The use of FRET technology for drug discovery

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Proteases play important roles in the pathogenesis of several major diseases; as a result, they have been the subject of much research in both the academia and the industrial sectors. The discovery that an HIV protease inhibitor was effective in the treatment of AIDS (1) has spurred similar research for the identification of protease involvement in other diseases; and more importantly, research into potential protease inhibitors. In the last decade or so, the use of fluorescence resonance energy transfer (FRET) technology has made the continuous assay of protease activity and high throughput screening (HTS) of protease inhibitors faster and easier. This article presents a short summary of the biological roles of proteases, the different proteases and the diseases they are involved in, the use of FRET technology for the study of proteases, and data comparison of two FRET pairs, EDANS/DABCYL and 5-FAM/QXL™ 520.

BIOLOGICAL ROLES OF PROTEASES

One of the most important mechanisms modulating the properties and functions of proteins is the proteolytic cleavage of peptide bonds as mediated by proteases. Proteases (also known as peptidases), are enzymes that catalyze the hydrolysis of peptide bonds (2). Based on their reaction mechanisms and nature of active sites, proteases can be classified into serine proteases, cysteine proteases, aspartyl proteases, and zinc (metallo) proteases. Proteases can also be classified as exopeptidases, enzymes that hydrolyze amino acid residues from the terminus of a peptide, or endopeptidases, enzymes that cleave internal peptide bonds (3). Ubiquitously distributed in all tissues and biological fluids, they play key roles in protein activation, cell regulation and signaling, as well as in the generation of amino acids for protein synthesis or utilization in metabolic pathways (4-7). Over 50 hereditary disorders have been attributed to a loss-of-function mutation in protease genes (8). The detection of proteases and screening of specific protease inhibitors are critical factors in the discovery of potential drugs for treatment and management of protease-related diseases. Listed below are four groups of proteases that play significant roles in the pathogenesis of some of the world's worst killer diseases.

PROTEASES

Matrix Metalloproteinases, MMPs

The matrix metalloproteinases (MMPs) constitute a family of zinc-dependent endopeptidases that function within the extracellular matrix. These enzymes are responsible for the breakdown of connective tissues and are important in bone remodeling, the menstrual cycle and repair of tissue damage. While the exact contribution of MMPs to certain pathological processes is difficult to assess, MMPs appear to play a key role in the development of arthritis as well as in the invasion and metastasis of cancer (9-12). MMPs tend to have multiple substrates, with most family members having the ability to degrade different types of collagen along with elastin, gelatin and fibronectin.

Hepatitis C Virus Protease

The Hepatitis C Virus (HCV) NS3/4A serine protease is essential for HCV replication and the formation of infectious viral particles. This virus belongs to the Flaviviridae family of positive, single stranded RNA. Approximately 170 million people worldwide are infected with this virus, and infection results in liver diseases such as chronic hepatitis, cirrhosis, and hepatocellular carcinoma (13). The HCV NS3/4A protease is considered one of the most attractive targets for anti-HCV therapy.

HIV Protease

The 1012 kD aspartic protease of human

published by B5 srl
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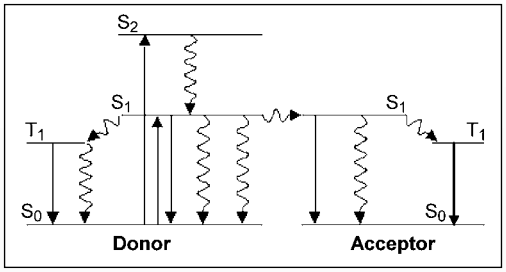

Figure 1 – Diagram of a FRET process from the donor to acceptor molecule. Horizontal lines represent discrete electron energy levels for each molecule. Energy levels are labeled as either singlet states (S) or triplet states (T) with subscripts numbered zero, one or two (representing the ground state, first excited electronic state or second excited electronic state). Electrons generally reside in the ground state, S_0, they may be excited to higher energy levels by a number of processes, including light absorption and chemical reaction

immunodeficiency virus-1 (HIV-1) is active when it forms a homodimer. It is required for post-translational cleavage of the precursor polyproteins, Pr*gag* and Pr*gag-pol* (14). With an estimated 39.5 million people living with AIDS in 2006, and an estimated 2.9 million deaths due to AIDS in 2006 (15), this protease, essential for the maturation of HIV infectious particles, is one of the key targets for developing anti-AIDS drugs.

Renin

The renin–angiotensin system (RAS) plays a central role in the regulation of blood pressure and electrolyte homoeostasis (16). At the first and rate-limiting step of the RAS cascade, renin (EC 3.4.23.15), a highly specific aspartyl protease, cleaves angiotensinogen, produced in the liver, to yield angiotensin I, which is further converted into angiotensin II by ACE (Angiotensin Converting Enzyme). Angiotensin II constricts blood vessels leading to increased blood pressure. It also increases the secretion of ADH and aldosterone, and stimulates the hypothalamus to activate the thirst reflex. In the US alone, about 72 million people over the age of 20 and older have high blood pressure, and nearly one in three adults have high blood pressure (17). Since an overactive renin-angiotensin system leads to hypertension, renin is an attractive target for the treatment of this disease.

FRET SUBSTRATES FOR THE ANALYSIS OF PROTEASES

Numerous methods are used in the analysis of proteases that are present in solutions, cells or tissues; however, spectrophotometric methods have been favored due to their high speed, better accuracy and ease of use. This method has been predominantly used in high throughput screening of protease activities and inhibitors. The spectral and enzymatic properties of chromogenic and fluorogenic substrates play a critical role in the successful use of spectrophotometric methods for analyzing proteases. Since fluorogenic substrates are generally several orders of magnitude more sensitive than chromogenic substrates, have a wide linear dynamic range and good reproducibility (18), the use of these substrates is on the increase. In recent years, FRET (fluorescence or Förster resonance energy transfer) based assays have found broad applications, one of which is in the detection of different proteases. Briefly, in the process of FRET, when a donor and an acceptor molecule are within a specified range called the Förster radius (usually within 10 to 100 Å), the excited-state energy of a donor is transferred to an acceptor molecule, without the transfer of photons of light. As illustrated in Figure 1, the pathway leading from the S_1 level of the donor to the S_1 level of the acceptor represents FRET. Once excited, the acceptor can return to the ground state by the same pathways described for the donor. If the acceptor molecule is also fluorescent, it can emit light at its characteristic wavelength, which is always longer than the emission wavelength of the donor (reviewed in 19-21). FRET efficiency falls dramatically as the distance between the donor and acceptor exceeds the Förster radius, making it an important technique for investigating a variety of biological phenomena in which tracking physical proximity is important. If the fluorescence of the donor molecule is quenched, it indicates that the donor and acceptor molecules are within the Förster radius; whereas if the donor fluoresces at its characteristic wavelength, it indicates that the distance between the donor and acceptor molecules has increased beyond the Förster radius. A schematic diagram is shown in Figure 2. When the HCV FRET peptide is intact, the fluorescence of the donor molecule, which in this case is 5-FAM, is quenched by the acceptor, QXL™ 520; however, when the protease recognizes the sequence and cleaves the peptide, the fluorescence of 5-FAM is recovered and can be measured at its emission wavelength of 520 nm.

FRET DONOR AND ACCEPTOR PAIRS

FRET is characterized by the appearance of sensitized fluorescence of the acceptor, the intensity ratio change of donor/acceptor (if the acceptor is fluorescent), or the fluorescence decrease of the donor (the acceptor can be either fluorescent or non-fluorescent). Probes incorporating fluorescent donor / non-fluorescent acceptor (*e.g.* Dabcyl) combinations have been developed primarily for detection of proteolysis (22-24) and nucleic acid hybridization (25-28).

QXL™ Quenchers

EDANS/DABCYL and MCA/DNP are two FRET pairs traditionally used in the development of a variety of FRET probes; however, their short absorption wavelengths and low extinction coefficients have limited their use in developing sensitive fluorogenic probes. QXL™ quenchers, a novel series of nonfluorescent dyes manufactured by AnaSpec Inc., have eliminated these limitations. Spanning the full visible spectrum (Figure 3), these dyes exhibit unusually high quenching efficiency. They are excellent dark quenchers individually optimized to pair with all of the popular fluorescent dyes such as fluoresceins and rhodamines (data not shown). QXL™ 520 has absorption

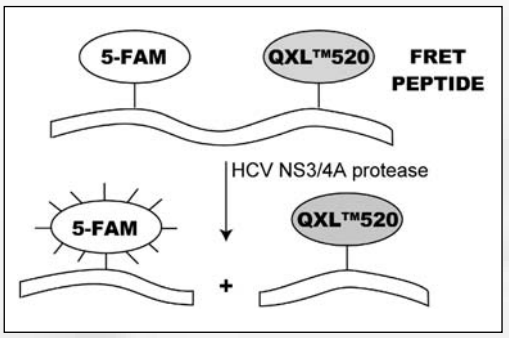

Figure 2 – A schematic diagram showing the proteolytic cleavage of 5-FAM/QXL™ 520 FRET peptide by HCV NS3/4A protease

maximum matching the emission of FAM and HiLyte Fluor™ 488, while QXL™ 570 is proven to be the best quencher for TAMRA and HiLyte Fluor™ 555. QXL™ 670 and 680 are the most effective quenchers for HiLyte Fluor™ 647 and Cy™5 fluorophores.

EDANS/DABCYL OR 5-FAM/QXL™ 520?

The use of fluorogenic FRET peptide substrates has resulted in the development of fluorimetric protease activity assays. Among

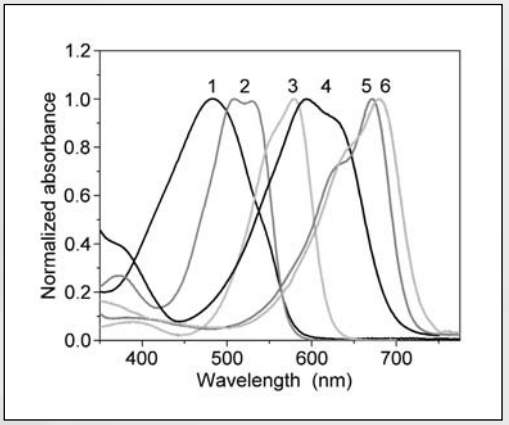

Figure 3 – The normalized absorption spectra of QXL™ dark quenchers (free acids) in MeOH. 1. QXL™ 490; 2. QXL™ 520; 3. QXL™ 570; 4. QXL™ 610; 5. QXL™ 670; 6. QXL™ 680

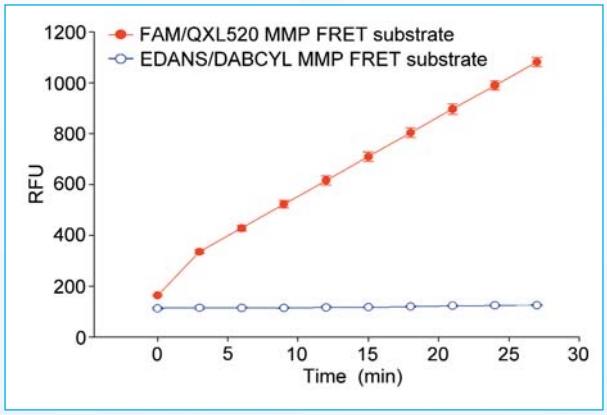

Figure 4 –5-FAM/QXL™ 520 MMP generic substrate showed higher sensitivity than Edans/Dabcyl based FRET peptide

them, EDANS/DABCYL and MCA/DNP are the most commonly used. However, these existing FRET pairs have a few limitations such as lower sensitivity and shorter wavelengths. MMP, HCV, HIV and Renin substrates incorporating 5-FAM (donor) and QXL™ 520 (quencher) have been developed. This design offers several advantages: i) Fluorescence signal can be continuously monitored at Ex/Em = 490 nm/520 nm; ii) 5-FAM has stronger absorption and fluorescent signals than EDANS and MCA; iii) 5-FAM has longer absorption and emission wavelengths than EDANS and MCA.

5-FAM/QXL™ 520 FRET peptides are more readily adapted to HTS since this pair shows less interference from autofluorescence and absorbance of test compounds and cellular components. Among the sixteen 5-FAM/ QXL™ 520 MMP FRET peptides screened, one peptide showed high cleavage kinetics with all the MMPs tested. A submicromolar concentration of this 5-FAM/ QXL™ 520 FRET peptide is adequate to detect picomolar level of MMPs. On other hand, the EDANS/DABCYL substrate is not as sensitive (Figure 4).

For the HCV 5-FAM/QXL™520 FRET peptide, its enzyme detection dynamic range is from 8.27 to 0.064 pmole, while that of EDANS/DABCYL FRET peptide is from 8.27 to 0.52 pmole. This demonstrates that 5-FAM/QXL™ 520 FRET peptide is eight times more sensitive than EDANS/DABCYL FRET peptide (Figure 5).

For the Renin 5-FAM/QXL™ 520 FRET peptide, its sensitivity over EDANS/DABCYL FRET peptide is even more impressive. It is over 40 fold more sensitive than the EDANS/DABCYL FRET peptide and can detect 0.8 ng/ml of renin. The DNP/MC-Ala FRET substrate can detect 8 ng/ml of renin, which is 4 fold more sensitive than the DABCYL/EDANS FRET substrate (Figure 6).

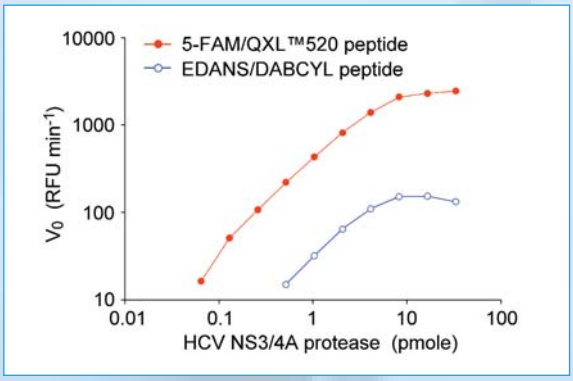

Figure 5 – The sensitivity comparison of 5-FAM/QXL™ 520 FRET peptide and EDANS/DABCYL FRET peptide

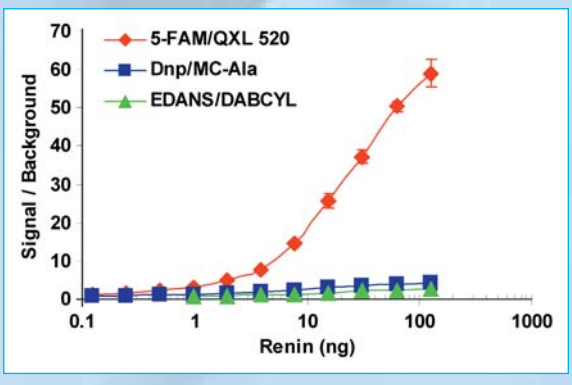

Figure 6 – Compared to the EDANS/DABCYL FRET substrate, the 5-FAM/QXL™ 520 FRET substrate is 40 fold more sensitive and can detect 0.8 ng/ml of renin. The MC-Ala/ DNP FRET substrate can detect 8 ng/ml of renin, which is 4 fold more sensitive than the EDANS/DABCYL FRET substrate

CONCLUSION

In the past decade, a number of structurally different synthetic protease inhibitors of excellent potency, especially for HCV (29-30), HIV (31-32) and renin (33) proteases have been described. However, to further accelerate the drug-discovery process, continuous, homogeneous assays with high sensitivity are still needed. To facilitate high throughput screening of inhibitors, we have developed highly sensitive FRET peptide substrates containing 5-FAM as the donor and QXL™ 520 as the non-fluorescent acceptor. Among the proteases tested, these substrates have been verified to be more sensitive that the traditional EDANS/DABCYL FRET substrates.

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