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Bioorganic & Medicinal Chemistry Letters 18 (2008) 2028–2031
Bioorganic & Medicinal Chemistry Letters

Synthesis and biological evaluation of PEG-tirofiban conjugates

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Received 16 November 2007; revised 29 January 2008; accepted 29 January 2008
Available online 2 February 2008

Abstract—We have conjugated tirofiban, an antagonist of the GPIIb/IIIa integrin receptor, to PEG, and shown that these polymers effectively inhibit platelet aggregation. This inhibition decreased with the size of the polymer. Our goal was to develop new cryoprotective agents to store frozen platelets. Surprisingly, tirofiban-conjugated PEG did not exhibit any protection.

Platelets cannot be conserved more than five days, which causes regularly some shortage in the stocks of blood banks. Consequently, there is an ongoing need to develop a method to store platelets for a longer time. Their storage in the frozen state has been limited by the toxicity and the lack of efficiency of cryoprotective agents.[1] DMSO protects platelets from the stress of freezing and thawing, however, residual DMSO in platelet concentrate induces nausea, vomiting, local vasospasm and garlic-like taste, which makes it unsuitable for clinical purposes.[1,2] On the other hand, polyethylene glycol (PEG), which has been used for many years as a cryoprotectant for several tissues and cells, is well tolerated by the organism. However, its cryoprotective efficiency is not sufficient for platelets.[3] The main damages occur at the level of the plasmic membrane that loses its integrity due to formation of ice crystals during freezing and thawing.

We aimed at improving this cryoprotection by conjugating PEG to the ligand of a membrane receptor concentrate the polymer close to the membrane where it could display its cryoprotective effects (Fig. 1). We expected this layer of cryoprotective polymer to maintain membrane integrity during freezing and thawing. The membrane receptor that we selected is the GPIIb/IIIa integrin receptor because it is the most abundant one at the platelet surface (more than 40,000 copies per platelet).[4] The ligand had to be an antagonist to prevent it from triggering the aggregation of platelets.

Keywords: Poly(ethylene glycol), PEG; Cryopreservation, Platelet aggregation; Antithrombotic; GPIIb/IIIa receptor.
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doi:10.1016/j.bmcl.2008.01.118

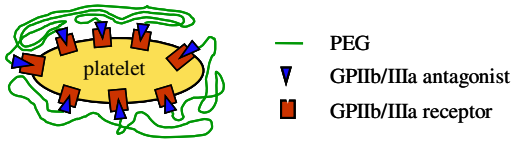


Figure 1. Platelet wrapping by PEG conjugated to a GPIIb/IIIa antagonist.

The first task of our research program was to design and synthesize a GPIIb/IIIa antagonist that could be easily conjugated to PEG. Tirofiban (**1**) is an antithrombotic drug developed by Merck scientists that binds with a high affinity to GPIIb/IIIa receptor even when platelets are not activated (K_d = 13 nM).[5] On the basis of SAR studies that showed that the butylsulfonamide moiety could be replaced by various arylsulfonamides,[6] we envisioned compound **2** to be a suitable ligand for conjugation to PEG (Fig. 2).

The general approach to prepare **2** was based on the synthesis of tirofiban developed by Merck scientists (Scheme 1).[7] Deprotonation and alkylation of picoline **3** with bromide **4** followed by deprotection afforded pyridinylbutanol **5** (71% from **3**). Hydrogenation over Adams' catalyst and N-Boc protection gave **6** (57%, 2 steps). This alcohol was used to alkylate protected tyrosine **7** by a Mitsunobu reaction (76%). Removal of the Cbz and sulfonylation of the free amine completed the synthesis of the GpIIb/IIIA-binding moiety of the ligand.

Completion of the synthesis required the introduction of the linker moiety by a Sonogashira's reaction with

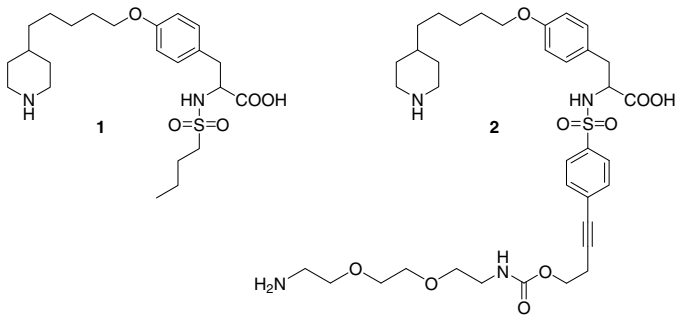
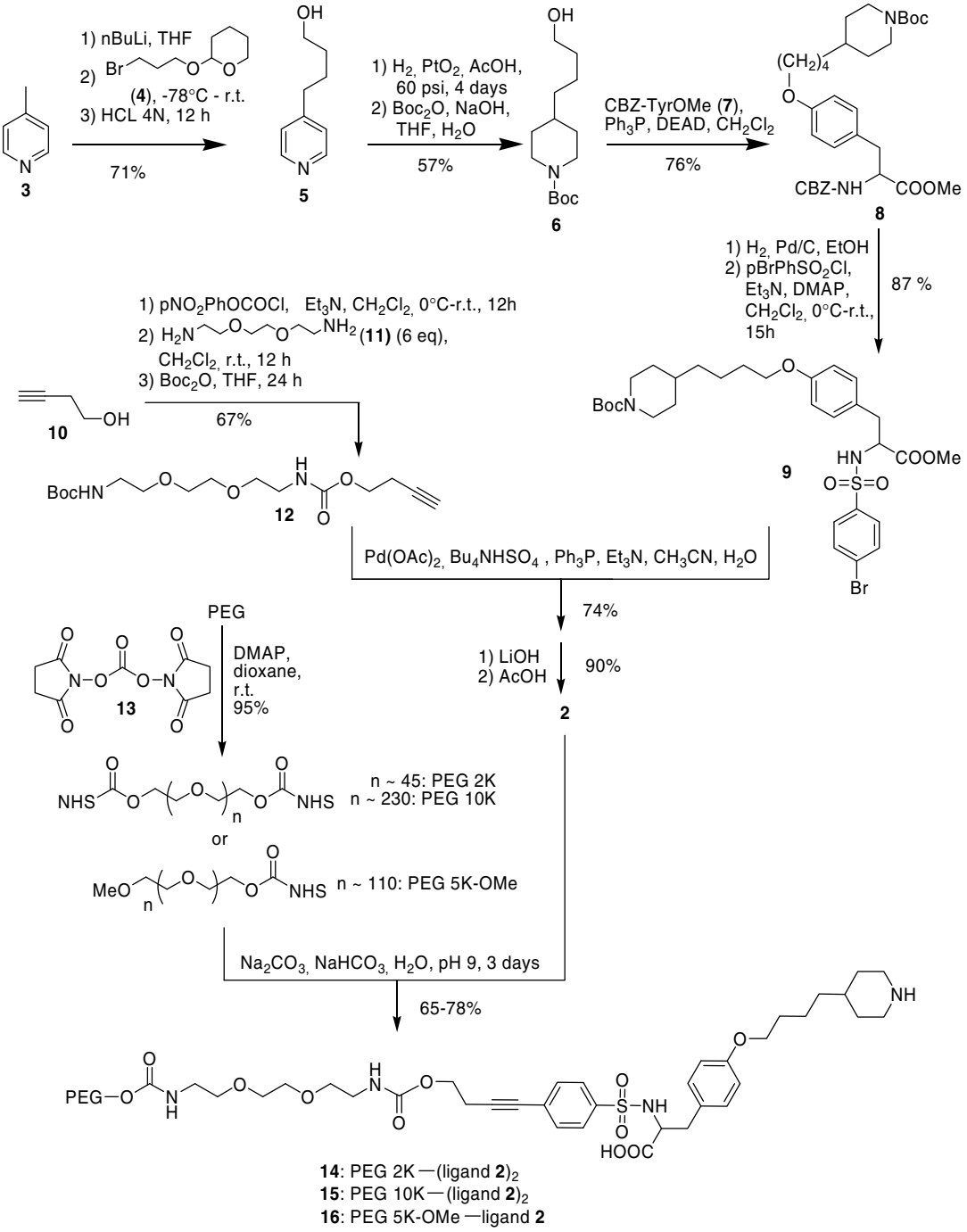


Figure 2. Tirofiban (**1**) and its analogue to be conjugated (**2**).



Scheme 1. Synthesis of ligand **2** and its conjugation to PEG.

alkyne **12**. This reagent was easily prepared in 3 steps from butynol **10**. Reaction of this alcohol with an excess of 4-nitrophenylchloroformate cleanly provided an activated carbonate, whose reaction with an excess of diamine **11** and subsequent protection as a Boc efficiently afforded **12** (67%, 3 steps).

Optimization of the Sonogashira coupling between **9** and **12** led to a satisfying yield of 74% under Jeffery's conditions.[8] Saponification of the ester and deprotection of the N-Boc afforded the ligand **2** (90%, 2 steps).

Initial attempts to conjugate **2** to PEG using 4-nitrophenylchloroformate according to Schiavon's procedure gave low yields.[9] However, the use of di-succinimidyle carbonate **13**[10] in presence of DMAP in dioxane afforded the corresponding activated PEG in 95% yields. First attempts to conjugate these activated carbonates with ligand **2** in chloroform or pyridine gave poor yields. Finally, we found that performing this conjugation with ligand **2** in an aqueous buffer at pH 9 efficiently afforded the expected adducts **14**–**16** (65–78% yields).[11,12]

Before examining the cryoprotective properties of **14**–**16**, we tested whether PEG conjugates retain the binding and pharmacological properties of tirofiban. We checked that these conjugates inhibit the aggregation of platelets activated by ADP, collagen or TRAP (a thrombin receptor agonist) according to established procedures (Fig. 3).[13] We showed that better inhibition of platelet aggregation is achieved with a shorter polymer conjugate: PEK 2K conjugate **14** abolished totally the aggregation at a 10 μM concentration, while PEG 5K and 10K conjugates **15** and **16** required a 10-fold higher concentration to achieve the same effect. This could be due to the repulsive effect of PEG that tends to repel these conjugated polymers from the membrane and its receptors.

We observed that the aggregation induced by collagen and TRAP was less sensitive to the inhibition by PEG conjugates than the one induced by ADP, however, the activity profile remained the same. Overall, these data confirmed that PEG conjugates **14**–**16** efficiently bind to the GPIIb/IIIa receptor and do not exhibit deleterious effects on platelet function.

Next, we examined the cryoprotective properties of **14**, **16** and regular PEG using Dayan's method for freezing platelets.[14] Briefly, a solution of the polymer was added to a suspension of human platelets in a glycerol–glucose medium. This preparation was frozen in liquid nitrogen using Dayan's statically controlled cooling rate device, carefully thawed in a bath at 37 °C, and washed by centrifugation. We examined the swirling and the aggregating properties and compared them to a non-frozen sample. In all frozen samples, swirling was slightly reduced, which indicates that a portion of the platelets changed their shape and were not able to aggregate anymore. Platelet frozen in the presence of regular PEG 2K and PEG 10K retained partially their ability to aggregate in response to ADP (Table 1).

Surprisingly, this ability was totally lost with **14** and **16**, indicating that conjugating tirofiban analogue **2** to PEG abolishes cryoprotective properties of PEG. This might be explained by a destabilization of the membrane bilayer by PEG due to a depletion interaction.[15]

None of the platelet samples responded to collagen, which shows that the activation by collagen is more sensitive to freezing stress, than the one by ADP.

Several drugs have been conjugated to PEG in order to modify their bioavailability.[16] None of them were designed for cryopreservation. We provided therein the first experimental evidence that such an approach is unlikely to be successful. We also demonstrated that PEG-tirofiban conjugates effectively inhibit platelet aggregation, and that this inhibition decreases with the size of the polymer.

Table 1. Aggregation properties of platelet samples that were frozen in the presence of 0.2 mM PEG or PEG-**2** conjugates (expressed as a percentage relative to a non-frozen sample)

	% Aggregation induced by ADP	% Aggregation induced by collagen
Control (no polymers)	0	0
PEG 2K	40	0
14	0	0
PEG 10K	32	0
16	0	0

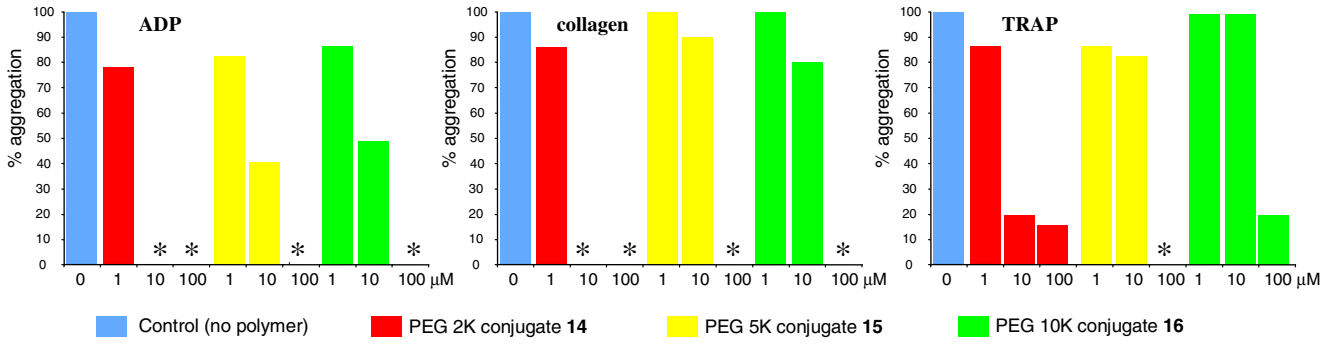


Figure 3. Effect of PEG conjugates **14**–**16** on human platelet aggregation induced by ADP (5 μM), collagen (2.5 mg/ml) or TRAP (1 μM). The concentrations of polymers are based on the quantity of conjugated tirofiban (* 0% aggregation).

Acknowledgment

Financial support from 'Délégation Générale pour l'Armement' (Grant No. 0334037) is gratefully acknowledged.

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12. Compound **2** (2 equiv) reacted with succinimidyl carbonate PEG in an aqueous buffer at pH 9 according to Ref. 11. After 72 h, the solution was filtered, dialyzed three times and lyophilized to afford the adduct as a white solid, which was analyzed by ^{1}H NMR in DMSO-d_6.
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