A SIMPLE AND RAPID SCREEN FOR INHIBITORS OF FACTOR XIIIa

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Many biologically active agents have been discovered through screening of crude natural product extracts. While traditional pharmaceutical screens have focussed on the search for novel antibiotics and antitumor agents, an increasing number are being applied to other therapeutic areas. Recent examples include the identification of an angiogenesis inhibitor,[1] anticoagulants[2] and various receptor antagonists.[3~6] During our studies of thrombosis in cardiovascular disease, we identified factor XIIIa inhibition as a potential target for therapeutic intervention that was suitable for natural product screen development.

Factor XIIIa is a cysteine transamidase which is important in the process of normal human blood coagulation.[7] This enzyme exists as a free plasma zymogen which, once activated by thrombin in the presence of calcium, can rapidly crosslink α and γ chains of fibrin *via* amide bond formation at specific glutamine and lysine residues. This crosslinking increases both the resistance to plasmin degradation as well as the mechanical stability of a clot. Although congenital factor XIIIa deficiency is known to occur infrequently, it is characterized by few side effects outside of the potential for moderate to severe bleeding in homozygous individuals. Administration of crude factor XIIIa from plasma is an effective treatment for this deficiency.[8] Thus, temporary inhibition of factor XIIIa appears to be a potentially safe adjunct to other thrombolytic therapies and has been investigated in animal models.[9,10]

A simple assay for factor XIIIa activity *in vitro* can be based on the extent of clot dissolution by denaturants,[7] as stability to denaturing conditions reflects the number of fibrin crosslinks in a clot (Scheme 1). In this note, we describe a convenient high throughput method for detecting factor XIIIa inhibitors based on the urea solubility of mini clots formed in microtiter plates. The method is amenable to testing both synthetic compounds and natural products extracts, and can be used to generate estimates of IC_{50} values. Various enzymatic assays which are suitable for more precise characterization of factor XIIIa inhibitors have been described elsewhere.[11~14]

In each well, a test substance (25 μl in 10% aqueous DMSO) is premixed with either distilled water or 200 mM dithiothreitol (5 μl). To this, 30 μl of crude bovine fibrinogen containing contaminating factor XIII (Sigma F-8630, 10 mg/ml in 0.1 M sodium phosphate buffer, pH 7.2) is added. The clotting reaction is then started by adding 40 μl of a Ca/thrombin solution, prepared by diluting 25 μl of bovine plasma thrombin (1,000 U/ml) with 3.825 ml distilled water and then adding 150 μl of 500 mM $CaCl_2$ immediately prior to use. Clots begin forming immediately, and may be treated with urea after two hours at room temperature but are also stable under sealing tape for longer periods of time. Before initiating the solubility test, wells are checked visually to ensure that all have formed clots. Incomplete clot formation at this point is suggestive of thrombin inhibition.

To determine stability in urea, the clots are overlayed with 160 μl of 8 M urea and allowed to stand, sealed, overnight at room temperature. The entire sealed microtiter plate is then subjected to sonication for 30 seconds. Control (crosslinked) clots remain intact after sonication. Clots formed in the presence of factor XIIIa inhibitors show increasing levels of visible shredding and ultimately full dissolution as a function of inhibitor dose. Clot solubility can be scored visually with 10 μM iodoacetamide serving as a convenient reference standard for complete clot dissolution. The minimum concentration of a substance required to cause any detectable clot dissolution (MDC), such as visible shredding of the clot or appearance of clear patches, can be used to estimate the relative

Scheme 1. Overview of the clot dissolution screen for factor XIIIa inhibitors.

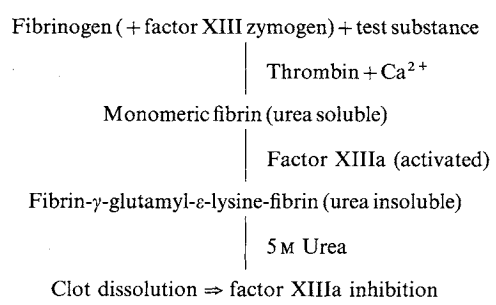


Table 1. Detection levels for reference factor XIIIa inhibitors[15] and peptidic substrate analogs in the clot dissolution assay.

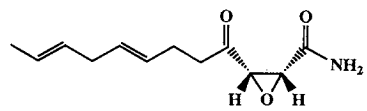
Compound	Minimum detectable concentration (μM)	
	−DTT	+DTT
Iodoacetamide	1	50
Cerulenin (**1**)	10	10~25
2	1	25
3	>1,000	>1,000
LSLSQSKVLG-NH_2[a]	100	50
LSLSFSKVLG-NH_2[a]	500	1,000
LSLSESKVLG-NH_2[a]	500	>1,000
LSLS(ORN)SKVLG-NH_2	500	>1,000
NQEQVSPLTLLK-NH_2[b]	50	25
NCEQVSPLTLLK-NH_2[b]	100	50
NREQVSPLTLLK-NH_2[b]	100	500
NYEQVSPLTLLK-NH_2[b]	1,000	500

[a] Substrate analogs based on casein.[19]
[b] Analogs of the *N*-terminus of α2 antiplasmin.[16]

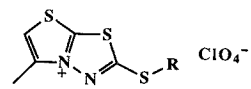
potency of factor XIIIa inhibitors. Alternatively, the extent of clot dissolution can be quantified with a microtiter plate reader by measuring differential turbidity (405 mm) before and after urea treatment. In our hands, manual scoring of MDCs proved adequate for side-by-side comparisons of inhibitor potencies.

As a cysteine transamidase, factor XIIIa is susceptible to inhibition by general sulfhydryl trapping agents such as iodoacetamide. Inhibitors operating by this nonspecific mechanism have little value for drug discovery and so a simple way to rule them out is desirable. Such agents can be made less effective against factor XIIIa when the assay is carried out in the presence of high levels of thiols. Several thiols were evaluated for this purpose, and dithiothreitol (DTT) at concentrations below 100 μM was found to be most useful. Testing samples in the presence and absence of 10 μM DTT proved to be a simple method for discriminating against nonspecific alkylating agents that can appear in screening. Lower sensitivity to DTT reversal than iodoacetamide was imposed as a requirement for identifying potential inhibitors. With this criterion, the assay could detect a natural product inhibitor of factor XIIIa, cerulenin,[15] at concentrations achievable in crude fermentation extracts (Table 1). Cerulenin, however, can be easily dereplicated during screening by applying a bioautographic check for antifungal activity in any factor XIIIa inhibiting extract.

Only one factor XIIIa inhibitor lead was detected out of 500 solid and liquid fermentation extracts screened by this method. None of the extracts showed significant thrombin inhibition. Thus, the assay appears to be robust and selective. Preliminary characterization of peptides related to fibrin and α2-antiplasmin[16] showed that only very specific peptide sequences interfere with factor XIIIa crosslinking of fibrin (Table 1). This method, although only semi-quantitative, has an advantage over spectrophotometric and radiometric assays in that Factor XIIIa inhibition is estimated in the presence of its natural substrate. Recent evidence suggests that certain functions of factor XIIIa are not detectable with assays measuring transamidase ligation of small substrates.[17,18] A further refinement of the general screen protocol described here could be achieved by including human sources of factor XIIIa and fibrinogen.



Cerulenin (**1**)



2 R=CH_2COCH_3
3 R=$CH_2CH_2CH_3$

A simple, high throughput microclot assay for measuring factor XIIIa activity has been described. Our results indicate that this functional assay is suitable for screening natural products extracts and synthetic compounds for new factor XIIIa inhibitors.

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References

1) OTSUKA, T.; T. SHIBATA, Y. TSURUMI, S. TAKASE, M. OKUHARA, H. TERANO, M. KOHSAKA & H. IMANAKA: A new angiogenesis inhibitor, FR-111142. J. Antibiotics 45: 348~354, 1992
2) KAMIYAMA, T.; T. UMINO, N. NAKAYAMA, Y. ITEZONO, T. SATOH, Y. YAMASHITA, A. YAMAGUCHI & K. YOKOSE: Ro 09-1679, a novel thrombin

inhibitor. J. Antibiotics 45: 424~427, 1992

3) LAM, Y. K. T.; D. BOGEN, R. S. CHANG, K. A. FAUST, O. D. HENSENS, D. L. ZINK, C. D. SCHWARTZ, L. ZITANO, G. M. GARRITY, M. M. GAGLIARDI, S. A. CURRIE & H. B. WOODRUFF: Novel and potent gastrin and brain cholecystokinin antagonists from *Streptomyces olivaceus*. Taxonomy, fermentation, isolation, chemical conversions, and physico-chemical and biochemical properties. J. Antibiotics 44: 613~625, 1991
4) TSUJII, E.; Y. TSURUMI, S. MIYATA, K. FUJIE, A. KAWAKAMI, M. OKAMOTO & M. OKUHARA: WF11605, an antagonist of leukotriene B_4 produced by a fungus. I. Producing strain, fermentation, isolation and biological activity. J. Antibiotics 45: 698~703, 1992
5) WEBER, W.; W. FISCHLI, E. HOCHULI, E. KUPFER & E. K. WEIBEL: Anantin—A peptide antagonist of the atrial natriuretic factor (ANF). I. Producing organism, fermentation, isolation and biological activity. J. Antibiotics 44: 164~171, 1991
6) KOJIRI, K.; M. IHARA, S. NAKAJIMA, K. KAWAMURA, K. FUNAISHI, M. YANO & H. SUDA: Endothelin-binding inhibitors, BE-18257A and BE-18257B. I. Taxonomy, fermentation, isolation and characterization. J. Antibiotics 44: 1342~1347, 1991
7) COOKE, R. D. & J. J. HOLBROOK: Calcium and the assays of human plasma clotting factor XIII. Biochem. J. 141: 71~78, 1974
8) FEAR, J. D.; K. J. A. MILOSZEWSKI & M. S. LOSOWSKY: The half life of factor XIII in the management of inherited deficiency. Thromb. Haemostasis 49: 102~105, 1983
9) LEIDY, E. M.; A. M. STERN, P. A. FRIEDMAN & L. R. BUSH: Enhanced thrombolysis by a factor XIIIa inhibitor in a rabbit model of femoral artery thrombosis. Thromb. Res. 59: 15~26, 1990
10) SHEBUSKI, R. J.; G. R. SITKO, D. A. CLAREMON, J. J. BALDWIN, D. C. REMY & A. M. STERN: Inhibition of factor XIIIa in a canine model of coronary thrombosis: effect on reperfusion and acute reocclusion after recombinant tissue-type plasminogen activator. Blood 75: 1455~1459, 1990
11) RETZINGER, G. S.; B. C. COOK, R. E. SMITH & M. C. MCGINNIS: Quantitation of plasma factor XIIIa activity using fibrin-coated microscopic latex beads. Anal. Biochem. 195: 18~23, 1991
12) FICKENSCHER, K.; A. AAB & W. STUBER: A photometric assay for blood coagulation factor XIII. Thromb. Haemostasis 65: 535~540, 1991
13) DEMPFLE, C.-E.; J. HARENBERG, K. HOCHREUTER & D. L. HEENE: Microtiter assay for measurement of factor XIII activity in plasma. J. Lab Clin. Med. 119: 522~528, 1992
14) FINK, M. L.; Y. Y. SHAO & G. J. KERSH: A flourometric, high-performance liquid chromatographic assay for transglutaminase activity. Anal. Biochem. 201: 270~276, 1992
15) ATKINSON, J. G.; J. J. BALDWIN, D. A. CLAREMON, P. A. FRIEDMAN, D. C. REMY & A. M. STERN (Merk): Factor XIIIa inhibitor compounds useful for thrombolytic therapy. Eur. Pat. Appl. 88347690. Mar. 21, 1988
16) KIMURA, S.; T. TAMAKI & N. AOKI: Acceleration of fibrinolysis by the *N*-terminal peptide of alpha 2-plasmin inhibitor. Blood 66: 157~160, 1985
17) HORNYAK, T. J. & J. A. SHAFER: Interactions of factor XIII with fibrin as substrate and cofactor. Biochemistry 31: 423~429, 1992
18) FUKUE, H.; K. ANDERSON, P. MCPHEDRAN, L. CLYNE & J. MCDONAGH: A unique factor XIII inhibitor to a fibrin-binding site on factor XIIIa. Blood 79: 65~74, 1992
19) GORMAN, J. J. & J. E. FOLK: Structural features of glutamine substrates for transglutaminases. J. Biol. Chem. 259: 9007~9010, 1984