A Novel Fluorimetric Assay for the Detection of TACE (α-Secretase) Activity Using a Long Wavelength FRET Peptide Substrate

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Introduction

TACE (TNF- α converting enzyme), also called ADAM17 or α-secretase, is involved in myogenesis, neurogenesis, and fertilization through the process of shedding of cell surface proteins. TACE is the predominant 'sheddase' responsible for the generation of soluble mature TNF.[1] Considerable efforts have been made for the research and development of anti-TNF- α agents to reduce the severity of inflammatory responses in disease states. The inhibition of TACE by a pharmacological agent may represent an alternative approach to modulate the effect of TNF-α .[2]

To facilitate high throughput screening of TACE inhibitors, we synthesized a novel peptide substrate for TACE using QXL™ 520/5-FAM FRET pair. Using this FRET substrate, we developed a new kit – the SensoLyte™ 520 TACE Activity Assay. This kit can be used to detect the activity of the enzyme and for screening of TACE inhibitors. It is highly sensitive and can detect subnanogram amounts of enzyme.

Assay Principle

The SensoLyte™ 520 TACE Activity Assay is based on FRET (Fluorescence or Förster resonance energy transfer) principle. 5-FAM and QXL™ 520 is a novel donor - acceptor pair for FRET peptides. The absorption spectrum of QXL™ 520 perfectly overlaps with the emission spectrum of 5-FAM (Figure 1). Unlike the hydrophobic Dnp, QXL™ 520 is a hydrophilic compound. This property of QXL™ 520 increases the solubility of the peptide substrate; thus the problem caused by the hydrophobic nature of many fluorescent donors and quenchers is alleviated.

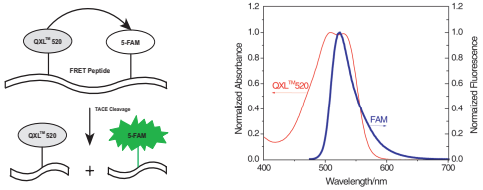

Figure 1. Proteolytic cleavage of QXL™ 520/5-FAM FRET peptide by TACE. QXL™520 is an excellent quencher when paired with 5-FAM. Upon cleavage into two separate fragments by protease, the fluorescence of 5-FAM is recovered and can be monitored at Ex/Em = 490 nm/520 nm.

Materials and Methods

➢SensoLyte™ 520 TACE Activity Assay Kit (Cat# 72085)

✓TACE (QXL™ 520)-peptide-(5-FAM) FRET Substrate - designed and synthesized by Fmoc solid phase synthesis method.

✓TACE Reaction Buffer

✓TACE Inhibitor (TAPI-0 is a patented product of Research Corporation Technologies)

➢Recombinant Human TACE (Calbiochem, San Diego, CA)

➢ TACE Abz/Dpa (Abz-LAQAVRSSSR-Dpa-NH_2) and Mca/Dpa (Mca-KPLGL-Dpa-AR-NH_2) FRET substrates - synthesized by Fmoc solid phase synthesis method [Dpa=Dap(Dnp)].

SensoLyte™ 520 TACE Assay Kit was used as recommended by the protocol. The reaction volumes for this kit are 40 µl of enzyme, 10 µl of test compound/buffer, 50 µl of substrate. All incubations were performed at 37ºC. Assays were configured in 96-well black plates.

Results

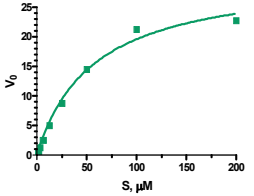

Figure 2. Michaelis-Menten plot. For the QXL™ 520/5-FAM TACE FRET substrate, initial velocities (Vo) of hydrolysis by enzyme were determined after incubation of 10 ng TACE with a range of concentrations of substrates. The resulting data were analyzed by non-linear regression. The K_m value obtained was 32 µM.

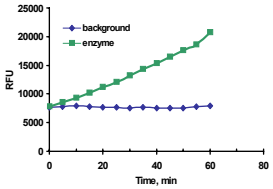

Figure 3. Assay kinetics. QXL™ 520/5-FAM substrate was incubated with 10 ng of TACE. Fluorescent signal was continuously monitored at Ex/Em = 490 nm/ 520 nm for 60 min.

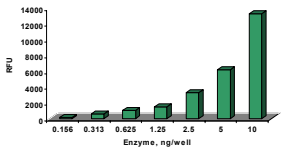

Figure 4. Sensitivity of SensoLyte™ 520 TACE Assay Kit. Fluorescence was measured 60 min after incubation of FRET substrate with serial dilutions of TACE. Sensitivity of assay at these conditions was 0.31 ng of enzyme.

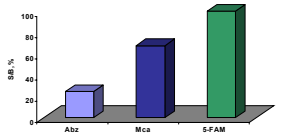

Figure 5. Comparison of signal/background ratio for different TACE substrates. TACE substrates (Abz/Dpa, Mca/Dpa and QXL™520/5-FAM) at a final concentration of 5 µM were incubated with 10 ng of enzyme for 1 hour. Signal/background ratio for QXL™ 520/5-FAM labeled substrate is presented as 100%.

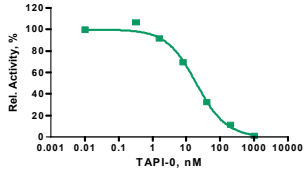

Figure 6. Inhibitor studies. To validate assay for inhibitor screening, the QXL™ 520/5-FAM substrate was incubated with 10 ng of enzyme in the presence of TACE inhibitor TAPI-0 (10^{-2} to 10^3 nM). Kinetic readings were taken every 5 min for 60 min. The calculated IC_{50} was 19 nM.

Conclusions

- We have developed a new TACE assay kit – the SensoLyte™ 520 TACE assay kit, which uses a highly sensitive QXL™ 520/5-FAM FRET substrate.
- The longer excitation and emission wavelengths of 5-FAM minimize the interference from autofluorescence and absorbance of test compounds and cell components.
- This SensoLyte™ 520 TACE assay kit is capable of continuous, homogeneous monitoring of the enzymatic reaction.
- IC_{50} value for an inhibitor determined with SensoLyte™ 520 TACE assay kit are consistent with published data.

References:

1. Moss, ML. et al. Nature 385, 733 (1997).
2. Levin, JI. et al. Bioor. Med. Chem. Lett. 13, 2799 (2003).