Engineered Thrombin Aims to Take on Heparin

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Blood clots may not look like much, just tiny blobs of cells, lipids, and fibrin. But clots can either save our lives or end our lives, depending on when and where they form. The process that brings about clotting, thrombosis, is a highly regulated cascade of protein interactions, with thrombin running the show. Thrombin helps regulate coagulation by living a yin-yang life, acting both as a procoagulant or an anticoagulant depending on which protein it binds or doesn't bind to. Thrombin can even turn itself off in a negative feedback loop. These features give us a natural ability to keep blood liquid enough to flow through the circulatory system and gelatinous enough to form a clot so that we don't bleed out. If thrombin is activated under the wrong conditions, though, clotting can lead to debilitating or fatal consequences such as heart attack, stroke, or deep vein thrombosis.

To treat or prevent such conditions, physicians use drugs called antithrombotics, which come in two categories depending on their action: anticoagulants or antiplatelets. Thrombin is also a primary activator of platelets, tiny cells that help blood clot.

Many medical conditions call for anticoagulant drugs, administered either short or long term. People on dialysis need anticoagulants so blood doesn't clot in the port that links the dialysis machine to their blood supply. People with atrial fibrillation are at higher risk of stroke because a fast, irregular heartbeat slows blood flow and thus raises the risk of clotting. Orthopedic and cardiac surgery can raise the risk of clotting in the legs, called deep vein thrombosis.

Two of the oldest anticoagulants on the market are warfarin (Coumadin) and heparin. Coumadin is given orally and takes a few days to work; it's commonly used to prevent stroke associated with atrial fibrillation. Heparin is injected and works in minutes; it's used in cardiac bypass surgery and in orthopedic surgery such as hip replacement.

Both drugs are tricky to dose because the so-called therapeutic window is narrow: too much and the drug causes bleeding, too little and the drug won't work. Although Coumadin and heparin are the most commonly used anticoagulants, the dosing problem found with them is true for any antithrombotic drug, because anticoagulants disable hemostasis, the system that keeps our blood flowing inside the circulatory system and plugs damaged vessels.

"We are bringing closer to the market something that exploits a natural pathway of anticoagulation in ways that do not produce bleeding." – Enrico Di Cera

Imagine clotting working like a two-phase system: an injured blood vessel brings a procoagulant response followed quickly by an anticoagulant response. If drugs boost the anticoagulant side of the response, the procoagulant side is shut down faster, so the net effect tilts toward anticoagulation. That's the down side of the use of the current drugs, because all drugs like Coumadin stop the procoagulation side.

"Until the safety of anticoagulation is solved, we'll have no solution for stroke and heart attack, and these diseases will remain the leading causes of death, says Andras Gruber, M.D., a thrombin expert at Oregon Health & Science University School of Medicine. An anticoagulant that can be administered to patients with severe acute thrombotic diseases such as heart attack and stroke, without the risk of causing hemorrhage, would be so significant as to revolutionize the treatment of cardiovascular and cerebrovascular diseases."

In 2010, two new oral anticoagulants hit the market: dabigatran, which is a direct thrombin inhibitor, and rivaroxaban, which directly inhibits a protein called factor Xa. In addition, many oral anticoagulants are in the pipeline. All target either thrombin or Xa, both late roadblocks in the in the coagulation cascade that leads to fibrin. Blocking fibrin formation would stop clots, so these new drugs may minimize bleeding risk.

Gruber has a different approach. In the 1990s, he wondered whether the part of thrombin that clotted blood could be disabled and the part that caused anticoagulation could be kept. Such an engineered molecule could work well as a safe anticoagulant.

Developing engineered thrombin as a therapeutic approach was not easy, says Gruber, because injecting thrombin at a little bit higher concentration than normally enters or resides in the blood stream would cause potentially fatal clotting. The most promising thrombin analog (mutant), says Gruber, is one created by Enrico Di Cera, chair of the Department of Biochemistry and Molecular Biology at Saint Louis University School of Medicine.

Di Cera and colleagues used protein engineering to mutate the thrombin molecule. By 2000, the group created a thrombin with mutations at tryptophan 215 (W) and glutamate 217 (E); they dubbed the W215A/E217A thrombin mutant WE-thrombin. In a test tube, WE-thrombin was able to activate protein C, the natural anticlotting pathway that controls and inhibits the coagulation. At the same time, WE's ability to clot blood was reduced by several thousand-fold (Cantwell and Di Cera, 2000).

By 2002, the researchers were able to make enough WE-thrombin to test in a baboon model. The mutant enzyme activated protein C and had very little activity against fibrinogen, the protein that promotes clotting (Gruber et al., 2002). More recently, Gruber and Di Cera reported that low doses of WE activate protein C on the endothelial surface,

exactly as thrombin does under physiological conditions. WE brought about an anticoagulant response and no bleeding was detected. This effect held true when compared head-to-head with giving activated protein C directly and was also safer than the administration of low molecular weight heparins (Gruber et al., 2007). ''Importantly, no bleeding was detected at doses of WE-thrombin that produced significant antithrombotic effects,'' says Di Cera.

The group now has a new generation of anticoagulant thrombin mutants produced by further protein engineering in Di Cera's lab. In this latest effort, the procoagulant activity of the mutant can abolish any possible side effects due to fibrinogen clotting or platelet aggregation (Marino et al., 2010). ''With this research, we optimized the mutant so that there is no clotting at all. Furthermore, we generated a new mutant with exclusive prothrombotic activity, thereby demonstrating that the individual functions of thrombin can be dissociated by replacing a single amino acid in the protein,'' says Di Cera.

The financial stakes are high. Worldwide sales of anticoagulants are in the tens of billions of dollars. In 2007, Gruber founded a biotech company called Aronora, LLC (named after Gruber's son and daughter, combined) (http://www.aronorabio.com) to commercialize WE-thrombin for various thrombotic disease indications such as stroke and heart attack. ''I started Aronora because I was convinced that this fundamentally new method of anticoagulant treatment, activating protein C, was safe and, apparently, also effective, and because I was afraid that if I don't do it, nobody will,'' says Dr. Gruber.

For the first two years, Aronora, LLC existed only on paper. Then in 2009, the Company received notice of a Fast Track SBIR award from the NIH NHLBI, which allowed Gruber to hire a project manager. Today, Aronora is still small; the company has four employees (Gruber is not an employee but he expects the company to grow at a steady pace). ''We've since added other new technologies to our product portfolio and have been pretty successful to convince NIH that if they do not help us to go after the big killers, such as stroke, heart attack, sepsis, nobody in the private sector will, because these diseases are perceived as risky investments or outright no-nos since every drug in the field pretty much had serious draw backs or even failed so far,'' says Gruber.

Marketing is several years away, even if all goes well, and providing that financial hurdles do not limit the effort. Until then, the researchers are working to push WE-thrombin through development as fast and efficiently as they can, says Gruber. ''Every day of delay can be translated to lives. Of course, dealing with advanced, acutely high mortality diseases such as stroke and heart attack has a lot of risks, as in these patients everything goes south,'' says Gruber.

''I think it's exciting,'' says Nigel Mackman, an expert in the biochemistry coagulation at the University of North Carolina at Chapel Hill. ''Clotting is much more complicated than we appreciate and the fact that two new drugs are coming on the market says there's room for more anticoagulants in the market.'' Several drugs with different modes of actions may be needed, says Mackman, as thrombosis in different tissues induced by different pathologies may have a different event that starts them, and new anticoagulants may not work for all forms of thrombosis.

Mackman cautions that WE-thrombin may have a drawback in that it's boosting the anticoagulant side via a natural pathway. If a patient has any deficiencies in one of the three proteins that make up the protein C system, the WE-thrombin may not work well. Likewise, in a situation where the body is already making a lot of thrombin, the WE may not be effective.

As an anticoagulant for surgery, however, Mackman sees WE-thrombin as a good drug. ''You can put the drug onboard to dampen that thrombin response that happens during surgery,'' says Mackman.

Meanwhile, Di Cera is working to scale up production of WE-thrombin. He and his colleagues found ways to make the mutant efficiently in *Escherichia coli*; for some reason, he says, this version of WE-thrombin has a greater anticoagulant effect than the mutant made in mammalian cells. The group expects to file an IND by 2012.

''We are bringing closer to the market something that exploits a natural pathway of anticoagulation in ways that do not produce bleeding. This is an innovative and powerful tool to achieve anticoagulation without disruption of the hemostatic balance. Whether this is the 'holy grail' of anticoagulant therapy remains to be seen.'' says Di Cera. ''Regardless of what happens to WE-thrombin in the clinics, the mutant has provided us with a key reagent to dissect thrombin's functions and [with] unanticipated new information about the molecular mechanisms of activity and specificity of thrombin and related proteases. What amazes me is how much we still don't know about these enzymes.''

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