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(54) Title: A HIGH THROUGHPUT ASSAY USING FUSION PROTEINS (57) Abstract This application describes a high throughput assay for screening compounds which are capable of binding to a fusion protein which consists of a target protein and an FK506-binding protein. This application also describes an assay for screening compounds which inhibit a protease.		

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TITLE OF THE INVENTION

A HIGH THROUGHPUT ASSAY USING FUSION PROTEINS

BACKGROUND OF THE INVENTION

5 Several assays have been developed that screen compounds to determine if they are capable of binding to a fusion protein. One such method uses heterobifunctional cross-linking with diamino-europium-cryptate and a protein of interest. However, the activity of the protein is often affected by the modification with the
10 heterobifunctional cross-linking agents. These methods also require an additional purification for the heterogeneous products resulting from the covalent modifications of the proteins.

 A new method, Homogeneous Time-Resolved Fluorescence (HTRF), combines the benefits of a fluorescent label and a homogeneous
15 assay protocol in a high throughput screen. HTRF uses a pair of chemically stable labeling molecules, such as a lanthanide cryptate and a fluorescent molecule. Specifically, europium cryptate (Eu(K)) and XL-665 have been used to label biomolecules. XL-665 is a cross-linked derivative of allophycocyanin, a red-green algae pigment. HTRF works
20 by measuring the change in fluorescent energy. If XL-665 is in proximity to Eu(K), it will yield an amplified and long-lived signal. The amount of energy transfer is a function of proximity of the two molecules. Eu(K) has a long-lived fluorescent signal which facilitates the homogeneous nature of the assay. A time delay in reading the signal
25 eliminates the principal difficulty in applying fluorescence to screening formats. This technology is useful in both binding and protease assays. [Kolb et al., J. of Biomolecular Screening, 1, No. 4, 203-210 (1996); Kolb et al., Pharm. Manuf. Int., p. 31 (1996)]

SUMMARY OF THE INVENTION

 The instant invention relates to a method of screening that can be used to determine if compounds are capable of binding to a protein or are capable of blocking ligand-protein or protein-protein interactions. The instant invention covers a method of screening for

compounds capable of binding to a fusion protein which comprises combining a test compound, a biotinylated ligand, a fusion protein (target protein, peptide linker and FK506-binding protein), a donor-labeled ligand, and acceptor-labeled streptavidin, and then measuring the
5 fluorescence attributable to the binding of the biotinylated ligand to the fusion protein in the presence of the test compound relative to a control assay in the absence of the test compound, so as to determine the effect the test compound has on the binding of the biotinylated ligand. The instant invention also covers a method of screening for compounds
10 which inhibit a protease which comprises combining a test compound, a fusion protein substrate (reporter protein, peptide linker and FK506-binding protein), a protease, a donor-labeled ligand, and acceptor-labeled streptavidin, and then measuring the fluorescence attributable to the binding of the intact fusion protein substrate in the presence of the
15 test compound relative to a control assay in the absence of the test compound, so as to determine the effect the test compound has on proteolytic activity. This invention provides an immediate means of making use of HTRF technology for the functional assay of either ligand binding to a single or multiple signal transduction domain(s), tyrosine
20 phosphatases, nuclear receptors or bacterial tRNA synthetases in a signal increase assay or proteolytic activity in a signal decrease assay. The present invention is readily adaptable to robotic automation for high capacity screening for agonists, antagonists, and/or inhibitors.

25 BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1: Binding of the donor-labeled ligand (Eu(K) labeled ligand for FKBP), fusion protein (FKBP:SH2), biotinylated ligand and the acceptor-labeled streptavidin (SA-XL665), which
30 involves a fluorescent energy transfer that can be measured.

FIGURE 2: Cleaving of donor-labeled ligand (Eu(K) labeled ligand for FKBP), fusion protein substrate (FKBP:SH2:ITAM:B) and the acceptor-labeled streptavidin (SA-
35 XL665).

FIGURE 3: Cleaving of donor-labeled ligand (Eu(K) labeled ligand for FKBP), fusion protein substrate (FKBP:acetyl-CoA:B) and the acceptor-labeled streptavidin (SA-XL665).

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DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a method of screening for compounds which preferentially bind to a target protein or inhibit a protease.

10 As depicted in Figure 1, an embodiment of this invention is a method of screening for compounds capable of binding to a fusion protein which comprises the steps of:

- 15 a) mixing a test compound, a biotinylated ligand, the fusion protein, a donor-labeled ligand and acceptor-labeled streptavidin;
- b) incubating the mixture for an appropriate time;
- c) measuring the time-resolved fluorescence attributable to the binding of the biotinylated ligand to the fusion protein in the presence of the test compound; and
- 20 d) determining the binding of the biotinylated ligand to the fusion protein in the presence of the test compound relative to a control assay run in the absence of the test compound.

25 As depicted in Figures 2 and 3, another embodiment of this invention is a method of screening for compounds capable of inhibiting a protease which comprises the steps of:

- a) mixing a test compound, a fusion protein substrate, a donor-labeled ligand, acceptor-labeled streptavidin, and a protease;
- 30 b) incubating the mixture for an appropriate time;
- c) measuring the time-resolved fluorescence attributable to the binding of the intact fusion protein substrate to the acceptor-labeled streptavidin in the presence of the test compound; and

- d) determining the binding of the intact fusion protein substrate to the acceptor-labeled streptavidin in the presence of the test compound relative to a control assay run in the absence of the test compound.

5

For Figures 1-3, the following definitions apply to the terms used therein:

- Eu(K) = Europium Cryptate
10 "506" = FK506 analog
FKBP = FK506 Binding Protein
SH2 = Src homology domain 2
12-mer = phosphotyrosine-containing, 12 peptide
B = biotin
15 (SH2)₂ = tandem SH2 domains of ZAP70
ITAM = bisphosphotyrosine-containing, 25 residue peptide
SA = streptavidin

- The term "fusion protein" refers to a "target protein" fused
20 to an "FK506-binding protein" (FKBP), the two proteins being separated by a "peptide linker". The term "fusion protein substrate" refers to a "reporter protein" fused to an FKBP, the two proteins being separated by a peptide linker.

- A "peptide linker" may consist of a sequence containing
25 from about 1 to about 20 amino acids, which may or may not include the sequence for a protease cleavage site. An example of a peptide linker which is a protease cleavage site is represented by the amino acid sequence GLVPRGS. (SEQ. ID. NO. 1).

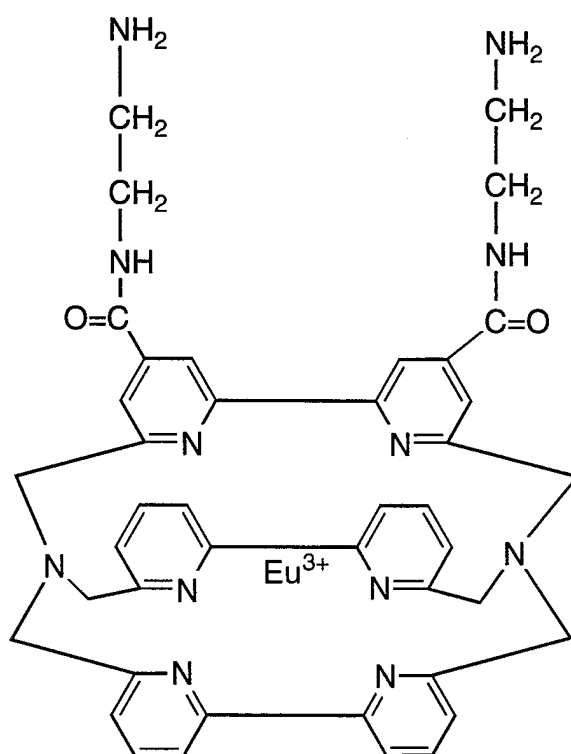
- The term "protease" refers to an enzyme that catalyzes the
30 hydrolytic breakdown of proteins and/or peptides. Examples of proteases may include, but are not limited to, thrombin, Human Immunodeficiency Virus (HIV) protease and Tumor Necrosis Factor (TNF) α converting enzyme.

The term "target protein" refers to any protein that has a defined ligand. Types of target proteins include, but are not limited to, tyrosine phosphatases (FKBP-phosphatase chimera as receptor and biotinylated-peptide containing a phosphonomethylene isostere as ligand), nuclear
5 receptors (FKBP-receptor and biotinylated DNA) and bacterial tRNA synthetases (FKBP-tRNA synthetase and biotinylated tRNA). Also included within this definition of target protein are single and multiple signal transduction domains, such as, but not limited to, Src homology 1 (SH1), Src homology 2 (SH2), Src homology 3 (SH3), and pleckstrin
10 homology (PH) domains [Hanks & Hunter, *FASEB J.*, 9, 576-596 (1995); Bolen, *Curr. Opin. Immunol.*, 7, 306-311 (1995); Kuriyan & Cowburn, *Curr. Opin. Struct. Biol.*, 3, 828-837 (1993); Cohen et al., *Cell*, 80, 237-248 (1995)]. The term "SH1 domain" refers to a family of homologous protein domains that bind ATP and catalyze tyrosine
15 phosphorylation of peptide and protein substrates. The term "SH2 domain" refers to a family of homologous protein domains that share the common property of recognizing phosphorylated tyrosine residues in specific peptide contexts. The term "SH3 domain" refers to a family of homologous protein domains that share the common property of
20 recognizing polyproline type II helices. The term "PH domain" refers to a family of homologous protein domains that mediate both protein-protein and protein-lipid interactions. Examples of SH2 domains which may be utilized in the method of the invention include, but are not limited to, the single and tandem SH2 domains present in the tyrosine
25 kinases ZAP70, Syk and Lck. The DNA sequences were obtained from GenBank, National Center for Biotechnology Information, National Library of Medicine, 8600 Rockville Pike, Bethesda, MD 20894. The Accession Numbers for the sequences are: human ZAP70 (L05148) human Syk (L28824) and human Lck (X13529). The sequences for
30 ZAP70, Syk and Lck are disclosed in the sequence listing as follows: the isolated DNA encoding for a fusion protein containing ZAP70 is (SEQ. ID. NO. 2); the isolated DNA encoding for a fusion protein containing Syk is (SEQ. ID. NO. 3); the isolated DNA encoding for a fusion protein containing Lck is (SEQ. ID. NO. 4); the sequence for the

FKBP-ZAP70:SH2 fusion protein is (SEQ. ID. NO. 5); the sequence for the FKBP-Syk:SH2 fusion protein is (SEQ. ID. NO. 6); and the sequence for the FKBP-Lck:SH2 fusion protein is (SEQ. ID. NO. 7).

5 The term "reporter protein" refers to a protein containing covalent biotin or a noncovalently bound biotinylated ligand. Examples of proteins containing covalent biotin are the C-terminal 87 residues of the biotin carboxy carrier protein of acetyl-CoA carboxylase from *Escherichia coli* and the biotin-carrier subunit of transcarboxylase from *Propionibacterium shermanii*. An example of a protein containing a
10 noncovalently bound biotinylated ligand is the tandem SH2 domains of ZAP70 bound to a biotinylated phosphopeptide derived from an Immunoreceptor Tyrosine-based Activation Motif (ITAM) sequence of the human T-cell receptor, the B-cell receptor or a high affinity Immunoglobulin E. (IgE) receptor. Specifically, the Zeta (ζ) 1
15 sequence of the human T-cell receptor may be utilized. (SEQ. ID. NO. 8) (California Peptide Research Inc., 918 Enterprise Way, Suite 1, Napa, CA 94558)

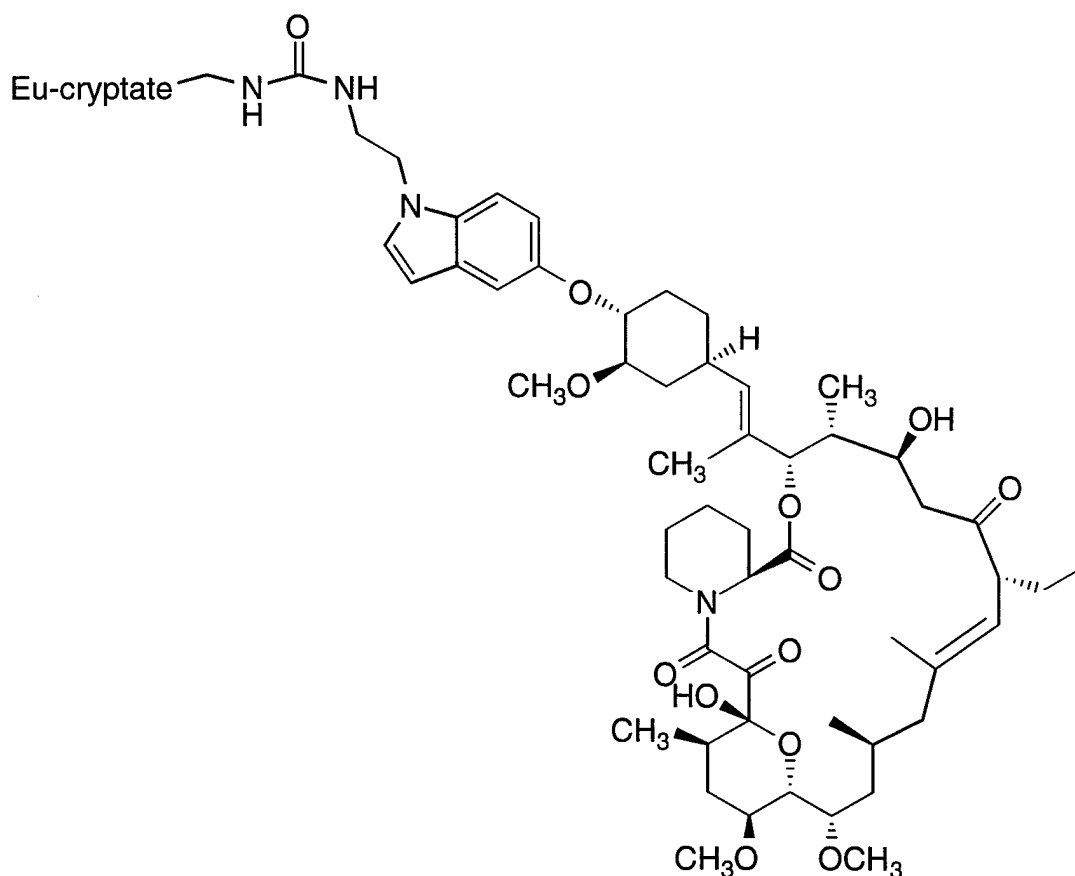
The term "donor-labeled ligand" refers to an organic cryptate-containing molecule loaded with a fluorescent lanthanide
20 metal which binds to the FKBP. An example of a fluorescent lanthanide metal is europium. The europium-cryptate molecule was obtained from CIS Bio International, Subsidiary of Compagnie ORIS INDUSTRIE SA, Boite Postale 175, F30203 Bagnols sur Ceze Cedex, France, is depicted below.



5

Europium Cryptate (Eu(K))

An example of a donor-labeled ligand useful in the instant invention is



5 The term "acceptor-labeled streptavidin" refers to streptavidin coupled to one or more light harvesting molecules. An example of a light harvesting molecule is allophycocyanin and an example of an acceptor-labeled streptavidin useful in the instant invention is SA-XL665. (CIS Bio International, Subsidiary of Compagnie ORIS INDUSTRIE SA, Boite Postale 175, F30203 Bagnols sur Ceze Cedex, France)

10 The term "control assay" refers to the assay when performed in the presence of the donor-labeled ligand, acceptor-labeled streptavidin, and either fusion protein substrate or fusion protein plus biotinylated ligand, but in the absence of the test compound.

15 The term FK506-binding proteins may include, but are not limited to, the below listed FKBP and FKBP homologues, which

include a citation to the references which disclose them. This list is not intended to limit the scope of the invention.

Mammalian

- | | | |
|----|-----------|--|
| 5 | FKBP-12 | Galat et al., <i>Eur. J. Biochem.</i> , 216:689-707 (1993). |
| | FKBP-12.6 | Wiederrecht, G. and F. Etzkorn
<i>Perspectives in Drug Discovery and Design</i> , 2:57-84 (1994). |
| 10 | FKBP-13 | Galat et al., <i>supra</i> ; Wiederrecht and Etzkorn, <i>supra</i> . |
| | FKBP-25 | Galat et al., <i>supra</i> ; Wiederrecht and Etzkorn, <i>supra</i> . |
| | FKBP-38 | Wiederrecht and Etzkorn, <i>supra</i> . |
| 15 | FKBP-51 | Baughman et al., <i>Mol. Cell. Biol.</i> , 8, 4395-4402(1995) . |
| | FKBP-52 | Galat et al., <i>supra</i> . |

Bacteria

- | | | |
|----|---------------------------|---|
| 20 | Legionella pneumophila | Galat et al., <i>supra</i> . |
| | Legionella micadei | Galat et al., <i>supra</i> . |
| | Chlamydia trachomatis | Galat et al., <i>supra</i> . |
| | E. coli fkpa | Horne, S.M. and K.D. Young, <i>Arch. Microbiol.</i> , 163:357-365 (1995). |
| 25 | E. coli slyD | Roof et al., <i>J. Biol. Chem.</i> 269:2902-2910 (1994). |
| | E. coli orf149 | Trandinh et al., <i>FASEB J.</i> 6:3410-3420 (1992). |
| | Neisseria meningitidis | Hacker, J. and G. Fischer, <i>Mol. Micro.</i> , 10:445-456 (1993). |
| 30 | Streptomyces chrysomallus | Hacker and Fischer, <i>supra</i> . |

Fungal

- | | | |
|---|------------------|---|
| | yeast FKBP-12 | Cardenas et al., <i>Perspectives in Drug Discovery and Design</i> , 2:103-126 (1994). |
| 5 | yeast FKBP-13 | Cardenas et al., <i>supra</i> . |
| | yeast NPR1(FPR3) | Cardenas et al., <i>supra</i> . |
| | Neurospora | Galat et al., <i>supra</i> . |

10 A variety of host cells used to produce the FKBP
chimeras may be used in this invention, which include, but are not
limited to, bacteria, yeast, bluegreen algae, plant cells, insect cells
and animal cells.

15 Expression vectors are defined herein as DNA sequences
that are required for the transcription of cloned copies of genes and
the translation of their mRNAs in an appropriate host. Such vectors
can be used to express genes in a variety of host cells, such as,
bacteria, yeast, bluegreen algae, plant cells, insect cells and animal
cells.

20 Specifically designed vectors allow the shuttling of DNA
between hosts such as bacteria-yeast or bacteria-animal cells. An
appropriately constructed expression vector may contain: an origin of
replication for autonomous replication in host cells, selectable markers,
a limited number of useful restriction enzyme sites, a potential for high
copy number, and active promoters. A promoter is defined as a DNA
25 sequence that directs RNA polymerase to bind to DNA and initiate RNA
synthesis. A strong promoter is one which causes mRNAs to be initiated
at high frequency. Expression vectors may include, but are not limited
to, cloning vectors, modified cloning vectors, specifically designed
plasmids or viruses. Commercially available vectors suitable for FKBP
30 fusion protein expression include, but are not limited to pBR322
(Promega), pGEX (Amersham), pT7 (USB), pET (Novagen), pIBI
(IBI), pProEX-1 (Gibco/BRL), pBluescript II (Stratagene), pTZ18R and
pTZ19R (USB), pSE420 (Invitrogen), pVL1392 (Invitrogen), pBlueBac
(Invitrogen), pBacPAK (Clontech), pHIL (Invitrogen), pYES2

(Invitrogen), pCDNA (Invitrogen), pREP (Invitrogen) or the like. The expression vector may be introduced into host cells via any one of a number of techniques including but not limited to transformation, transfection, infection, protoplast fusion, and electroporation.

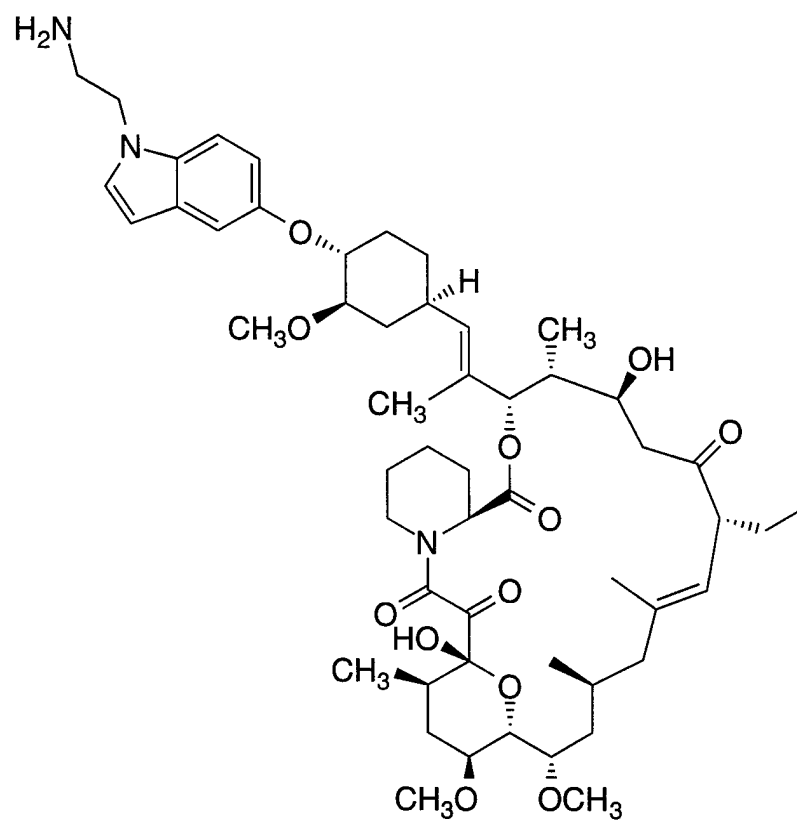
5 *E. coli* containing an expression plasmid with the gene for the target or reporter protein fused to FKBP are grown and appropriately induced. The cells are then pelleted and resuspended in a suitable buffer. Although FKBP-12 lacks sequences that specifically direct it to the periplasm, FKBP fusions may be located there and can be released by a standard freeze/thaw treatment of the cell pellet.
10 Following centrifugation, the resulting supernatant contains >80% pure FKBP fusion, which if desired can be purified further by conventional methods. Alternatively, the assay is not dependent on pure protein and the initial periplasmic preparation may be used directly.

15 A fusion protein which contains a single or multiple SH2 domain(s) may be purified by preparing an affinity matrix consisting of biotinylated phosphopeptide coupled to avidin or streptavidin immobilized on a solid support. A freeze/thaw extract is prepared from the cells which express the fusion protein and is loaded onto the affinity
20 matrix. The desired fusion protein is then specifically eluted with phenyl phosphate.

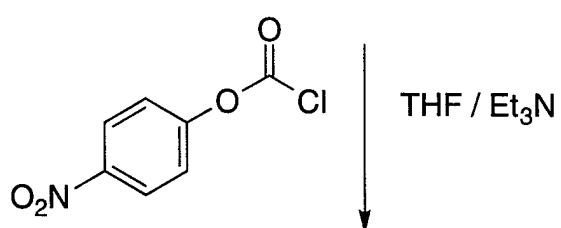
 To assay the formation of a complex between a target protein and its ligand, the biotinylated ligand is mixed with the FKBP fusion protein in a suitable buffer in the presence of the donor-labeled
25 ligand in the well of a black microplate. After a suitable incubation period to allow complex formation to occur, acceptor-labeled streptavidin is added to capture the tagged ligand and any bound fusion protein. The plate is incubated for a sufficient period to allow the capture to go to completion and then the time resolved fluorescent signal
30 is measured. Screening for agonists/antagonists/inhibitors is carried out by performing the initial incubation prior to the acceptor-labeled streptavidin capture step in the presence of a test compound(s) to determine whether they have an effect upon the binding of the

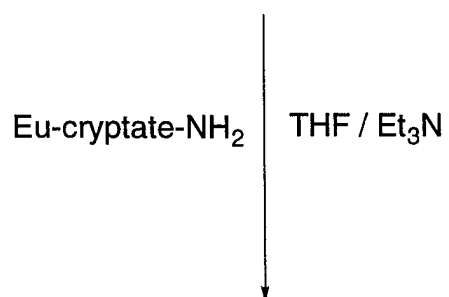
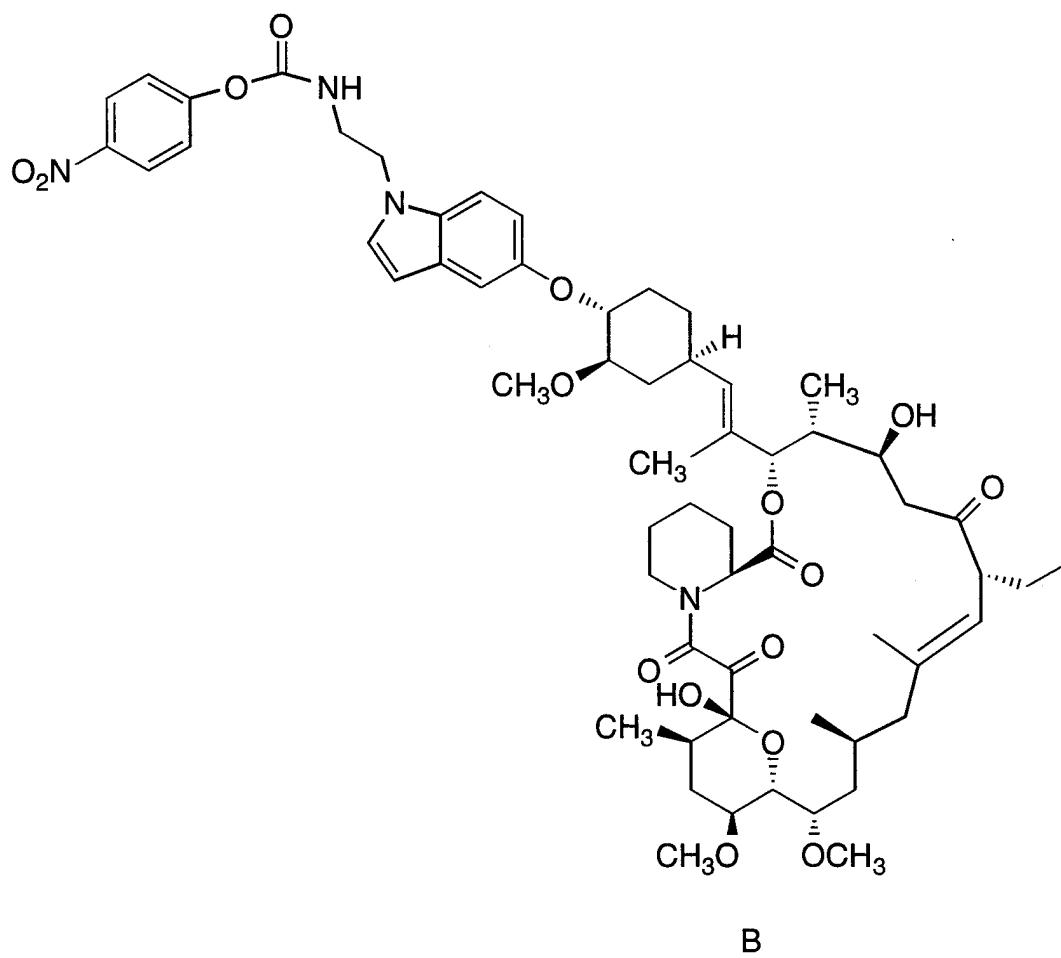
biotinylated ligand to the fusion protein. This principle is illustrated by Figure 1.

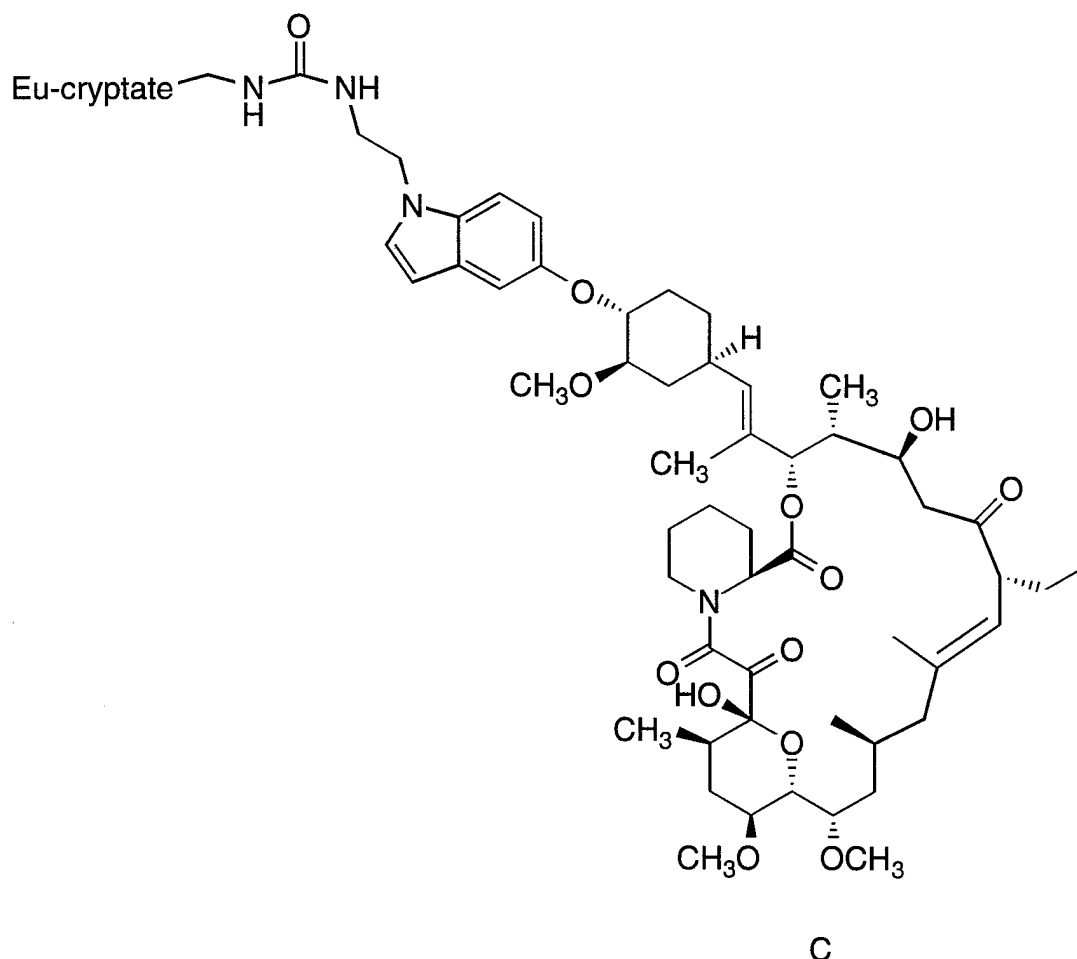
5 To assay the catalytic activity of a protease, a FKBP fusion protein substrate is combined with the protease in a suitable buffer in the presence of the donor-labeled ligand and acceptor-labeled streptavidin in the well of a black microplate. After a suitable incubation period, the time resolved fluorescent signal of the remaining intact fusion protein substrate is measured. Screening for inhibitors is carried out by performing the incubation in the presence of a test
10 compound(s) to determine whether they have an effect upon the cleavage of the fusion protein substrate. This principle is illustrated by Figures 2 and 3.

SCHEME 1

A



SCHEME 1 (cont'd.)

SCHEME 1 (cont'd.)

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Scheme 1 depicts the treatment of Compound A, an analog of FK506, with 1 equivalent of p-nitrophenylchloroformate in THF in the presence of a tertiary amine, which gives the p-nitrophenylcarbamate, Compound B. The nitrophenylcarbamate is isolated by silica gel chromatography or used without further purification. The nitrophenylcarbamate is an activated acylating group and will undergo attack by a variety of nucleophiles. The cryptate has two primary amino groups (nucleophiles). Treatment of the p-nitrophenylcarbamate with the europium cryptate in a non-protic

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solvent (such as THF) in the presence of a tertiary amine should afford the corresponding urea. Compound C, the cryptate hybrid of the FK506 analog, will be purified by either normal phase silica gel chromatography on prep TLC plates or by reverse phase HPLC chromatography.

The cryptate can be linked to Compound A, other FK506 analogs, and related molecules by a variety of linkages, such as carbamates, amides, ureas, amines, etc. One of ordinary skill in the art would be familiar with the standard procedures used to prepare the FK506 analog containing the cryptate molecule.

The present invention can be understood further by the following examples, which do not constitute a limitation of the invention.

EXAMPLE 1

Process for Preparing the FKBP fusion cloning vector

General techniques for modifying and expressing genes in various host cells can be found in Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. Current Protocols in Molecular Biology (John Wiley & Sons, New York, New York, 1989). Sequences for a 3'- altered FKBP fragment that contained a glycine codon (GGT) in place of the stop (TGA) codon followed by a sequence encoding a thrombin site (Leu-Val-Pro-Arg) and *Bam*HI restriction site (GGATCC) were amplified using the polymerase chain reaction (PCR). The PCR reaction contained the following primers: 5'-GATCGCCATGGGAGTGCAGGTGGAAACCATCTCCCCA-3' (SEQ. ID. NO. 9) and 5'-TACGAATTCTGGCGTGGATCCAC GCGGAACCAGACCTTCCAGTTTTAG-3' (SEQ. ID. NO. 10) and a plasmid containing human FKBP-12 as the template. The resulting 367 base pair amplification product was ligated into the vector pCRII (Invitrogen) and the ligation mixture transformed into competent *Escherichia coli* cells. Clones containing an insert were identified using PCR with flanking vector primers. Dideoxy DNA sequencing confirmed the nucleotide sequence of one positive isolate. The altered

338 base pair FKBP fragment was excised from the pCRII plasmid using *NcoI* and *BamHI* and ligated into *NcoI* and *BamHI* digested pET9d (Novagen) plasmid. Competent *E. coli* were transformed with the ligation mixture, and colonies containing the insert were identified using
5 PCR with primers encoding for flanking vector sequences. The FKBP fusion cloning vector is called pET9dFKBPt.

EXAMPLE 2

10 Process for Preparing the FK-ZAP70 fusion expression vector

A DNA fragment encoding for the tandem SH2 domains of ZAP70 was prepared by PCR to contain a *BamHI* site at the 5'-end such that the reading frame was conserved with that of FKBP in the fusion vector. At the 3'-end, the fragment also incorporated a stop codon
15 followed by a *BamHI* site. The PCR reaction contained Molt-4 cDNA (Clontech) and the following primers:
5'-ATTAGGATCCATGCCAGATCCTGCAGCTCACCTGCCCT-3'
(SEQ. ID. NO. 11) and 5'-ATATGGATCCTTACCAGAGGCGTTGCT-3'
(SEQ. ID. NO. 12). The fragment was cloned into a suitable vector,
20 sequenced, digested with *BamHI*, and the insert containing the SH2 domains ligated to *BamHI* treated pET9dFKBPt, and transformed into *E. coli*. Clones containing inserts in the correct orientation were identified by PCR or restriction enzyme analysis. Plasmid DNA was prepared and used to transform BL21(DE3) cells.

25

EXAMPLE 3

Process for Preparing the FK-Syk fusion expression vector

The expression vector for the tandem SH2 domains of Syk fused to FKBP was prepared as in Example 2 except that the PCR
30 reaction contained Raji cell cDNA (Clontech) and the following primers: 5'-CAATAGGATCCATGGCCAGCAGCGGCATGGCTGA-3'
(SEQ. ID. NO. 13) and
5'-GACCTAGGATCCCTAATTAACATTTCCCTGTGTGCCGAT-3'
35 (SEQ. ID. NO. 14).

EXAMPLE 4

Process for Preparing the FK-Lck fusion expression vector

- 5 The expression vector for the SH2 domain of Lck fused to FKBP was prepared as in Example 2 except that the PCR reaction contained Molt-4 cDNA (Clontech) and the following primers:
- 5'-ATATGGATCCATGGCGAACAGCCTGGAGCCCCGAACCCT-3'
 (SEQ. ID. NO. 15) and
- 10 5'-ATTAGGATCCTTAGGTCTGGCAGGGGCGGCTCAACCGTGT
 GCA-3' (SEQ. ID. NO. 16).

EXAMPLE 5

FK-ZAP70

- 15 Step A: Process for Expression of FK-ZAP70
 E. coli BL21(DE3) cells containing the pET9dFKBPt/
 ZAP70SH2 plasmid were grown in Luria-Bertani (LB) media
 containing 50 microgram/ml kanamycin at 37 degrees C until the optical
20 density measured at 600 nm was 0.5-1.0. Expression of the FK-ZAP70
 fusion protein was induced with 0.1 mM isopropyl beta-
 thiogalactopyranoside and the cells were grown for another 3-5 hr at 30
 degrees C. They were pelleted at 4400 x g for 10 min at 4 degrees C
 and resuspended in 2% of the original culture volume with 100 mM tris
25 pH 8.0 containing 1 microgram/ml each aprotinin, pepstatin, leupeptin,
 and bestatin. The resuspended pellet was frozen at -20 degrees C until
 further purification.

Step B: Process for Purification of FK-ZAP70

- 30 The affinity matrix for purification of FK-ZAP70 was
 prepared by combining agarose-immobilized avidin with excess
 biotinylated phosphopeptide derived from the ζ 1 ITAM sequence of the
 human T-cell receptor, biotinyl-GSNQLpYNELNLGRREEpYDVLDK,
 (SEQ. ID. NO. 8) and washing out unbound peptide. Frozen cells
35 containing FK-ZAP70 were thawed in warm water, refrozen on dry ice

for about 25 min., then thawed again. After the addition of 0.1% octyl glucoside, 1 mM dithiothreitol (DTT) and 500 mM NaCl, the extract was centrifuged at 35,000 x g for approximately 30 minutes. The supernatant was loaded onto the phosphopeptide affinity column, at about 4° and washed with phosphate buffered saline containing 1 mM DTT and 0.1% octyl glucoside. FK-ZAP70 was eluted with 200 mM phenyl phosphate in the same buffer at about 37°. The protein pool was concentrated and the phenyl phosphate removed on a desalting column. The purified FK-ZAP70 was stored at about -30° in 10 mM HEPES/150 mM NaCl/1 mM DTT/0.1 mM EDTA/10% glycerol.

EXAMPLE 6

FK-Syk

E. coli BL21(DE3) cells containing the pET9dFKBPt/SykSH2 plasmid were grown, induced, and harvested as described in Example 5. FK-Syk was purified using the same affinity matrix and methodology described in Example 5.

EXAMPLE 7

FK-Lck

E. coli BL21(DE3) cells containing the pET9dFKBPt/LckSH2 plasmid were grown, induced, and harvested as described in Example 5. The affinity matrix for purification of FK-Lck was prepared by combining agarose-immobilized avidin with excess biotinyl-EPQpYEEIPIYL, (SEQ. ID. NO. 17) and washing out unbound peptide. The remaining methodology for purification was the same as Example 5.

EXAMPLE 8

Method of Screening for Inhibitors of FK-ZAP

A DMSO solution of test compound(s) and biotinyl-phosphopeptide stock solution in a suitable buffer are dispensed into the

- wells of a 96-well black microplate. Next, a mixture of FK-ZAP protein and Eu(K)-labeled FK506 analog are added to each test well. Finally, a solution of SA-XL665 is dispensed to each well and the plate is incubated for an appropriate time. The fluorescence ratio is then
- 5 measured in a Packard Discovery HTRF analyzer. (Packard Instrument Company, 800 Research Parkway, Meridan, CT 06450)

EXAMPLE 9

10 Method of Screening for Inhibitors of Thrombin

- A DMSO solution of test compound(s) and a suitable buffer containing a mixture of FK-ZAP protein, Eu(K)-labeled FK506 analog, and biotinyl-phosphopeptide are dispensed into the wells of a 96-well black microplate. Next, thrombin is added to each test well. After an
- 15 appropriate incubation period, a solution of SA-XL665 is dispensed to each well. The fluorescence ratio is then measured in a Packard Discovery HTRF analyzer. (Packard Instrument Company, 800 Research Parkway, Meridan, CT 06450)

SEQUENCE LISTING

(1) GENERAL INFORMATION

- (i) APPLICANT: HERMES, JEFFREY D.
SALOWE, SCOTT P.
SINCLAIR, PETER J.
- (ii) TITLE OF THE INVENTION: A HIGH THROUGHPUT ASSAY USING
FUSION PROTEINS
- (iii) NUMBER OF SEQUENCES: 17
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Merck & Co., Inc.
 - (B) STREET: P.O. Box 2000, 126 E. Lincoln Ave.
 - (C) CITY: Rahway
 - (D) STATE: NJ
 - (E) COUNTRY: USA
 - (F) ZIP: 07065-0900
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Diskette
 - (B) COMPUTER: IBM Compatible
 - (C) OPERATING SYSTEM: DOS
 - (D) SOFTWARE: FastSEQ for Windows Version 2.0
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: 60/040,795
 - (B) FILING DATE: 14 March 1997
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Camara, Valerie J
 - (B) REGISTRATION NUMBER: 35,090
 - (C) REFERENCE/DOCKET NUMBER: 19916
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: 732-594-3902
 - (B) TELEFAX: 732-594-4720
 - (C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 7 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Gly Leu Val Pro Arg Gly Ser
 1 5

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1137 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```

ATGGGAGTGC AGGTGGAAAC CATCTCCCCA GGAGATGGAC GCACCTTCCC CAAGCGCGGC 60
CAGACCTGCG TGGTGCAC TA CACCGGGATG CTTGAAGATG GAAAGAAATT TGATTCCTCC 120
CGGGACAGAA ACAAGCCCTT TAAGTTTATG CTAGGCAAGC AGGAGGTGAT CCGAGGCTGG 180
GAAGAAGGGG TTGCCCAGAT GAGTGTGGGT CAGAGAGCCA AACTGACTAT ATCTCCAGAT 240
TATGCCTATG GTGCCACTGG GCACCCAGGC ATCATCCCAC CACATGCCAC TCTCGTCTTC 300
GATGTGGAGC TTCTAAAACT GGAAGGTCTG GTTCCGCGTG GATCCATGCC AGATCCTGCA 360
GCTCACCTGC CCTTCTTCTA CGGCAGCATC TCGCGTGCCG AGGCCGAGGA GCACCTGAAG 420
CTGGCGGGCA TGGCGGACGG GCTCTTCTTG CTGCGCCAGT GCCTGCGCTC GCTGGGCGGC 480
TATGTGCTGT CGCTCGTGCA CGATGTGCGC TTCCACCACT TTCCCATCGA GCGCCAGCTC 540
AACGGCACCT ACGCCATTGC CGGCGGCAAA GCGCACTGTG GACCGGCAGA GCTCTGCGAG 600
TTCTACTCGC GCGACCCCGA CGGGCTGCCC TGCAACCTGC GCAAGCCGTG CAACCGGCCG 660
TCGGGCCTCG AGCCGCAGCC GGGGGTCTTC GACTGCCTGC GAGACGCCAT GGTGCGTGAC 720
TACGTGCGCC AGACGTGGAA GCTGGAGGGC GAGGCCCTGG AGCAGGCCAT CATCAGCCAG 780
GCCCCGAGG TGGAGAAGCT CATTGCTACG ACGGCCACG AGCGGATGCC CTGGTACCAC 840
AGCAGCCTGA CGCGTGAGGA GGCCGAGCGT AAACCTTACT CTGGGGCGCA GACCGACGGC 900
AAGTTCCTGC TGAGGCCGCG GAAGGAGCAG GGCACATACG CCCTGTCCCT CATCTATGGG 960
AAGACGGTGT ACCACTACCT CATCAGCCAA GACAAGGCGG GCAAGTACTG CATTCCCAG 1020
GGCACCAAGT TTGACACGCT CTGGCAGCTG GTGGAGTATC TGAAGCTGAA GGCGGACGGG 1080
CTCATCTACT GCCTGAAGGA GGCCTGCCCC AACAGCAGTG CCAGCAACGC CTCTTAA 1137

```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1155 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```

ATGGGAGTGC AGGTGGAAAC CATCTCCCCA GGAGATGGAC GCACCTTCCC CAAGCGCGGC 60
CAGACCTGCG TGGTGCAC TA CACCGGGATG CTTGAAGATG GAAAGAAATT TGATTCCTCC 120
CGGGACAGAA ACAAGCCCTT TAAGTTTATG CTAGGCAAGC AGGAGGTGAT CCGAGGCTGG 180
GAAGAAGGGG TTGCCCAGAT GAGTGTGGGT CAGAGAGCCA AACTGACTAT ATCTCCAGAT 240
TATGCCTATG GTGCCACTGG GCACCCAGGC ATCATCCCAC CACATGCCAC TCTCGTCTTC 300
GATGTGGAGC TTCTAAAACT GGAAGGTCTG GTTCCGCGTG GATCCATGGC CAGCAGCGGC 360

```

```

ATGGCTGACA GCGCCAACCA CCTGCCCTTC TTTTTCGGCA ACATCACCCG GGAGGAGGCA 420
GAAGATTACC TGGTCCAGGG GGGCATGAGT GATGGGCTTT ATTTGCTGCG CCAGAGCCGC 480
AACTACCTGG GTGGCTTCGC CCTGTCCGTG GCCACGGGA GGAAGGCACA CCACTACACC 540
ATCGAGCGGG AGCTGAATGG CACCTACGCC ATCGCCGGTG GCAGGACCCA TGCCAGCCCC 600
GCCGACCTCT GCCACTACCA CTCCCAGGAG TCTGATGGCC TGGTCTGCCT CCTCAAGAAG 660
CCCTTCAACC GGCCCCAAGG GGTGCAGCCC AAGACTGGGC CCTTTGAGGA TTTGAAGGAA 720
AACCTCATCA GGAATATGT GAAGCAGACA TGGAACCTGC AGGGTCAGGC TCTGGAGCAG 780
GCCATCATCA GTCAGAAGCC TCAGCTGGAG AAGCTGATCG CTACCACAGC CCATGAAAAA 840
ATGCCTTGGT TCCATGGAAA AATCTCTCGG GAAGAATCTG AGCAAATTGT CCTGATAGGA 900
TCAAAGACAA ATGGAAAGTT CCTGATCCGA GCCAGAGACA ACAACGGCTC CTACGCCCTG 960
TGCCCTGCTGC ACGAAGGGAA GGTGCTGCAC TATCGCATCG ACAAAGACAA GACAGGGAAG 1020
CTCTCCATCC CCGAGGGAAA GAAGTTCGAC ACGTCTGGC AGCTAGTCGA GCATTATTTCT 1080
TATAAAGCAG ATGGTTTGT T AAGAGTTCTT ACTGTCCCAT GTCAAAAAAT CGGCACACAG 1140
GGAAATGTTA ATTAG                                     1155

```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 675 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```

ATGGGAGTGC AGGTGGAAAC CATCTCCCA GGAGATGGAC GCACCTTCCC CAAGCGCGGC 60
CAGACCTGCG TGGTGCACCTA CACCGGGATG CTTGAAGATG GAAAGAAATT TGATTCTCTC 120
CGGGACAGAA ACAAGCCCTT TAAGTTTATG CTAGGCAAGC AGGAGGTGAT CCGAGGCTGG 180
GAAGAAGGGG TTGCCCAGAT GAGTGTGGGT CAGAGAGCCA AACTGACTAT ATCTCCAGAT 240
TATGCCTATG GTGCCACTGG GCACCCAGGC ATCATCCAC CACATGCCAC TCTCGTCTTC 300
GATGTGGAGC TTCTAAAACT GGAAGGTCTG GTTCCGCGTG GATCCATGGC GAACAGCCTG 360
GAGCCCGAAC CCTGGTTCTT CAAGAACCTG AGCCGCAAGG ACGCGGAGCG GCAGCTCCTG 420
GCGCCCGGGA ACACTCACGG CTCCTTCCTC ATCCGGGAGA GCGAGAGCAC CGCGGGATCG 480
TTTTCACTGT CGGTCCGGGA CTTGACCAG AACCAGGGAG AGGTGGTGAA ACATTACAAG 540
ATCCGTAATC TGGACAACGG TGGCTTCTAC ATCTCCCCTC GAATCACTTT TCCCGGCCTG 600
CATGAACTGG TCCGCCATTA CACCAATGCT TCAGATGGGC TGTGCACACG GTTGAGCCGC 660
CCCTGCCAGA CCTAA                                     675

```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 378 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```

Met Gly Val Gln Val Glu Thr Ile Ser Pro Gly Asp Gly Arg Thr Phe
 1           5           10           15
Pro Lys Arg Gly Gln Thr Cys Val Val His Tyr Thr Gly Met Leu Glu
          20           25           30

```

Asp	Gly	Lys	Lys	Phe	Asp	Ser	Ser	Arg	Asp	Arg	Asn	Lys	Pro	Phe	Lys
		35					40					45			
Phe	Met	Leu	Gly	Lys	Gln	Glu	Val	Ile	Arg	Gly	Trp	Glu	Glu	Gly	Val
	50					55					60				
Ala	Gln	Met	Ser	Val	Gly	Gln	Arg	Ala	Lys	Leu	Thr	Ile	Ser	Pro	Asp
65					70					75					80
Tyr	Ala	Tyr	Gly	Ala	Thr	Gly	His	Pro	Gly	Ile	Ile	Pro	Pro	His	Ala
			85						90					95	
Thr	Leu	Val	Phe	Asp	Val	Glu	Leu	Leu	Lys	Leu	Glu	Gly	Leu	Val	Pro
			100					105					110		
Arg	Gly	Ser	Met	Pro	Asp	Pro	Ala	Ala	His	Leu	Pro	Phe	Phe	Tyr	Gly
		115					120					125			
Ser	Ile	Ser	Arg	Ala	Glu	Ala	Glu	Glu	His	Leu	Lys	Leu	Ala	Gly	Met
	130					135					140				
Ala	Asp	Gly	Leu	Phe	Leu	Leu	Arg	Gln	Cys	Leu	Arg	Ser	Leu	Gly	Gly
145					150					155					160
Tyr	Val	Leu	Ser	Leu	Val	His	Asp	Val	Arg	Phe	His	His	Phe	Pro	Ile
				165				170						175	
Glu	Arg	Gln	Leu	Asn	Gly	Thr	Tyr	Ala	Ile	Ala	Gly	Gly	Lys	Ala	His
			180					185					190		
Cys	Gly	Pro	Ala	Glu	Leu	Cys	Glu	Phe	Tyr	Ser	Arg	Asp	Pro	Asp	Gly
		195					200					205			
Leu	Pro	Cys	Asn	Leu	Arg	Lys	Pro	Cys	Asn	Arg	Pro	Ser	Gly	Leu	Glu
	210					215					220				
Pro	Gln	Pro	Gly	Val	Phe	Asp	Cys	Leu	Arg	Asp	Ala	Met	Val	Arg	Asp
225					230					235					240
Tyr	Val	Arg	Gln	Thr	Trp	Lys	Leu	Glu	Gly	Glu	Ala	Leu	Glu	Gln	Ala
				245					250					255	
Ile	Ile	Ser	Gln	Ala	Pro	Gln	Val	Glu	Lys	Leu	Ile	Ala	Thr	Thr	Ala
			260					265					270		
His	Glu	Arg	Met	Pro	Trp	Tyr	His	Ser	Ser	Leu	Thr	Arg	Glu	Glu	Ala
		275				280						285			
Glu	Arg	Lys	Leu	Tyr	Ser	Gly	Ala	Gln	Thr	Asp	Gly	Lys	Phe	Leu	Leu
	290					295					300				
Arg	Pro	Arg	Lys	Glu	Gln	Gly	Thr	Tyr	Ala	Leu	Ser	Leu	Ile	Tyr	Gly
305					310					315					320
Lys	Thr	Val	Tyr	His	Tyr	Leu	Ile	Ser	Gln	Asp	Lys	Ala	Gly	Lys	Tyr
				325					330					335	
Cys	Ile	Pro	Glu	Gly	Thr	Lys	Phe	Asp	Thr	Leu	Trp	Gln	Leu	Val	Glu
			340					345					350		
Tyr	Leu	Lys	Leu	Lys	Ala	Asp	Gly	Leu	Ile	Tyr	Cys	Leu	Lys	Glu	Ala
	355						360					365			
Cys	Pro	Asn	Ser	Ser	Ala	Ser	Asn	Ala	Ser						
	370						375								

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 384 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met	Gly	Val	Gln	Val	Glu	Thr	Ile	Ser	Pro	Gly	Asp	Gly	Arg	Thr	Phe
1				5					10					15	
Pro	Lys	Arg	Gly	Gln	Thr	Cys	Val	Val	His	Tyr	Thr	Gly	Met	Leu	Glu
			20					25					30		
Asp	Gly	Lys	Lys	Phe	Asp	Ser	Ser	Arg	Asp	Arg	Asn	Lys	Pro	Phe	Lys
		35				40					45				
Phe	Met	Leu	Gly	Lys	Gln	Glu	Val	Ile	Arg	Gly	Trp	Glu	Glu	Gly	Val
	50					55					60				
Ala	Gln	Met	Ser	Val	Gly	Gln	Arg	Ala	Lys	Leu	Thr	Ile	Ser	Pro	Asp
65					70					75					80
Tyr	Ala	Tyr	Gly	Ala	Thr	Gly	His	Pro	Gly	Ile	Ile	Pro	Pro	His	Ala
				85					90					95	
Thr	Leu	Val	Phe	Asp	Val	Glu	Leu	Leu	Lys	Leu	Glu	Gly	Leu	Val	Pro
			100					105					110		
Arg	Gly	Ser	Met	Ala	Ser	Ser	Gly	Met	Ala	Asp	Ser	Ala	Asn	His	Leu
		115					120					125			
Pro	Phe	Phe	Phe	Gly	Asn	Ile	Thr	Arg	Glu	Glu	Ala	Glu	Asp	Tyr	Leu
	130					135					140				
Val	Gln	Gly	Gly	Met	Ser	Asp	Gly	Leu	Tyr	Leu	Leu	Arg	Gln	Ser	Arg
145					150					155					160
Asn	Tyr	Leu	Gly	Gly	Phe	Ala	Leu	Ser	Val	Ala	His	Gly	Arg	Lys	Ala
				165					170					175	
His	His	Tyr	Thr	Ile	Glu	Arg	Glu	Leu	Asn	Gly	Thr	Tyr	Ala	Ile	Ala
			180					185					190		
Gly	Gly	Arg	Thr	His	Ala	Ser	Pro	Ala	Asp	Leu	Cys	His	Tyr	His	Ser
		195					200					205			
Gln	Glu	Ser	Asp	Gly	Leu	Val	Cys	Leu	Leu	Lys	Lys	Pro	Phe	Asn	Arg
	210					215					220				
Pro	Gln	Gly	Val	Gln	Pro	Lys	Thr	Gly	Pro	Phe	Glu	Asp	Leu	Lys	Glu
225					230					235					240
Asn	Leu	Ile	Arg	Glu	Tyr	Val	Lys	Gln	Thr	Trp	Asn	Leu	Gln	Gly	Gln
				245					250					255	
Ala	Leu	Glu	Gln	Ala	Ile	Ile	Ser	Gln	Lys	Pro	Gln	Leu	Glu	Lys	Leu
			260					265					270		
Ile	Ala	Thr	Thr	Ala	His	Glu	Lys	Met	Pro	Trp	Phe	His	Gly	Lys	Ile
		275					280					285			
Ser	Arg	Glu	Glu	Ser	Glu	Gln	Ile	Val	Leu	Ile	Gly	Ser	Lys	Thr	Asn
	290					295					300				
Gly	Lys	Phe	Leu	Ile	Arg	Ala	Arg	Asp	Asn	Asn	Gly	Ser	Tyr	Ala	Leu
305					310					315					320
Cys	Leu	Leu	His	Glu	Gly	Lys	Val	Leu	His	Tyr	Arg	Ile	Asp	Lys	Asp
			325						330				335		
Lys	Thr	Gly	Lys	Leu	Ser	Ile	Pro	Glu	Gly	Lys	Lys	Phe	Asp	Thr	Leu
			340					345				350			
Trp	Gln	Leu	Val	Glu	His	Tyr	Ser	Tyr	Lys	Ala	Asp	Gly	Leu	Leu	Arg
		355					360					365			
Val	Leu	Thr	Val	Pro	Cys	Gln	Lys	Ile	Gly	Thr	Gln	Gly	Asn	Val	Asn
	370						375					380			

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 224 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Met	Gly	Val	Gln	Val	Glu	Thr	Ile	Ser	Pro	Gly	Asp	Gly	Arg	Thr	Phe
1				5					10					15	
Pro	Lys	Arg	Gly	Gln	Thr	Cys	Val	Val	His	Tyr	Thr	Gly	Met	Leu	Glu
			20					25					30		
Asp	Gly	Lys	Lys	Phe	Asp	Ser	Ser	Arg	Asp	Arg	Asn	Lys	Pro	Phe	Lys
		35				40						45			
Phe	Met	Leu	Gly	Lys	Gln	Glu	Val	Ile	Arg	Gly	Trp	Glu	Glu	Gly	Val
	50					55					60				
Ala	Gln	Met	Ser	Val	Gly	Gln	Arg	Ala	Lys	Leu	Thr	Ile	Ser	Pro	Asp
65					70					75					80
Tyr	Ala	Tyr	Gly	Ala	Thr	Gly	His	Pro	Gly	Ile	Ile	Pro	Pro	His	Ala
			85						90					95	
Thr	Leu	Val	Phe	Asp	Val	Glu	Leu	Leu	Lys	Leu	Glu	Gly	Leu	Val	Pro
			100						105				110		
Arg	Gly	Ser	Met	Ala	Asn	Ser	Leu	Glu	Pro	Glu	Pro	Trp	Phe	Phe	Lys
		115					120					125			
Asn	Leu	Ser	Arg	Lys	Asp	Ala	Glu	Arg	Gln	Leu	Leu	Ala	Pro	Gly	Asn
	130					135					140				
Thr	His	Gly	Ser	Phe	Leu	Ile	Arg	Glu	Ser	Glu	Ser	Thr	Ala	Gly	Ser
145					150					155					160
Phe	Ser	Leu	Ser	Val	Arg	Asp	Phe	Asp	Gln	Asn	Gln	Gly	Glu	Val	Val
			165						170					175	
Lys	His	Tyr	Lys	Ile	Arg	Asn	Leu	Asp	Asn	Gly	Gly	Phe	Tyr	Ile	Ser
			180					185					190		
Pro	Arg	Ile	Thr	Phe	Pro	Gly	Leu	His	Glu	Leu	Val	Arg	His	Tyr	Thr
		195					200					205			
Asn	Ala	Ser	Asp	Gly	Leu	Cys	Thr	Arg	Leu	Ser	Arg	Pro	Cys	Gln	Thr
	210					215						220			

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(ix) FEATURE:

(A) NAME/KEY: Other

(B) LOCATION: 6...6

(D) OTHER INFORMATION: Xaa = Phosphorylated Tyrosine

(A) NAME/KEY: Other

(B) LOCATION: 17...17

(D) OTHER INFORMATION: Xaa = Phosphorylated Tyrosine

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Gly Ser Asn Gln Leu Xaa Asn Glu Leu Asn Leu Gly Arg Arg Glu Glu
1 5 10 15
Xaa Asp Val Leu Asp Lys
20

(2) INFORMATION FOR SEO ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GATCGCCATG GGAGTGCAGG TGGAAACCAT CTCCCCA

37

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 48 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TACGAATTCT GGCGTGGATC CACGCGGAAC CAGACCTTCC AGTTTTAG

48

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

ATTAGGATCC ATGCCAGATC CTGCAGCTCA CCTGCCCT

38

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

ATATGGATCC TTACCAGAGG CGTTGCT

27

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CAATAGGATC CATGGCCAGC AGCGGCATGG CTGA

34

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 39 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GACCTAGGAT CCCTAATTAA CATTTCCCTG TGTGCCGAT

39

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

ATATGGATCC ATGGCGAACA GCCTGGAGCC CGAACCCT

38

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 43 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

ATTAGGATCC TTAGGTCTGG CAGGGGCGGC TCAACCGTGT GCA

43

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 11 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(ix) FEATURE:

- (A) NAME/KEY: Other
- (B) LOCATION: 4...4
- (D) OTHER INFORMATION: XAA = PHOSPHORYLATED TYROSINE

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Glu	Pro	Gln	Xaa	Glu	Glu	Ile	Pro	Ile	Tyr	Leu
1				5				10		

WHAT IS CLAIMED IS:

1. A method of screening for compounds capable of binding to a fusion protein which comprises the steps of:

- 5 a) mixing a test compound, a biotinylated ligand, the fusion protein, a donor-labeled ligand and acceptor-labeled streptavidin;
- b) incubating the mixture for a suitable time period;
- 10 c) measuring the time-resolved fluorescence attributable to the binding of the biotinylated ligand to the fusion protein in the presence of the test compound; and
- d) determining the binding of the biotinylated ligand to the fusion protein in the presence of the test compound relative to a control assay run in the absence of the test compound.

15

2. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 1, wherein the acceptor-labeled streptavidin consists of streptavidin coupled to a light harvesting molecule.

20

3. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 2, wherein the acceptor-labeled streptavidin consists of SA-XL665.

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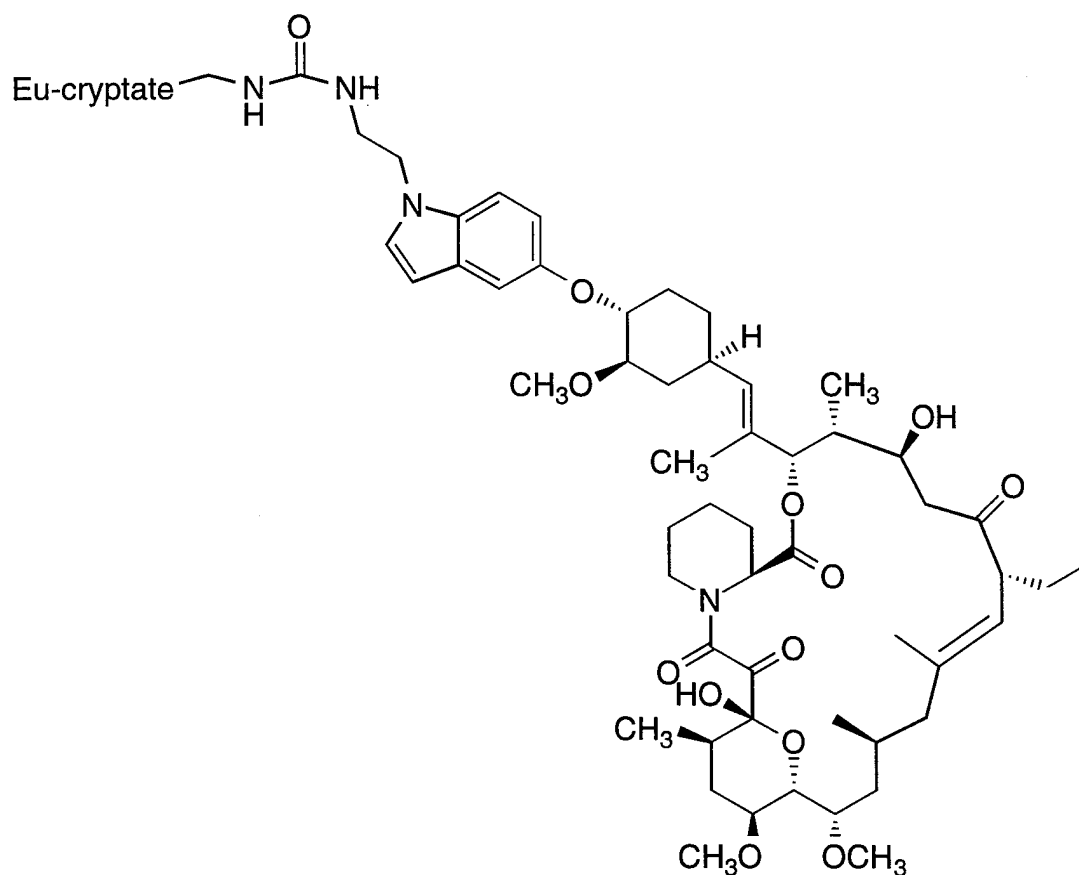
4. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 3, wherein the donor-labeled ligand comprises a cryptate-containing molecule coupled to a lanthanide metal.

30

5. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 4, wherein the donor-labeled ligand is selected from the group consisting of a Eu(K)-labeled analog of FK506 or and Eu(K)-labeled analog of rapamycin.

6. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 5, wherein the donor-labeled ligand is an Eu(K)-labeled analog of FK506.

5 7. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 6, wherein the donor-labeled ligand is



8. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 7, wherein the fusion protein comprises an FK506 binding protein, peptide linker and target protein.

9. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 8, wherein the FK506 binding protein is 12kDA human FK506 binding protein.

10. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 9, wherein the target protein is selected from tyrosine phosphatases, nuclear receptors,
5 bacterial tRNA synthetases or single and multiple signal transduction domains.

11. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 10, wherein the target
10 protein comprises a single or multiple signal transduction domain.

12. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 11, wherein the single or multiple signal transduction domain is selected from the group
15 consisting of: SH1, SH2, SH3 and PH domains.

13. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 12, wherein the target protein is a single or multiple SH2 domain.
20

14. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 13, wherein the target protein is a single or multiple SH2 domain selected from the group consisting of: ZAP70:SH2, Syk:SH2 and Lck:SH2.
25

15. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 14, wherein the target protein is the SH2 domain, ZAP70:SH2.

16. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 14, wherein the target protein is the SH2 domain, Syk:SH2.
30

17. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 14, wherein the target protein is the SH2 domain, Lck:SH2.

5 18. A method of screening for compounds capable of inhibiting a protease which comprises the steps of:

- a) mixing a test compound, a fusion protein substrate, a donor-labeled ligand, acceptor-labeled streptavidin, and a protease;
- 10 b) incubating the mixture for an appropriate time;
- c) measuring the time-resolved fluorescence attributable to the binding of the intact fusion protein substrate to the acceptor-labeled streptavidin in the presence of the test compound; and
- 15 d) determining the binding of the intact fusion protein substrate to the acceptor-labeled streptavidin in the presence of the test compound relative to a control assay run in the absence of the test compound.

20 19. The method of screening for compounds capable of inhibiting a protease, as recited in Claim 18, wherein the protease is selected from the group consisting of thrombin, HIV or TNF α converting enzyme.

25 20. The method of screening for compounds capable of inhibiting a protease, as recited in Claim 19, wherein the protease is thrombin.

30 21. The method of screening for compounds capable of inhibiting a protease, as recited in Claim 20, wherein the acceptor-labeled streptavidin consists of streptavidin coupled to a light harvesting molecule.

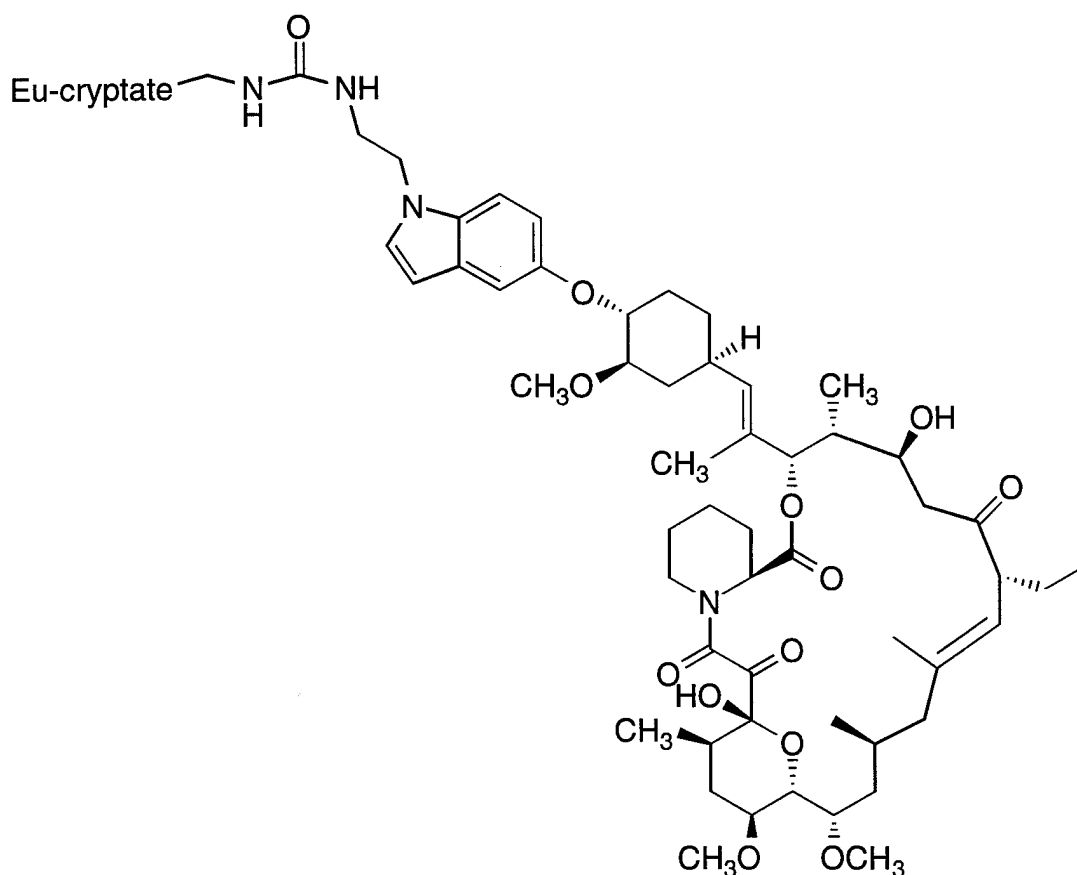
22. The method of screening for compounds capable of inhibiting a protease, as recited in Claim 21, wherein the acceptor-labeled streptavidin consists of SA-XL665.

5 23. The method of screening for compounds capable of inhibiting a protease, as recited in Claim 22, wherein the donor-labeled ligand comprises a cryptate-containing molecule coupled to a lanthanide metal.

10 24. The method of screening for compounds capable of inhibiting a protease, as recited in Claim 23, wherein the donor-labeled ligand is selected from the group consisting of an Eu(K)-labeled analog of FK506 or and Eu(K)-labeled analog of rapamycin.

15 25. The method of screening for compounds capable of inhibiting a protease, as recited in Claim 24, wherein the donor-labeled ligand is an Eu(K)-labeled analog of FK506.

20 26. The method of screening for compounds capable of inhibiting a protease, as recited in Claim 25, wherein the donor-labeled ligand is



27. The method of screening for compounds capable of inhibiting a protease, as recited in Claim 26, wherein the fusion protein comprises an FK506 binding protein, peptide linker and reporter protein.

28. The method of screening for compounds capable of inhibiting a protease, as recited in Claim 27, wherein the FK506 binding protein is 12kDA human FK506 binding protein.

29. The method of screening for compounds capable of inhibiting a protease, as recited in Claim 28, wherein the reporter protein is selected from the group consisting of SH2 domains or covalently biotinylated proteins.

30. The method of screening for compounds capable of inhibiting a protease, as recited in Claim 29, wherein the covalently biotinylated protein is selected from the group consisting of the C-terminal 87 residues of the biotin carboxy carrier protein of acetyl-CoA
5 carboxylase from *Escherichia coli* or the biotin-carrier subunit of transcarboxylase from *Propionibacterium shermanii*.

31. The method of screening for compounds capable of inhibiting a protease, as recited in Claim 29, wherein the SH2 domain is
10 the ZAP70 bound to a biotinylated phosphopeptide derived from an ITAM sequence.

32. The method of screening for compounds capable of inhibiting a protease, as recited in Claim 31, wherein the ITAM
15 sequence is selected from the group consisting of the human T-cell receptor, the B-cell receptor or a high affinity IgE receptor.

33. The method of screening for compounds capable of inhibiting a protease, as recited in Claim 32, wherein the ITAM
20 sequence is the Zeta 1 sequence of the human T-cell receptor. (SEQ. ID. NO. 8)

1/3

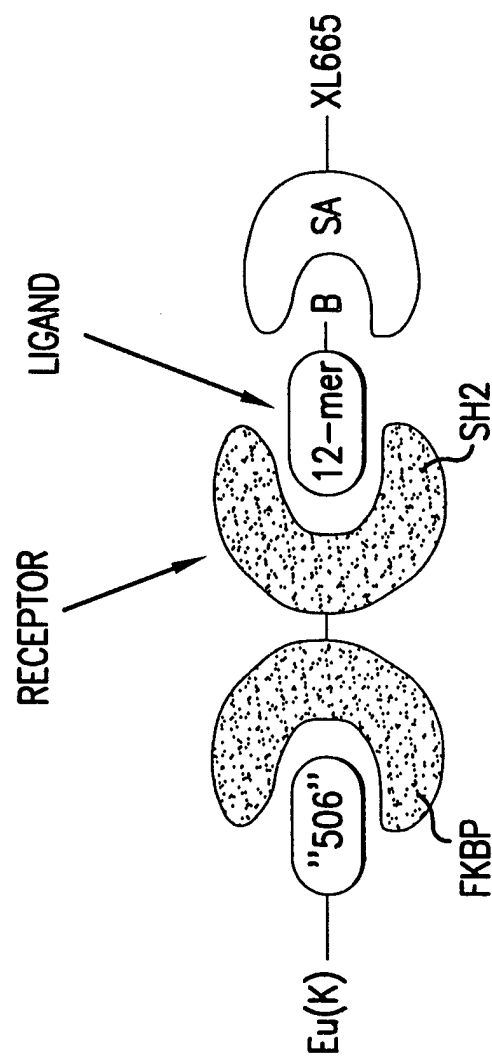


FIG. 1

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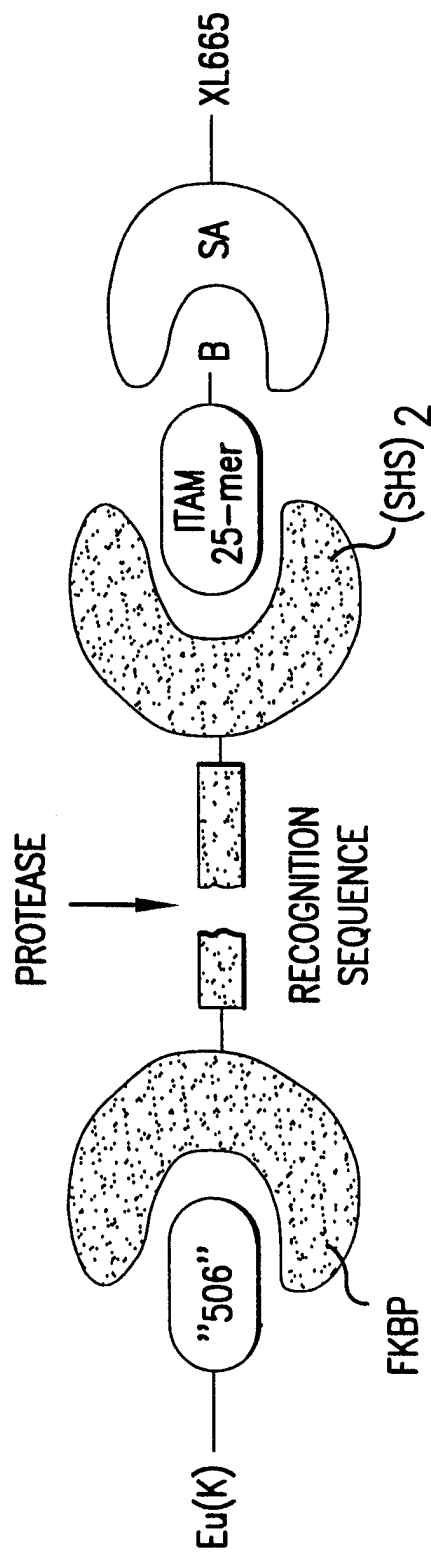


FIG.2

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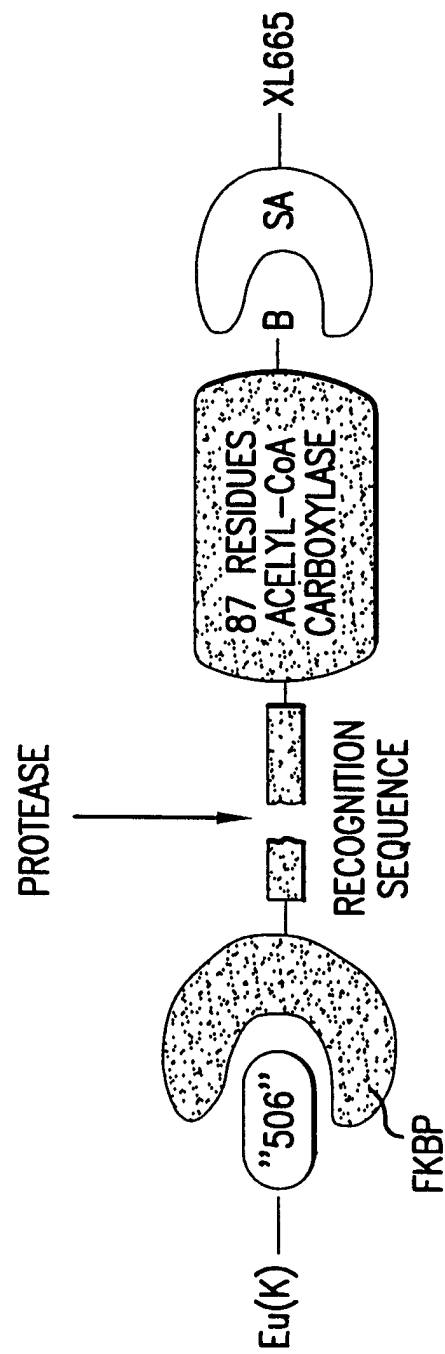


FIG.3

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US98/04610

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : G01N 33/53, 33/544, 33/531

US CL : 435/7.1, 7.5; 436/529, 543

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.1, 7.5; 436/543

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG, GENBANK, BIOTECHDS, BIOSIS, CA, MEDLINE, LIFESCI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	WO 97/10502 A1 (MERCK & CO., INC.) 20 March 1997, see entire document.	1-33
A	US 5,498,597 A (BURAKOFF et al) 12 March 1996, see entire document.	1-33
A	US 5,352,660 A (PAWSON) 04 October 1994, see entire document.	1-33
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Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*&* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

21 MAY 1998

Date of mailing of the international search report

16 JUL 1998

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Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

JENNIFER SHAVER

Telephone No. (703) 308-0196