Some Recent Papers Concerning Thrombin Which Got My Attention

I have worked on the chemistry and biology of thrombin for a number of years and I find that most of the continuing literature is either "salami slicing" or the repeating of work from the past which current investigators cannot find on their devices (changing nomenclature can confuse reviewers). I do, however, occasionally find papers which I consider are worthy of some comment. I found the two papers discussed below of interest. Both of these papers should stimulate future studies of some significance.

Active-Site Mutant of Human γ-Thrombin and Neutralize Dabigatran Anticoagulant

Sheffield and coworkers[1] have published an interesting paper where the S195A mutant of human γ-thrombin was used to neutralize dabigatran etexilate, a low-molecular inhibitor of thrombin which is used as an oral anticoagulant. Dabigatran etexilate is a pro-drug which releases dabigatran which is the actual inhibitor.[2,3] Dabigatran etexilate is one of several low-molecular weight coagulation inhibitors[4] which have been developed as substitutes for warfarin and, to a lesser extent, heparin. While there are accepted approaches to intentional or accidental overdoses of warfarin or heparin, it has been a challenge to find such therapeutic approaches to the non-vitamin K oral anticoagulants (NOAC).[5,6]

The work by Sheffield and coworkers[1] used a derivative of thrombin prepared by the limited proteolysis of a mutant where the serine residue at the enzyme active site has been replaced by alanine (S195A) where the alanine is considered to be structurally similar to serine but not totally isoteric.[7] The S195A derivative of serine proteases such as thrombin is essentially inactive but can still bind substrates and inhibitors.[8] Carter and Wells[8] replaced the three amino acids residues of the catalytic triad, histidine, serine, or aspartic acid, of subtilisin either in single, double, or a triple mutant. The engineering of a surface serine residue (S24) to a cysteine permitted the isolation of the mutant enzymes. The S221A mutant showed a decrease in k_{cat} of 10^6 for the hydrolysis of a tetrapeptide *p*-nitroanilide substrate as did the H64A mutant while a lesser decrease was observed for D32A. While there was some increase in K_m, the effect was not striking. Thus even the triple mutant binds substrate with an avidity similar to the native enzyme (230 μM for the triple mutant; 180 μM for the native enzyme). Thus, the concept underlying the approach of Sheffield and cooworkers[1] is that the inactive enzyme will bind dabigatran (presumably the prodrug has been processed to yield the amidine) preventing inhibition of thrombin. These investigators noted that a conceptually similar approach had been advanced for inhibitors of factor Xa.[9] These investigators did report that the mutant factor Xa did not have anticoagulant properties. This latter point is worth some further comment. Some years ago, David Stern and coworkers at Columbia University with the late Walt Kisiel from the University of New Mexico[10,11] showed that factor IXa inactivated by modification of the active site histidine residue with a peptide chloromethylketone was an anticoagulant by acting as a competitive inhibitor of factor IXa preventing binding to factor VIII. The recent work of Hosokawa and coworkers[12] is of more direct relevance to the Sheffield work. Hosokawa and coworkers[12] prepared a triple mutant of thrombin with alanine substitution at the active site histidine and serine and at Lys65 which was modified at carboxyl

groups by carbodiimide activation followed by reaction with glycine methyl ester.[13] This derivative had increased affinity for factor VIII and PAR1 but reduced affinity for fibrinogen. Thus, this derivative would inhibit the generation of thrombin but not the action of thrombin on fibrinogen. The derivative used by Sheffield and coworkers[1] was not based on α-thrombin but rather on γ-thrombin, a derivative obtained by limited digestion with trypsin. Sheffield and coworkers[1] observed that the S195A derivative of human α-thrombin could also mitigate the anticoagulant effect of dabigatran etexilate *in vitro* but was ineffective in correcting the *in vivo* effect (measured by ferric chloride occlusion). This would suggest that the S198A α-thrombin derivative could compete with thrombin for fibrinogen substrate. γ-Thrombin has been demonstrated to bind to fibrinogen with less avidity than α-thrombin[14] while the difference in the binding of low molecular substrates is far less striking.[15] These fragmented derivatives of thrombin were first described by Seegers and associates in 1974.[16]

It would be interesting to see how far the structure of thrombin could be reduced without losing effective binding of dabigatran. It might possible to develop a peptide mimetic that would be effective.

Thrombin and Pain Control

While it is clear that thrombin is pleiotropic,[17] I was surprised to find an article which described the combination of thrombin and a topical local anesthetic for pain management.[18] In this study, thrombin was admixed with the topical anesthetic and applied to the wound (hand surgery) prior to closure. The combination of thrombin with the local anesthetic provides for sustained post-operative pain control. The concept is similar to the use of fibrin sealant for sustained drug delivery[19,20] and takes advantages of endogenous fibrinogen to form a vehicle of sustained drug release. I thought that this was a very clever use of thrombin as a free-standing therapeutic product. There is a sense that thrombin and fibrin sealant are similar products with overlapping use - actually their relationship is more like that of a flathead screwdriver to a Phillips screwdriver. It should be noted that a substantial amount of thrombin remains bound to the fibrin and is released only with degradation of the clot.[21,22] There is a report on fibrin sealant reducing pain after tonsillectomy[23] and this effect may have been due to thrombin. There have been subsequent studies which support this observation.

I did a quick look at prior work on thrombin and pain and was surprised to find a number of studies on a role for coagulation proteases in nocioceptive processes which, while interesting, do not provide a consensus on effect but certainly support further study in this area. There appears to be a consensus that the pain effect can be mediated through protease-activated receptors (PARs) with interest in PAR-2 receptors which are not cleaved by thrombin but are cleaved by factor Xa, trypsin, tryptase and some other proteases.[24-29] There appears to be more interest in the potential role of tryptase, a mast cell protease.

There is lesser interest in other PARs and pain but enough to be intriguing.[30,31] There is one report on the use of argatroban, a thrombin inhibitor, on reducing pain in herpes zoster.[32] The most intriguing information comes from a recent study by Smith and coworkers[33] on differential effect of salmon thrombin and human thrombin. Salmon thrombin was observed to reduce pain while human thrombin did not have this effect; salmon thrombin has a reduced affinity for the PAR-1 receptor than human

thrombin. The role of PAR-1 in pain is complex and may be depend type of injury and location.[34] Smith and workers[33] also suggest the possible role for PAR-4 activation in analgesia as has been suggested by rodent studies.[35]

The above studies support a role for PARs in nocioception and a role for thrombin (and other proteases such as tryptase). It clear that work in this area will be challenging considering the multiple functions of thrombin and PARs. However, an antagonist of PAR-2 has shown promise in ulcerative colitis[36] and levels of dabigatran etexilate which do no inhibit coagulation have been shown to be effective in inhibiting fibrosis in an animal model.[37]

References

1. Sheffield, W.P., Lambourne, M.D., Eltringham-Smith, L.J., *et al*.,(2014). $_{\gamma T}$-S195A thrombin reduces the anticoagulant effects of dabigatran *in vitro* and *in vivo*. *J.Thromb.Haemost.* 12, 1110-11115

2. Sanford, M. and Plosker, G.L. (2008). Dabigatran etexilate. *Drugs* 68, 1699-1709.

3. Wienen, W., Stassen, J.-M., Priepke, H., Ries, U.J., and Hauel, N.(2007). In-vitro profile and ex-vivo anticoagulant activity of the direct thrombin inhibitor dabigatran and its orally active prodrug, dabigatran etexilate. *Thromb.Haemost.* 98, 155-167,

4. Levy, J.H., Spyropoulos, A.C., Samama, C.M., and Douketis, J.(2014). Direct oral anticoagulants: New drugs and new concepts. JACC Cardiovasc.Intervent. 7, 1333-1351.

5. Makris, M. (2014). Prothrombin complex concentrate for non-vitamin K oral anticoagulant reversal: good enough for now? *J.Thromb.Haemost.* 12, 1425-1427.

6. Costin, J., Ansell, J., Laulicht, B., Bakhru, S., and Steiner, S. (2014). Reversal agents in development for the new oral anticoagulants. *Postgrad.Med.* 126, 19-24.

7. Lewendon, A., Murrya, I.A., Shaw, W.V., Gibbs, M.R, and Leslie, A.G. (1990). Evidence for transition-state stabilization by serine-148 in the catalytic mechanism of chloramphenicol acetyltransferase. *Biochemistry* 29, 2075-2080.

8. Carter, P. and Wells, J.A. (1988). Dissecting the catalytic triad of a serine protease. *Nature* 332, 564-568.

9. Lu, G., De Guzman, F.R., Hollenbach, S.J., *et al*., (2013). A specific antidote for reversal of anticoagulation by direct and indirect inhibitors of coagulation factor Xa. *Nat.Med*. 19, 446-451.

10. Benedict, C.R., Ryan, J., Wolitzky, B., *et al*., (1991). Active site-blocked factor IXa prevents intravascular thrombus formation in the coronary vasculature without inhibiting extravascular coagulation in a canine thrombosis model. *J.Clin.Invest.* 88, 1760-1765.

11. Spanier, T. B., Chen, J.M., Oz, M.C., *et al*., Selective anticoagulation with active site-blocked factor IXA suggests separate roles for intrinsic and extrinsic coagulation pathways in cardiopulmonary bypass. *J.Thorac.Cardiovasc.Surg.* 116, 860-869.

12. Hosokawa, K., Matsuda, H., Ohnishi, T., Gonda, M., and Koide, T.(2010). M-AAA-thrombin: Potent anticoagulant and antiplatelet thrombin derivative with differential affinity for factor VIII and PAR1. *Thromb.Res.* 126, 447-453.

13. Lundblad, R.L. (2014), *Chemical Reagents for Protein Modification*, 4th edn., CRC Press, Boca Raton,Florida, USA.

14. Lewis, S.D., Lorand, L., Fenton, J.W.,II., and Shafer, J.A. (1987). Catalytic competence of human α- and γ-thrombin in the activation of fibrinogen and factor XIII. *Biochemistry* 26, 7597-7603.

15. Witting, J.I., Miller, T.M., and Fenton, J.W,II,(1987). Human α- and γ-thrombin specificity with tripeptide substrates. *Thromb.Res.* 46, 567-574.

16. Seegers, W.H., Walz, D.A., Reuterby, J. and McCoy, L.E. (1974). Isolation and some properties of thrombin-E and other prothrombin derivatives. *Thromb.Res.* 4, 829-860.

17. Siller-Matula, J.M., Schwameis, M., Blann, A., Mannhalter, C., and Jilma, B. (2011). Thrombin as a multi-functional enzyme. Focus on in vitro and in vivo effects. *Thromb.Haemost.* 106, 1020-1033.

18. Haddock, N.T., Weinstein, A.L., Sinno, S., and Chiu, D.T.W.(2014). Thrombin and topical local anesthetic for postoperative pain management. *Ann.Plastic Surg.* 73, 30-32.

19. Kitajiri, S., Tabuchi, K., Hiraumi, H., and Kaetsu, H.(2001). Relief of post-tonsillectomy pain by release of lidocaine from fibrin glue. *Laryngoscope* 111, 642-644.

20. Fu, J.Z., Li, J., and Yu, Z.L.(2009). Effect of implanting fibrin sealant with ropivacaine on pain after laparoscopic cholescystectomy. *World J.Gastroenterol.* 15, 5851-5854.

21. Francis, C.W., Markham, R.E.,Jr., Barlow, G.H., *et al.,* Thrombin activity of fibrin and soluble plasmic derivatives. *J.Lab.Clin.Med.* 102, 220-230.

22. Kinioh, K., Nakamura, M., Sunagawa, M., and Kosugi, T. (2002). Isolation of bound thrombin consisting of thrombin and fibrin N-terminal fragment from clot lysate. *Haematologica* 32, 457-465.

23. Gross, C.W., Gallagher, R., Schlosser, R.J., *et al*., (2001). Autologous fibrin sealant reduces pain after tonsillectomy. *Laryngoscope* 111, 259-263.

24. Vergnolle, N., Wallace, J.L., Bunnett, N.W., and Hollenberg, M.D.(2001). Protease-activated receptors in inflammation, neuronal signaling and pain. *Trends Pharmacol.Sci.* 22, 146-152.

25. Fiorucci, S. and Distrutti, E. (2002). Role of PAR2 in pain and inflammation. *Trends Pharmacol.Sci.* 23, 153-155.

26. Oliver, K.R. and Hill, R.G.(2002). Feeling below PAR: proteinase-activated receptors and the perception of neuroinflammatory pain. *Pharmacogenomics J.* 2, 10-11.

27. Dai, Y., Moriyama, T., Higashi, T., *et al*.,(2004). Proteinase-activated receptor 2-mediated potentiation of transient receptor potential vanilloid subfamily 1 activity reveals a mechanism for proteinase-induced inflammatory pain. *J.Neurosci.* 24, 4293-4299.

28. Poole, D.P., Amadesi, S., Veldhuis, N.A., *et al*.,(2013). Protease-activated receptor-2 (PAR2) protein and transient receptor potential vanilloid 4 (TRPV4) protein coupling is required for sustained inflammatory signaling. *J.Biol.Chem.* 288, 5790-5802.

29. Zhao, P., Lieu, T., Barlow, N., *et al*.,(2014). Cathepsin S causes inflammatory pain via biased agonism of PAR2 and TRPV4. *J.Biol.Chem.* 289, 27215-27234.

30. Vellani, V., Kinsey, A.M. Prandini, M., *et al*.,(2010). Protease activated receptors 1 and 4 sensitize TRPV1 in nocioceptive processes. *Mol.Pain.* 6:61.

31. Garcia, P.S., Gualti, A., and Levy, J.H.(2010). The role of thrombin and protease-activated receptors in pain mechanisms. *Thromb.Haemost.* 103, 1145-1151.

32. Fujii, K., Kanno, Y., Konishi, K., and Ohgou, N.(2001). A specific thrombin inhibitor, argatroban, alleviates herpes zoster-associated pain. *J.Dermatol.* 28, 200-207.

33. Smith, J.R., Syre, P.P., Oake, S.A., *et al*.,(2013). Salmon and human thrombin differentially regulate radicular pain, glial-induced inflammation and spinal neuronal excitablity through protease-activated receptor-1. *PLoS One* 8(11):e80006.

34. Dong, L., Smith, J.R., and Winklestein, B.A.(2013). Ketorolac reduces spinal astrocytic activation and PAR1 expression associated with attenuation of pain after facet joint injury. *J.Neurotrauma* 30, 818-825.

35. Annaházi, A.,Dabek, M. Gecse, K., *et al*.,(2012). Proteinase-activated receptor-4 evoked colorectal analgesia in mice: an endogenously activated feed-back loop in visceral inflammatory pain. *Neurogastroenterol Motil.* 24, 76-85.

36. Lohman, R.J., Cotterell, A.J., Suen, J., *et al*., (2012). Antagonism of protease-activated receptor 2 protects against experimental colitis. *J.Pharmacol.Exp.Ther.* 340, 256-265.

37. Bogatrkevich, G.S., Ludwicka-Bradley, A., Nietert, P.J., *et al*.,Antiinflammatory and antifibrotic effects of the oral direct thrombin inhibitor dabigatran etexilate in a murine model of interstitial lung disease. Arthritis Rheum. 63, 1416-1425.