), 1.88–1.65 (m, 4H), 1.58–1.18 ppm (m, 6H). ^{13}C NMR (126 MHz, [D$_6$]DMSO): δ = 171.5, 169.1, 169.0, 165.4, 162.1, 158.4, 158.1, 145.9, 132.5, 128.7, 128.0, 127.1, 126.4, 113.9, 69.9, 69.9, 69.4, 66.9, 55.6, 53.5, 46.5, 41.5, 37.9, 31.4, 29.0, 28.6, 24.3, 22.1 ppm. MS: m/z (%): 690.29 [M+H]$^+$. HPLC (start at 10% solvent B): t_R = 18.4 min.

4-MeO-bs-Lys(Adoa-Palmitoyl)-Pro-4-Amba·TFA (4): Compound **3** (100 mg, 0.11 mmol) and palmitic acid (28 mg, 0.11 mmol) were coupled by using BOP (48.6 mg, 0.11 mmol) and DIPEA (38 μL, 0.22 mmol) as described for the preparation of compound **3**. DMF was evaporated under reduced pressure, and the product was purified by preparative HPLC and lyophilized from *tert*-butyl alcohol to afford a white solid (75 mg, 65.4%). ^{1}H NMR (500 MHz, [D$_6$]DMSO): δ = 9.24 (s, 2H), 9.08 (s, 2H), 8.35 (t, J = 6.1, 1H), 7.78 (t, J = 5.6, 1H), 7.75–7.65 (m, 5H), 7.62 (t, J = 5.9, 1H), 7.41 (d, J = 8.4, 2H), 7.06 (d, J = 9.0, 2H), 4.35 (dd, J = 16.3, 6.1, 1H), 4.29 (dd, J = 16.2, 6.1, 1H), 3.94–3.80 (m, 7H), 3.58–3.46 (m, 5H), 3.40 (t, J = 6.0, 3H), 3.22–3.15 (m, 2H), 3.00 (dd, J = 13.0, 6.4, 2H), 2.04 (t, J = 7.5, 2H), 1.93–1.68 (m, 4H), 1.61–1.16 (m, 32H), 0.85 ppm (t, J = 6.9, 3H). ^{13}C NMR (126 MHz, [D$_6$]DMSO): δ = 172.2, 171.5, 169.2, 169.0, 165.4, 162.1, 158.4, 158.1, 146.0, 132.5, 128.7, 128.0, 127.0, 126.3, 113.9, 70.1, 70.0, 69.3, 69.1, 59.4, 55.6, 53.5, 46.6, 41.5, 38.4, 37.8, 35.3, 31.2, 29.0, 29.0, 28.95, 28.9, 28.9, 28.7, 28.6, 28.6, 25.2, 24.3, 22.1, 22.0, 13.9 ppm. MS: m/z (%): 928.63 [M+H]$^+$. HPLC (start at 50% solvent B): t_R = 20.88 min.

4-MeO-bs-Lys(Adoa-Biotinyl)-Pro-4-Amba·TFA (5): Inhibitor **3** (100 mg, 0.11 mmol) and biotin (27 mg, 0.11 mmol) were suspended in DMF (2 mL) and coupled with BOP (48 mg, 0.11 mmol) in the presence of DIPEA (37 μL, 0.22 mmol) as described for the preparation of compound **3**. The product was purified by HPLC and lyophilized from water to afford a white powder (68 mg, 66%). ^{1}H NMR (500 MHz, [D$_6$]DMSO): δ = 9.24 (s, 2H), 9.01 (s, 2H), 8.35 (t, J = 6.0, 1H), 7.80 (t, J = 5.6, 1H), 7.75–7.65 (m, 5H), 7.62 (t, J = 5.9, 1H), 7.41 (d, J = 8.4, 2H), 7.10–7.02 (m, 2H), 6.44–6.26 (m, 2H), 4.36–4.28 (m, 2H), 4.12 (dd, J = 7.7, 4.5, 1H), 3.93–3.81 (m, 7H), 3.57–3.46 (m, 6H), 3.43–3.37 (m, 3H), 3.21–3.07 (m, 3H), 3.00 (dd, J = 13.4, 6.7, 2H), 2.82 (dd, J = 12.5, 5.1, 1H), 2.60–2.56 (m, 1H), 2.07 (t, J = 7.4, 2H), 1.90–1.24 ppm (m, 16H). ^{13}C NMR (126 MHz, [D$_6$]DMSO): δ = 172.1, 171.5, 169.2, 169.0, 165.3, 162.7, 162.1, 158.2, 157.9, 146.0, 132.5, 128.7, 128.0, 127.0, 126.3, 113.9, 70.1, 70.0, 69.3, 69.1, 61.0, 59.2, 55.6, 55.4, 53.6, 53.5, 46.6, 41.8, 41.5, 38.4, 37.8, 36.4, 35.1, 31.3, 29.0, 28.6, 28.1, 28.0, 25.2, 24.3, 22.1 ppm. MS: m/z (%): 916.47 [M+H]$^+$. HPLC (start at 10% solvent B): t_R = 24.52 min.

4-MeO-bs-Lys(Palmitoyl)-Pro-4-Amba·TFA (6): 4-MeO-bs-Lys-Pro-4-Amba (**1**; 100 mg, 0.13 mmol) and palmitic acid (33.3 mg, 0.13 mmol) were coupled by using BOP (57.5 mg, 0.13 mmol) and DIPEA (44 μL, 0.26 mmol) as described for the preparation of compound **3**. The compound was purified by preparative HPLC and lyophilized from *tert*-butyl alcohol to afford a colorless solid (91.6 mg, 78.6%). ^{1}H NMR (500 MHz, [D$_6$]DMSO): δ = 9.25 (s, 2H), 9.20–9.11 (m, 2H), 8.38 (t, J = 6.1, 1H), 7.76–7.63 (m, 6H), 7.41 (d, J = 8.4, 2H), 7.10–7.04 (m, 2H), 4.35 (dd, J = 16.3, 6.0, 1H), 4.29 (dd, J = 16.2, 6.1, 1H), 3.93 (dd, J = 7.7, 3.8, 1H), 3.88 (td, J = 8.5, 5.7, 1H), 3.83 (s, 3H), 3.52–3.36 (m, 2H), 2.91 (dd, J = 12.4, 6.4, 2H), 2.00 (t, J = 7.4, 2H), 1.94–1.67 (m, 4H), 1.64–1.33 (m, 4H), 1.33–1.09 (m, 28H), 0.85 ppm (t, J = 6.9, 3H). ^{13}C NMR (126 MHz, [D$_6$]DMSO): δ = 171.9, 171.5, 169.2, 165.4, 162.1, 158.5, 158.2, 145.9, 132.6, 128.7, 128.0, 127.1, 126.3, 113.9, 59.4, 55.6, 53.4, 46.6, 41.6, 38.1, 35.4, 31.3, 31.2, 29.0, 28.9, 28.7, 28.6, 28.5, 25.2, 24.3, 22.1, 22.0, 13.9 ppm. MS: m/z (%): 783.45 [M+H]$^+$. HPLC (start at 10% solvent B): t_R = 60.36 min.

4-MeO-bs-Asp(Tota)-Pro-4-Amba·2 TFA (7): Compound **2**[11] (100 mg, 0.15 mmol) and Boc-Tota-OH (48 mg, 0.15 mmol) were coupled in DMF (2 mL) by using BOP (66 mg, 0.15 mmol) and DIPEA (51 μL, 0.3 mmol) as described for the preparation of compound **3**. The solvent was evaporated under reduced pressure, and the residue was treated for 30 min with TFA (1 mL). After concentration under reduced pressure, the product was purified by preparative HPLC and lyophilized from water to afford a colorless solid (60 mg, 42%). ^{1}H NMR (500 MHz, [D$_6$]DMSO): δ = 9.30 (s, 2H), 9.20 (s, 2H), 8.17 (t, J = 6.2, 1H), 8.09 (d, J = 9.2, 1H), 8.03 (t, J = 5.5, 1H), 7.83–7.63 (m, 7H), 7.34 (d, J = 8.5, 2H), 7.06 (d, J = 9.0, 1H), 4.33–4.18 (m, 3H), 3.85–3.78 (m, 4H), 3.59 (dd, J = 11.9, 5.5, 1H), 3.51–3.35 (m, 13H), 3.26–3.20 (m, 2H), 2.92–2.73 (m, 4H), 2.62 (dd, J = 15.4, 9.4, 1H), 2.32 (dd, J = 15.5, 5.3, 1H), 1.83–1.69 (m, 4H), 1.41 ppm (quint., J = 6.9, 2H). ^{13}C NMR (126 MHz, [D$_6$]DMSO): δ = 171.1, 169.4, 169.0, 165.4, 162.2, 158.5, 158.3, 145.8, 132.6, 128.6, 127.9, 126.9, 126.3, 118.3, 115.9, 114.0, 69.6, 69.6, 69.4, 69.4, 67.8, 67.3, 59.9, 55.7, 50.2, 46.4, 41.6, 36.7, 35.7, 29.0, 28.8, 27.1, 23.7 ppm. MS: m/z (%): 734.37 [M+H]$^+$. HPLC (start at 10% solvent B): t_R = 19.65 min.

4-MeO-bs-Asp(Tota-Palmitoyl)-Pro-4-Amba·TFA (8): Compound **7** (50 mg, 52 μmol) and palmitic acid (13.3 mg, 52 μmol) were coupled by using BOP (23 mg, 52 μmol) and DIPEA (17.7 μL, 0.1 mmol) as described for the preparation of compound **3**. The product was purified by preparative HPLC and lyophilized from *tert*-butyl alcohol to afford a colorless solid (30 mg, 53%). ^{1}H NMR (500 MHz, [D$_6$]DMSO): δ = 9.28–9.16 (m, 2H), 9.07 (s, 2H), 8.16 (t, J = 6.2, 1H), 8.10 (d, J = 9.1, 1H), 8.07–7.99 (m, 1H), 7.79–7.63 (m, 5H), 7.45 (d, J = 8.4, 1H), 7.34 (d, J = 8.4, 1H), 7.09–6.98 (m, 2H), 4.36–4.21 (m, 2H), 3.85–3.77 (m, 3H), 3.59 (dd, J = 14.6, 8.1, 1H), 3.49–3.19 (m, 14H), 3.05–2.73 (m, 4H), 2.64 (dd, J = 15.5, 9.8, 1H), 2.32 (dd, J = 15.5, 5.0, 1H), 2.04–1.65 (m, 5H), 1.61–1.36 (m, 6H), 1.20 (s, 24H), 0.82 ppm (t, J = 6.9, 3H). ^{13}C NMR (126 MHz, [D$_6$]DMSO): δ = 172.0, 171.0, 169.4, 169.0, 165.3, 162.2, 158.4, 158.1, 145.9, 132.7, 128.5, 128.1, 127.9, 127.2, 126.9, 126.2, 114.3, 114.0, 69.7, 69.5, 69.4, 68.1, 67.8, 60.0, 55.7, 50.2, 46.4, 41.7, 35.6, 35.3, 31.2, 29.4, 29.2, 29.0, 28.7, 28.6, 25.2, 23.6, 22.0, 13.9 ppm. MS: m/z (%): 972.41 [M+H]$^+$. HPLC (start at 10% solvent B): t_R = 62.57 min.

4-MeO-bs-Asp(Tota-Biotinyl)-Pro-4-Amba·TFA (9): Compound **7** (100 mg, 0.1 mmol) and biotin (24 mg, 0.1 mmol) were suspended in DMF (2 mL) and coupled by treatment with BOP (44 mg, 0.1 mmol) and DIPEA (34 μL, 0.2 mmol) as described for the preparation of compound **3**. The product was purified by preparative HPLC and lyophilized from water to afford a colorless solid (68 mg, 63%). ^{1}H NMR (500 MHz, [D$_6$]DMSO): δ = 9.22 (s, 2H), 9.12 (s, 2H), 8.20 (t, J = 6.1, 1H), 8.14 (d, J = 9.1, 1H), 8.06 (t, J = 5.4, 1H), 7.74–7.67 (m, 5H), 7.38 (d, J = 8.2, 2H), 7.09 (d, J = 8.9, 2H), 6.36 (d, J = 24.2, 2H), 4.37–4.21 (m, 4H), 4.16–4.10 (m, 1H), 3.89–3.81 (m, 4H), 3.63 (t, J = 7.8, 1H), 3.52–3.35 (m, 13H), 3.25 (t, J = 6.3, 2H), 3.12–3.03 (m, 3H), 2.90 (dq, J = 12.9, 6.6, 1H), 2.86–2.76 (m, 2H), 2.67 (dd, J = 15.5, 9.8, 1H), 2.61–2.55 (m, 1H), 2.35 (dd, J = 15.4, 5.0, 1H), 2.04 (t, J = 7.4, 2H), 1.85–1.68 (m, 4H), 1.63–1.41 (m, 8H), 1.34–1.24 ppm (m, 2H). ^{13}C NMR (126 MHz, [D$_6$]DMSO): δ = 171.9, 171.0,

169.4, 169.0, 165.3, 162.7, 162.2, 158.4, 158.1, 132.7, 128.5, 127.9, 126.9, 126.2, 114.0, 69.7, 69.5, 69.4, 68.1, 67.8, 61.0, 60.0, 59.2, 55.7, 55.4, 50.2, 46.4, 41.7, 35.7, 35.2, 29.4, 28.8, 28.2, 28.0, 25.2, 23.7 ppm. MS: *m/z* (%): 960.53 $[M+H]^+$. HPLC (start at 10% solvent B): $t_R = 26.03$ min.

Biological evaluation

Enzyme kinetic measurements: Kinetic measurements were performed with a Fluoroskan Ascent Microplate reader (Thermo Fisher Scientific Inc.) by using 50 mM Tris·HCl buffer (pH 8.0 at room temperature, containing 0.1 M NaCl and the inhibitor, 100 μL), substrate (20 μL) diluted with water and enzyme (20 μL) (total assay volume 140 μL). Measurements with bovine α-thrombin (prepared according to Walsmann,[30] 15 pM in assay) and uPA (Medac, ≈1.7 nM in assay) were performed with the substrate Tos-Gly-Pro-Arg-AMC (K_M for thrombin: 4 μM; K_M for uPA: 41 μM), whereas Mes-D-Ser(Bzl)-Phe-Arg-AMC (Mes = methylsulfonyl, Bzl = benzyl) was used for human plasmin (Calbiochem, ≈350 pM in assay, $K_M = 25$ μM) and Mes-D-Arg-Pro-Arg-AMC[31] was used for factor Xa (FXa; Enzyme Research South Bend, IN, USA, ≈100 pM in assay, $K_M = 22$ μM). In all measurements, linear progress curves were observed. The K_i values of the inhibitors summarized in Tables 1 and 2 were calculated from Dixon plots.

The inhibitory potency of the prepared liposomes against thrombin, given as IC_{50} values, was determined as described above by using a single substrate concentration of 11 μM Tos-Gly-Pro-Arg-AMC. The data shown in the IC_{50} curves (Figure 4) were fitted to the three-parameter Equation (1),[32] in which *v* is the steady-state velocity at different inhibitor concentrations, v_0 is the constant velocity in the absence of inhibitor, *I* is the inhibitor concentration, and *s* is a slope factor. The inhibitor concentration on the *x* axis was calculated on the basis of the total inhibitor amount used for the preparation of the liposomes, assuming that it was completely incorporated and distributed on the surface.

$$v = \frac{v_0}{1 + \left(\frac{I}{IC_{50}}\right)^s} \quad (1)$$

Anticoagulant activity in plasma: The aPTT measurements were performed in a Thrombotrack 8 (Immuno GmbH, Heidelberg, Germany) by using a commercial test kit (Diagnostica Stago, Düsseldorf, Germany). Platelet-poor plasma (100 μL, German Red Cross, Frankfurt, prior incubated at 37 °C), a mixture of phospholipid cephalin and contact activator Kaolin (100 μL), and a inhibitor- or liposome-containing solution in 50 mM Tris-buffer (pH 7.8 containing 0.1 M NaCl, 80 μL) was incubated at 37 °C for 6 min and clotting was initiated by adding 0.02 M $CaCl_2$ (100 μL) prewarmed to 37 °C. The determined clotting times were plotted as a function of the inhibitor concentrations.

Neutralization of bifunctional liposomes by avidin: Avidin-based neutralization of the thrombin inhibitory potency was performed in microtiter plates in the same buffer as that described above for the enzyme kinetic measurements by using 15 pM thrombin and 11 μM Tos-Gly-Pro-Arg-AMC in the assay. The amounts of liposomes and inhibitors used were adjusted to provide approximately 90% thrombin inhibition. Briefly, the substrate (20 μL) dissolved in water, liposomes or inhibitors in buffer (50 μL), and avidin (50 μL, 0.055–3.6 μM in assay, avidin obtained from Calbiochem) in buffer were mixed and the measurement was started by the addition of thrombin (20 μL). The determined residual thrombin activities (in percentage relative to a control without inhibitor) were plotted against the avidin concentration.

Preparation of antithrombotic liposomes

Liposomes containing thrombin inhibitors **4** and **6** were prepared by using a thin-film hydration method as described previously.[16,17] The membrane was composed of a mixture of DPPC, CH, and palmitoylated inhibitors **4** and **6** in a ratio of 65:30:5 mol%. Stock solutions of the lipids and inhibitors were prepared in a mixture of chloroform/methanol (2:1 *v/v*). For the preparation of the anticoagulant liposomes, the components were mixed from the stock solutions in a round-bottomed flask to a total amount of 10 mg lipid and inhibitor. The lipid and inhibitor mixture was dried to a thin film by using a rotary evaporator under vacuum at 40 °C. The resulting film was rehydrated with phosphate-buffered saline (PBS, 1 mL), pH 7.4 (0.15 M). After vigorous shaking, the lipid dispersions were sonicated in a bath-type sonicator at 55 °C for 2 min and stored by 4 °C. This method provided liposomes with an anticoagulant surface. Bifunctionalized liposomes used for the neutralization assay with avidin were obtained in the same manner by the addition of biotinylated compound **13** in the lipid and inhibitor mixture. The concentration of compound **13** in the mixture was 10% of inhibitor **4** or **6**, respectively. Additional bifunctionalized liposomes with increasing concentrations of biotin derivative **13** (20, 50, and 100%) were prepared from inhibitor **4**.

Physicochemical characterization of liposomes

The physicochemical properties of the liposomes including size, charge, shape, and morphology were investigated by using dynamic light scattering (DLS), laser Doppler velocimetry (LDV), and atomic force microscopy (AFM).

Dynamic light scattering: The hydrodynamic diameter of lipid formulations, prepared and stored as described above, were determined by DLS by using a Zetasizer Nano ZS (Malvern Instruments, Herrenberg, Germany) equipped with a 10 mW HeNe laser at a wavelength of 633 nm at 25 °C, as described previously.[33] Scattered light was detected at an angle of 173° with laser attenuation and measurement position adjusted automatically by the Malvern software. Values given are the means ± standard deviation of three independent experiments with each experiment comprising three measurements of the same sample with at least 10 runs, as determined by the Zetasizer. The average value was calculated with the volume distribution data of the samples ± standard deviation.

Laser Doppler velocimetry: The ζ potential was measured with a Zetasizer Nano ZS (Malvern Instruments, Herrenberg, Germany) at 25 °C and a scattering angle of 17° by measuring electrophoretic mobility with LDV. Values given are the means ± standard deviation of three independent experiments with each experiment comprising three measurements of the same sample with at least 10 runs, as determined by the Zetasizer. The average value was calculated with data of the samples ± standard deviation.

Atomic force microscopy: The lipid formulations (20 μL) were transferred onto a silicon chip and left to dry. AFM was performed with a vibration-damped Nanoscope IV Bioscope (Veeco Instruments, Mannheim, Germany) as described in detail elsewhere.[34] Commercial pyramidal Si_3N_4 tips (NSC16 AlBS, Micromash, Estonia) were mounted on a cantilever (length 230 μm, resonance frequency 170 kHz, and nominal force constant 40 N m^{-1}) and measurements were performed in tapping mode to minimize damage to

the specimens. The scan speed was proportional to the scan size and the scan frequency was between 0.5 and 1.5 Hz. Images were obtained by displaying the amplitude signal of the cantilever in the trace direction, and the height signal in the retrace direction, both signals being simultaneously recorded. The results were visualized either in height or in amplitude mode.

Keywords: anticoagulants · inhibitors · liposomes · peptidomimetics · thrombin

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