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8. For Southern blot analysis, 10 μg of genomic DNA was digested with Bsu 36I and the fragments separated by electrophoresis on a 0.7% agarose gel. After the DNA fragments were transferred to a nylon membrane, hybridization was performed with a ^{32}P-labeled, 1.2-kbp Bsu 36I β^A-globin fragment cloned in pBR328 as the probe.
9. For analysis of the β-globin sequence, the 345-bp PCR fragment was prepared by amplification from a crude cell lysate with primers BG02 (5′-TCCTAAGCCAGTGCCAGAAGA) and BG05 (5′-CTATTGGTCTCCTTAAACCTG) and Expand Taq polymerase (Boehringer Mannheim). For analysis of the δ-globin gene, the same cell extracts were used in amplification reactions with primers DG06 (5′-CTCACAAACTAATGAAACCCTGC) and DG07 (5′-GAAAACAGCCCAAGGGACAG) to generate a 335-bp fragment. Gels were stained with SYBR green (FMC Bioproducts), and fluorescence intensities were quantitated with a Molecular Dynamics fluorimager. DNA sequencing was performed in both directions with an ABI 373A sequencer.
10. There is a documented polymorphism in the third base of codon 2. A switch from C to T has been observed in this position and is evident in the β^A sequence [S. H. Orkin *et al.*, *Nature* **296**, 627 (1982)] outside the targeted region.
11. Four flasks were seeded with 24 ml of β^A cells at a concentration of 1×10^5 cells per milliliter. Two of the flasks were transfected for 16 hours with 47 nM SC2 chimeric oligonucleotide after which the medium was replaced. The cells rested for 3 days to allow for the expression of the HPRT-resistant phenotype. On day 0, 6-thioguanine was added (5 μg/ml) to each of the SC2-treated and untreated flasks. The media of these flasks was exchanged twice during the experiment. All cells were counted on day 0 and frequently thereafter for 14 days. The numbers are presented in Fig. 4C on a logarithmic scale, illustrating the kill curve of the 6-thioguanine–selected flasks. PCR analysis was performed on the control flasks on days 0 and 14 of the experiment, showing the persistence of the conversion event [H. L. Liber and W. G. Tilley, *Mutat. Res.* **94**, 467 (1982)].
12. We are indebted to members of E.B.K.'s laboratory for helpful suggestions throughout the course of this work, to H. Alder (Thomas Jefferson University) for assistance in the direct DNA sequencing protocols, and to A. Andrus (Applied Biosystems, Inc.) for providing the chimeric molecules. Supported by a grant from Kimeragen, Inc.

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Enhanced Protein C Activation and Inhibition of Fibrinogen Cleavage by a Thrombin Modulator

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A modulator of the enzymatic activity of human thrombin, designated LY254603, was identified that enhances the thrombin-catalyzed generation of the anticoagulant factor activated protein C, yet inhibits thrombin-dependent fibrinogen clotting. By means of mutant substrates, it was shown that LY254603 mediates the change in enzymatic substrate specificity through an alteration in thrombin's S3 substrate recognition site, a mechanism that appeared to be independent of allosteric changes induced by either sodium ions or by thrombomodulin. This compound may represent the prototype of a class of agents that specifically modulates the balance between thrombin's procoagulant and anticoagulant functions.

The serine protease thrombin is the pivotal controlling factor of all thrombosis, a leading cause of death as well as of morbidity associated with vascular disease. This enzyme plays a dual role in the regulation of blood coagulation. Thrombin's classical role is as a procoagulant enzyme that catalyzes the conversion of fibrinogen to fibrin, but thrombin also acts as an anticoagulant enzyme by the proteolytic activation of protein C, a key feedback regulator of the coagulation cascade (*1*). These opposing functions are physiologically controlled by allosteric changes induced by Na^+ (*2*) and cell-surface thrombomodulin (TM) (*3*), which results in a change in thrombin's substrate specificity. TM functions by directly blocking thrombin's procoagulant activities (*3*) and stimulating the thrombin-catalyzed generation of activated protein C (aPC) in the presence of Ca^{2+} (*4*, *5*).

Thrombomodulin's interaction with thrombin via anion-binding site I (*6*) results in an allosteric change that alters thrombin's substrate specificity (*7*). This allosteric change, at least in part, eliminates repulsive interactions between Glu^{39} and Glu^{192} in thrombin (*3*) and the P3 and P3′ acidic residues in protein C, which have been shown to contribute to the slow rate of activation by free α-thrombin (*5*, *8*). In addition to these allosteric changes in active-site substrate specificity induced by macromolecular interactions at anion-binding site 1, recent studies by Di Cera *et al.* (*2*) have demonstrated that thrombin exists in two forms allosterically controlled by Na^+ binding. The "fast" form of thrombin (Na^+-bound) cleaves fibrinogen with higher specificity, whereas the "slow" form preferentially cleaves protein C.

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We reasoned that if thrombin existed in multiple forms capable of recognizing different substrates, then it might be possible to identify agents that would shift the equilibrium from one form to the other. Therefore, we tested approximately 8300 synthetic organic compounds from the Lilly Research Laboratories files (*9*) for agents that might increase the rate of thrombin-dependent activation of human protein C. Compounds that had a stimulating effect on protein C activation by thrombin were further analyzed for their effect on fibrinogen cleavage. Compounds with both thrombin activities were inspected for structural similarities, and, on the basis of that analysis, additional analogs were obtained from Lilly Research Laboratories files for comparative biochemical evaluation. After two iterations of this approach, LY254603 was identified (Fig. 1A, inset). At the screening concentration of 25 μg/ml, the rate of thrombin-catalyzed aPC generation was substantially increased in the presence of LY254603, as shown by an approximate eightfold increase in the amount of aPC generated (Fig. 1A). The compound had no effect on aPC amidolytic activity, as demonstrated in control experiments with preactivated protein C. Whereas the presence of LY254603 resulted in a marked stimulation in the ability of thrombin to cleave the protein C substrate, it also resulted in a marked reduction in the ability of thrombin to cleave fibrinogen (Fig. 1B). These data suggested that we identified a compound capable of altering the substrate specificity of thrombin.

The effects of LY254603 on stimulation of protein C cleavage and reduction of fibrinogen cleaving ability were dependent on concentration. The concentration required to double the rate of aPC generation in our assays was approximately 500 nM, and the maximal effect in this experiment reached a plateau at approximately a 10-fold increase (Fig. 1C). This plateau appeared to be a result of the solubility of the compound in the reaction, as the maximal water solubility was observed to be approximately 25 μM. The inhibitory effect of LY254603 on thrombin cleavage of fibrinogen was concentration-dependent within the same range, as observed for the stimulating effect on protein C cleavage (Fig. 1D). In repeated experiments, the median inhibitory concentration (IC_{50}) for fibrinogen clotting and the concentration for half-maximal stimulation of protein C cleavage were both ~3 μM. The ~10-fold increase in aPC generation with the more than 95% decrease in fibrinogen clotting (at the same concentrations of both thrombin and LY254603) effectively yields a shift of at least several hundredfold in thrombin's differential cleavage specificity. LY254603 significantly extended the activated partial thromboplastin clotting time in human plasma (16 ± 5 s at 40

μM); however, further study will be required to obtain a more potent and soluble compound for detailed mechanistic studies in such complex systems.

Because the activation of protein C by thrombin is inhibited by Ca^{2+}-induced conformational change in protein C (*3–5*), we tested the effect of LY254603 on protein C activation as a function of Ca^{2+} concentration. Although at each concentration of Ca^{2+} the rate of aPC generation was increased about sixfold by 6 μM LY254603, we observed no significant difference in the Ca^{2+} dependency. Both in the presence and absence of LY254603, the IC_{50} Ca^{2+} concentrations were about 0.2 mM (Fig. 2A), which is consistent with previous results with recombinant protein C (*10*). Thus, the effect of LY254603 on enhancing protein C substrate recognition by thrombin is independent of the Ca^{2+}-induced conformational change in protein C previously shown to alter thrombin recognition.

We speculated that one potential mechanism by which LY254603 could increase protein C activation while inhibiting fibrinogen clotting would be alteration of the equilibrium between the slow and fast forms in favor of the slow form. If this were the case, we might expect that LY254603 would not significantly increase the rate of aPC activation under conditions of low Na^+ concentration, where the thrombin was essentially all in the slow form. We determined the rates of activation and effect of LY254603 in NaCl and choline chloride (ChCl) both in the presence and absence of Ca^{2+}. There was no difference in the stimulatory effect of LY254603 whether thrombin was in the slow form (no Na^+ present) or in the fast form (bound to Na^+), or if Ca^{2+} was present or absent (Fig. 2B). These data showed that the effect of LY254603 was independent of cation-mediated allosteric changes in thrombin and suggested that the compound does not affect substrate specificity by altering the transition from the fast form to the slow form.

To determine if the alteration in substrate specificity seen in the presence of LY254603 was mediated through changes in thrombin recognition at either or both of the critical residues in protein C (Asp residues P3 and P3′), we analyzed the effect of LY254603 on the rates of thrombin-catalyzed activation of wild-type human protein C (wtHPC), D167F (*5*), and D172K (*10*). Both D167F (Asp to Phe change at P3) and D172K (Asp to Lys change at P3′) were activated by thrombin 15- to 20-fold faster than wtHPC as described (*5*, *10*). However, we observed no significant stimulation of the thrombin-catalyzed D167F activation by LY254603, whereas D172K was stimulated to a similar degree as wtHPC (Fig. 3A). Even at concentrations at which the effect on wtHPC was maximal, there was only a slight stimulation in the rate of D167F activation in the presence of LY254603 (Fig. 3B). These data strongly suggest that LY254603 affects thrombin through a change that favors the P3 acidic residue in protein C, without altering the preference at P3′.

Because TM also alters the P3 as well as the P3′ subsite specificity of thrombin, we were interested in determining if LY254603 affected aPC generation by thrombin in complex with TM. LY254603 also stimulated the rate of aPC generation by thrombin in complex with TM (Fig. 4A) from 7- to 10-fold, similar to the increase observed with thrombin alone. The rate of aPC generation by the physiologically relevant

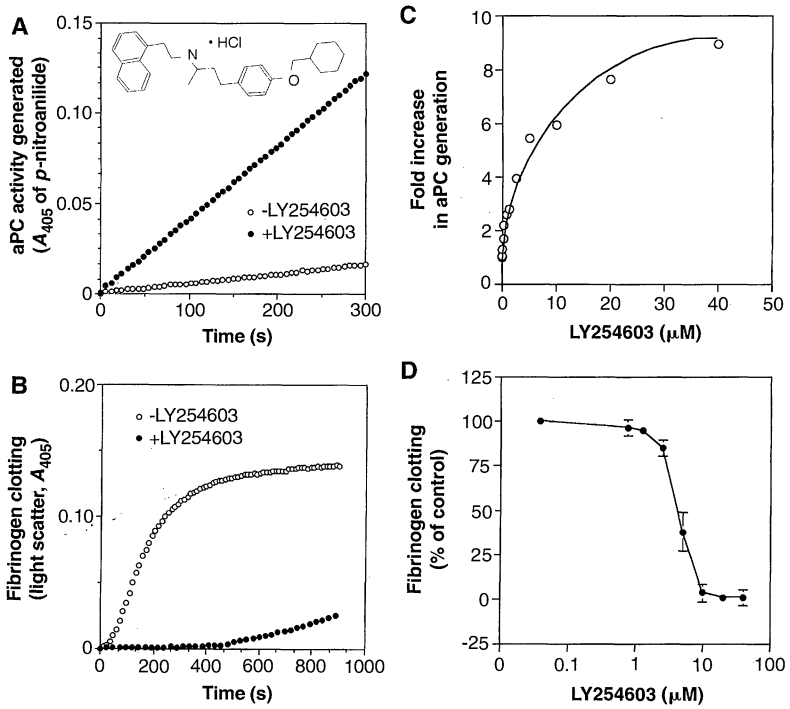

Fig. 1. Identification of LY254603 and determination of its effects on thrombin-catalyzed protein C activation and fibrinogen clotting. (**A**) Effect of LY254603 on protein C activation (*14–16*). The inset shows the structure of LY254603 as determined by nuclear magnetic resonance spectroscopy, mass spectroscopy, and elemental analysis. (**B**) Effect of LY254603 on the kinetics of fibrinogen cleavage by thrombin (*17*). (**C**) Concentration response for the effect of LY254603 on thrombin-catalyzed protein C activation. Reaction conditions were as described (*14*). The results are expressed as the fold increase in aPC generated relative to reactions containing no LY254603, which showed a rate of aPC generation of 1.2 mmol min^{-1} mol^{-1}. (**D**) Concentration response for the effect of LY254603 on fibrinogen cleavage. Reactions containing 10 nM thrombin and varying levels of LY254603 were performed as described (*17*). Clotting times were determined from the kinetic plots as the time to maximum velocity. The effect of LY254603 (mean ± SD, $n = 4$) on the inhibition of thrombin-dependent fibrinogen cleavage was determined from standard curves as the percent of thrombin activity remaining. A_{405}, absorbance at 405 nm.

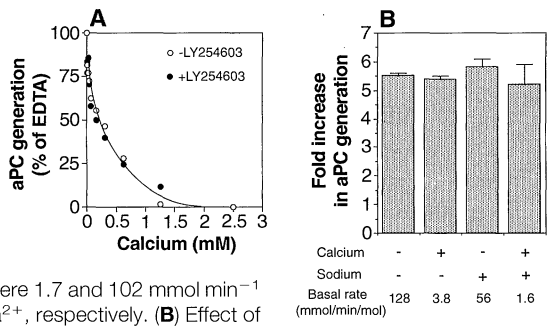

Fig. 2. (**A**) The effect of LY254603 on Ca^{2+}-dependent inhibition of aPC generation by thrombin. Reactions containing 6 μM LY254603, 3 nM thrombin, and 400 nM protein C zymogen were performed in the presence of varying levels of Ca^{2+}, and the amount of aPC generated was determined as the percent of aPC generated relative to that obtained in the absence of Ca^{2+} (5 mM EDTA) as 100%. Rates of aPC generation in the absence of LY254603 were 1.7 and 102 mmol min^{-1} mol^{-1} in the presence and absence of Ca^{2+}, respectively. (**B**) Effect of LY254603 on thrombin in the presence and absence of Na^+ and Ca^{2+}. The conditions were with either 200 mM NaCl (+) or 195 mM choline chloride and 5 mM NaCl (−) to maintain ionic strength as described (*2*). The fold increase was determined from the difference between experiments with and without 6 μM LY254603. The initial rates of aPC generation in the absence of LY254603 are indicated.

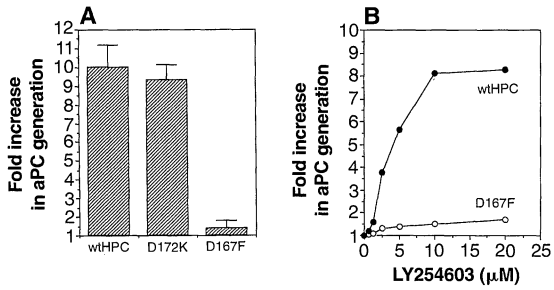

Fig. 3. (**A**) Effect of LY254603 on α-thrombin–catalyzed aPC generation by use of protein C molecules with amino acid changes at the P3 and P3′ residues. We incubated wtHPC, P3′ derivative D172K, and P3 derivative D167F with 2 nM thrombin in the presence and absence of LY254603 (10 μM), and the amount of aPC generated after a 30-min incubation at 37°C was determined (*14*). The results, mean ± SD (n = 3), are expressed as the fold increase in aPC generated in the presence of LY254603 relative to the control. Rates of aPC generation in the absence of LY254603 were 2.6, 47, and 37 mmol min^{-1} mol^{-1} for wtHPC, D167F, and D172K, respectively. (**B**) Concentration response comparing wtHPC and D167F.

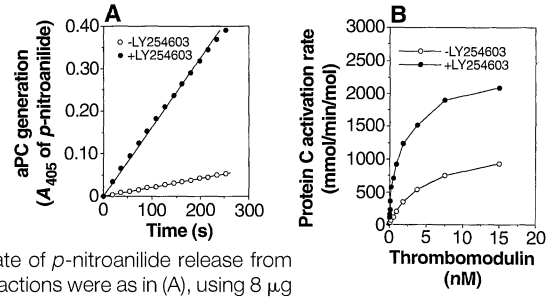

Fig. 4. Effect of LY254603 on the activation of protein C by thrombin in complex with TM. (**A**) Reactions were performed in 20 mM tris, pH 7.4, 150 mM NaCl, 5 mM $CaCl_2$, and 300 μg of bovine serum albumin per milliliter, with 0.3 nM human thrombin and 3 nM chondroitin sulfate–modified soluble recombinant human TM (*18*). We determined the amount of aPC generated after 10 min at 37°C by measuring the rate of *p*-nitroanilide release from synthetic peptide substrate S2366. (**B**) Reactions were as in (A), using 8 μg of LY254603 per milliliter, 150 nM protein C, 2 nM human thrombin, and varying amounts of TM.

thrombin-TM complex was about 300 times that of thrombin alone at the TM concentration used (near its dissociation constant). In other words, the rate with thrombin-TM plus LY254603 was about 2000 to 3000 times that of thrombin alone. LY254603 increased the rate of TM-stimulated aPC generation at all concentrations of TM (Fig. 4B). We observed a decrease in half-maximal saturating TM concentration to one-fifth original levels and a twofold increase in aPC at saturating TM levels. Although these data suggest that LY254603 may increase the affinity of the thrombin-TM complex, detailed kinetic studies will be required to distinguish the effects of LY254603 on the thrombin interaction with TM and on thrombin-TM interactions with protein C. Further studies will also be required to define the physical binding site of this compound. However, the multiplicative nature of the increase in aPC generation by LY254603 suggests that the stimulating effects of TM and LY254603 on thrombin-catalyzed protein C cleavage may be mediated through different allosteric mechanisms, although both ultimately affect P3 specificity.

We describe a compound capable of modulating the substrate cleavage specificity of human thrombin. Although our data would suggest that the mechanism for the modulation by LY254603 may be distinct from that of TM, it appears to functionally modulate the activity in a similar manner, especially with regard to altering the P3 cleavage specificity of thrombin. Musci and Berliner (*11*) made the first observation demonstrating that small ligands could alter thrombin substrate activity. They demonstrated that tryptamine analogs (1 mM) enhanced the ability of thrombin to activate protein C in the absence of Ca^{2+}; however, these compounds also increased the activation of thrombin-catalyzed fibrinogen clotting, while inhibiting both functions at higher concentrations (*12*). LY254603 appears to be distinct from these compounds in its ability to differentially alter thrombin substrate specificity, and with far greater potency.

Recently, in a commentary on the paradoxical activities of thrombin, Griffin (*13*) speculated that future agents might be discovered that would enhance the protein C pathway. The results of our study, in fact, demonstrate the possibility of identifying agents that modulate the balance between the pro- and anticoagulant activities of thrombin. Whereas future studies are required to identify a more potent and soluble pharmacological agent that is effective in plasma, our results demonstrate a concept that may lead to future strategies for developing antithrombotic agents.

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