Alexa Fluor 680-NH-CO-CH_2-S-CH_2-Phe-Pro-Arg-CH_2-prothrombin

AF680-ProT

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Created: March 15, 2012; Updated: May 15, 2012.

Chemical name:	Alexa Fluor 680-NH-CO-CH_2-S-CH_2-Phe-Pro-Arg-CH_2-prothrombin	
Abbreviated name:	AF680-ProT	
Synonym:		
Agent Category:	Proteins	
Target:	Staphylocoagulase	
Target Category:	Bacterial enzyme	
Method of detection:	Optical imaging	
Source of signal / contrast:	Alexa Fluor 680	
Activation:	No	
Studies:	• *In vitro* • Rodents	No structure is available.

Background

[PubMed]

The fluorescently labeled prothrombin analog, Alexa Fluor 680-NH-CO-CH_2-S-CH_2-Phe-Pro-Arg-CH_2-prothrombin, abbreviated as AF680-ProT, is a probe developed by Panizzi et al. for detecting *Staphylococcus aureus* (*S. aureus*) endocarditis by targeting staphylocoagulase (1).

Infective endocarditis is a life-threatening condition that is commonly caused by *S. aureus*, and the hallmark of *S. aureus* endocarditis is the development of vegetations on

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NLM Citation: Shan L. Alexa Fluor 680-NH-CO-CH_2-S-CH_2-Phe-Pro-Arg-CH_2-prothrombin. 2012 Mar 15 [Updated 2012 May 15]. In: Molecular Imaging and Contrast Agent Database (MICAD) [Internet]. Bethesda (MD): National Center for Biotechnology Information (US); 2004-2013.

heart valves (2). After entering into the bloodstream, *S. aureus* proliferates quickly, colonizes on either damaged or normal heart valves, and leads to a rapid progressive endocarditis with destruction of the heart valves (3-5). In this pathological process, adherence of *S. aureus* to heart valves is the key to initiate the vegetation formation, and SC is one of the most important stimulators of the initiation. SC is an extracellular protein secreted by coagulase-positive S. aureus, and it can bind prothrombin with a high affinity (K_d = ~17–72 pM) in a 1:1 stoichiometry (6-8). This binding forms an active SC-prothrombin complex, which induces plasma coagulation *via* specific cleavage of fibrinogen to fibrin (7, 9). The N-terminal D1 and D2 regions of SC are involved in the binding with prothrombin and its activation, and the C-terminal repeat region, comprising 27-amino-acid tandem repeats, is associated with the adherence of SC to fibrinogen (10, 11).

Panizzi et al. synthesized an engineered analog of human prothrombin in which the active site was modified by a thrombin inhibitor that possesses a protected thiol group (1, 6). Thus, the prothrombin analog could react with a thiol-specific Alexa Fluor 680 dye (AF680-ProT) for fluorescence imaging or with diethylenetriamine pentaacetic acid (DTPA) for ^{64}Cu-labeling (^{64}Cu-DTPA-ProT). The rationale underlying this design is the SC-dependent prothrombin recruitment, which simultaneously tethers the active SC-prothrombin analog complex into the fibrin(ogen)-rich *S. aureus* vegetations. Imaging studies by Panizzi et al. showed that the two probes could bind SC and intercalate into growing bacterial vegetations on the heart valves, with the potential to detect *S. aureus* endocarditis and monitor antibiotic therapy *via* noninvasive optical imaging and positron emission tomography (1).

This chapter summarizes the data obtained with AF680-ProT, and another chapter summarizes the data obtained with ^{64}Cu-DTPA-ProT.

Related Resource Links:

The nucleotide and protein sequences of staphylocoagulase in GenBank

The structure of staphylocoagulase

Synthesis

[PubMed]

Panizzi et al. described the synthesis of AF680-ProT in detail (1, 6). Prothrombin was purified from human plasma (absorption coefficient, 1.47 (mg/ml)$^{-1}$cm^{-1}; molecular weight, 71.6 kDa). Met-SC-(1–325)-His$_6$ fragment was expressed in E. coli strain BL21(DE3) plysS and purified from the soluble fraction after centrifugation. An active site was first induced through the binding of Met-SC-(1–325)-His$_6$ with prothrombin. This site was then inactivated *via* Ser^{195} and His^{57} alkylation with the thiol group-possessing thrombin inhibitor N$^{\alpha}$-[(acetylthio)acetyl]-D-Phe-Pro-Arg-CH_2Cl (ATA-FPR-CH_2Cl). The resulting Met-SC-(1–325)-His$_6$·ATA-FPR-ProT complex was fluorescently labeled

through the reaction with a 10-fold molar excess of thiol-reactive Alexa Fluor 680 C_2 maleimide. Finally, the Met-SC-(1–325)-His_6 was dissociated with NaSCN from the fluorescently labeled complex, and the desired product, AF680-ProT, was separated with gel filtration chromatography. The chemical purity of AF680-ProT and the number of dye moieties per probe were not described in detail.

In Vitro Studies: Testing in Cells and Tissues

[PubMed]

Panizzi et al. first determined the activity of the residual active prothrombin in the final product because the residual active prothrombin could trigger downstream activation of the clotting cascade *in vivo* (1). The results showed a residual activity of 0.3% for AF680-ProT, indicating less potential to trigger clotting cascade when used *in vivo*.

Panizzi et al. then verified whether the recombinant N-terminal fragment SC (1–325) was able to activate mouse prothrombin. The investigators found that the SC (1–325)-prothrombin complex hydrolyzed the chromogenic substrate of H-D-Phe-Pip-Arg-*p*NA (S2238) at a rate that was equivalent to that of mouse thrombin (k_{cat} = 26 ± 1 s^{-1}) (1, 6). The supernatants of various *S. aureus* strains were able to clot mouse plasma. These data indicate that the mouse is an appropriate model organism to study SC function and regulation *in vivo*.

To determine the molecular mechanism underlying prothrombin recruitment to vegetations, Panizzi et al. performed *in vitro* native gel binding experiments using either the N-terminal active fragment SC (1–325) or the full-length SC (1–660) (1, 6). After incubation of these SC forms with prothrombin and fibrinogen fragment D (FragD), SC (1–325) and prothrombin bound together but lacked the ability to interact with FragD. However, SC (1–660) formed an SC-prothrombin-FragD ternary complex, and multiple FragD subunits interacted with a single SC molecule. The SC recombinant fragment that contained only the pseudorepeat and the first SC repeat could bind FragD with a K_d of 36 ± 8 nM. These results suggest that SC could interact with at least four fibrinogen or fibrin molecules per SC molecule to form a megaprotein complex, and this complex anchored the active SC-prothrombin complex to the growing vegetation.

Animal Studies

Rodents

[PubMed]

To test the feasibility of AF680-ProT to image vegetations, Panizzi et al. established several mouse models of infective endocarditis by inserting a segment of suture material down the right carotid artery into the heart to induce damage of the aortic valve and by injecting bacteria (1 × 10^6 colony-forming units per 100 ìl phosphate-buffered saline (PBS)) *via* the tail vein 24 h later (1). Bacterial strains used to establish disease models

included SC-positive *S. aureus* strains (Newman D2 Tager 104, Xen29, and Xen8.1), SC-negative *S. epidermidis* FDA strain PCI 1200, and Newman SC and von Willebrand factor–binding protein (vWbp) double-knockout *S. aureus* (n = 6 mice/group). Newman D2 Tager 104, Xen29, and Xen8.1 are vancomycin-susceptible. Endocarditis was observed in >85% of the mice, but the size of formed vegetations and the extent of occlusion of the aortic valve varied, potentially correlating with the extent of denuded endothelium caused by the mechanical injury. On day 2 after suture insertion and 24 h after induction of bacteremia, 30–45 ìg AF680-ProT was injected *via* the tail vein, and imaging was performed 24 h later with fluorescence molecular tomography fused to X-ray computed tomography (FMT-CT). The isotropic spatial resolution was 110 ìm for CT and 1 mm for FMT.

FMT-CT imaging showed that AF680-ProT accumulated in the vegetations, which was consistent in mice induced with Tager 104, Xen29, and Xen8.1 strains, but not in mice infected with *S. epidermidis* or in mice without bacteremia. The fluorescence intensity in the aortic outflow tract in mice with *S. aureus*–induced vegetations was 20- to 28-fold higher than that in the mice without bacteremia and the mice with *S. epidermidis* challenge (1). AF680-ProT had a blood half-life of 79 ± 14 min.

The expression pattern of SC within vegetations was investigated in the excised aortas with vegetations (1). AF680-ProT was co-localized with SC at the interface of vegetations with the host's circulation, although bacteria were present throughout the vegetations. This result was consistent with the findings from immunoreactive staining for SC and vWbp and from *in situ* hybridization for SC RNA, which showed that the proteins and RNA were all limited to the periphery of vegetations. This SC expression pattern was considered to be the result of the differential expression of SC during vegetation development; as in younger lesions with lower bacterial burden, the entire *S. aureus* population stained positive for SC.

To image the efficiency of AF680-ProT for monitoring vancomycin therapy, mice were given daily intraperitoneal injections of 10 ìg vancomycin or PBS for 6 days (n = 8–10 mice/group) (1). FMT-CT imaging at 48 h after infection indicated that AF680-ProT was able to quantify the effect of vancomycin, showing that vancomycin treatment eliminated bacteria in vegetations and that termination of the therapy resulted in recurrence of the infection and a high risk of mortality, similar to the relapse observed in some patients with infective endocarditis.

To determine the specificity of AF680-ProT for endocarditic vegetations that contain SC, femoral artery thrombosis was first induced *via* topical application of $FeCl_3$ (1). No accumulation of AF680-ProT was observed in the thrombus after injection of 25 ìg AF680-ProT. Endocarditis was then induced with the *S. aureus* strain that is deficient of both SC and vWbp (n = 10–13 mice). Only background levels of AF680-ProT accumulation were detected in the vegetations. Improved survival was also observed in these mice, suggesting that SC increases the virulence of *S. aureus*. Histology revealed leukocyte infiltration and the absence of the protective fibrin barrier in the vegetations

induced by the SC and vWbp double-knockout *S. aureus*, indicating an impaired ability of the bacteria to evade the host defense. In the mice infected with an *S. aureus* strain that lacks only SC (n = 5 mice), AF680-ProT accumulation in the vegetations was reduced to 14% compared to mice that were infected with isogenic wild-type Newman strain bacteria ($P < 0.0001$).

Toxicity of the AF680-ProT was studied after three injections of 10 times (250 µg) the imaging dose over one week (n = 5 mice) (1). Pathological examination of the normal tissue sections revealed no abnormalities such as inflammatory foci, necrosis, clots, or bleeding. No differences were observed for the prothrombin time for either human or mouse plasma after incubation with the prothrombin analog (250 µg) at 37°C. Tail tip bleeding assay also showed no significant changes in clotting parameters in mice injected with the probe (n = 5 mice).

Other Non-Primate Mammals

[PubMed]

No references are currently available.

Non-Human Primates

[PubMed]

No references are currently available.

Human Studies

[PubMed]

No references are currently avaliable.

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