TACE Fluorometric Drug Discovery Kit

Catalog #: BML-AK310

Designed to screen TACE inhibitors using a quenched fluorogenic peptide.

NOTE: This version contains a change to the substrate storage conditions and form

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✦TACE Fluorometric Drug Discovery Kit - BML-AK310✦

BACKGROUND

TACE/ADAM17 (Tumor necrosis factor-alpha-converting enzyme; A Disintegrin And Metalloproteinase 17) is a soluble or membrane-bound metalloproteinase primarily responsible for activation of proTNF-α[1,2], while also targeting proteins such as fractalkine[3], amyloid precursor proteins[4], and CD40[5]. ADAM17/TACE is involved in cancer, vascular disorders, and inflammatory diseases such as rheumatoid arthritis and focal ischemic injury[6,7]. The catalytic domain of ADAM17/TACE is able to cleave proTNF-α[8] and is used in inhibitor screening[9-10].

The *TACE Fluorometric Drug Discovery Kit* is a complete assay system designed to screen TACE inhibitors using a quenched fluorogenic peptide: Mca-PLAQAV-Dpa-RSSSR-NH_2[11]. Mca fluorescence is quenched by the Dpa group until cleavage by proteases separates the two moieties. The assays are performed in a convenient 96-well microplate format. The kit is useful to screen inhibitors of TACE, a potential therapeutic target. The compound GM6001[12] is also included as a prototypic control inhibitor.

Please contact Enzo Life Sciences for kit components in bulk.

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7. *Inhibition of tumor necrosis factor-α-converting enzyme by a selective antagonist protects brain from focal ischemic injury*: X. Wang *et al.*; Molecular Pharmacol. **65**, 890 (2004)
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9. *The discovery of novel tartrate*-based TNF-α converting enzyme (TACE) inhibitors: K.E. Rosner *et al.*; Bioorg. Med. Chem. Lett. **20** 1189 (2010)
10. *Synthesis and activity of quinolinylmethyl P1' α-sulfone piperidine hydroxamate inhibitors of TACE:* C. Zhang *et al.*; Bioorg. Med. Chem. Lett. **19**, 3445 (2009)
11. *Design, synthesis, and structure-activity relationships of macrocyclic hydroxamic acids that inhibit tumor necrosis factor alpha release in vitro and in vivo*: C.-B. Xue *et al.*; J. Med. Chem. **44**, 2636 (2001)
12. *Membrane-anchored CD40 is processed by the tumor necrosis factor-alpha-converting enzyme. Implications for CD40 signalling*: C. Contin *et al.*; J. Biol. Chem. **278**, 32801 (2003)

PLEASE READ ENTIRE BOOKLET BEFORE PROCEEDING WITH THE ASSAY. CAREFULLY NOTE THE HANDLING AND STORAGE CONDITIONS OF EACH KIT COMPONENT. PLEASE CONTACT ENZO LIFE SCIENCES TECHNICAL SERVICES FOR ASSISTANCE IF NECESSARY. **DO NOT PERFORM THIS ASSAY IN WHITE PLATE DUE TO PLATE AUTOFLUORESCENCE AT THE WAVELENGTHS USED**

COMPONENTS OF BML-AK310

Note on storage: *Please note that all components, with the exception of the TACE enzyme (BML-SE268-9090), can be stored at either -80°C or - 20°C. The TACE enzyme must be stored at or below -70°C (i.e. -80°C) and should be handled carefully in order to retain maximal enzymatic activity. It is stable, in diluted or concentrated form, for several hours on ice. As tested, TACE enzyme is stable for at least 6 freeze/thaw cycles. To minimize the number of freeze/thaw cycles, aliquot the TACE into separate tubes, snap-freeze in liquid nitrogen or a dry ice bath, and store at or below -70°C. When setting up the assay, do not maintain diluted components at reaction temperature (e.g. 37°C) for an extended period of time prior to running the assay.*

BML-SE268-9090 TACE ENZYME (HUMAN, RECOMBINANT)

FORM: Recombinant human TACE catalytic domain. Purity >90% by SDS-PAGE.

UNIT DEFINITION: One unit is defined as the amount of enzyme that will hydrolyze 10 μM Mca-PLAQAV-Dpa-RSSSR-NH_2 (Cat. # BML-P132) at 1 pmol/min@ 37°C.

STORAGE: -80°C **Avoid freeze/thaw cycles**

QUANTITY: 3000 units

BML-P132-9090 SUBSTRATE (Mca-PLAQAV-Dpa-RSSSR-NH_2 fluorogenic substrate; MW=1638.7)

FORM: 1mM in DMSO

STORAGE: -80°C

QUANTITY: 305 μL (0.5 mg net peptide)

BML-P127-9090 CALIBRATION STANDARD (OmniMMP™ fluorogenic control peptide, MCA-Pro-Leu-OH; MW=444.5)

FORM: 40 μM in DMSO

STORAGE: -80°C

QUANTITY: 50 μL

BML-EI300-9090 INHIBITOR (GM6001; MW=388.5)

FORM: 80μM in DMSO

STORAGE: -80°C

QUANTITY: 10 μL

BML-KI585-0020 ASSAY BUFFER

FORM: 50mM Tris-HCl pH 9.0

STORAGE: Ambient

QUANTITY: 20 mL

80-2409 96-WELL BLACK NBS MICROPLATE

STORAGE: Ambient

OTHER MATERIALS REQUIRED

Fluorescent microplate reader capable of excitation at 328nm and emission at 420nm. The following Ex/Em have also been used: 320,340/393, 360/40,400,405, 460/40

Single or multi-channel pipet capable of pipetting 1-100 µL

accurately. Microcentrifuge tubes for reagent dilutions.

Ice bucket to keep reagents cold until use.

Water bath or incubator for component temperature equilibration.

EXPERIMENTAL METHODS

To start assay:

1. Briefly warm kit components BML-KI597-0400, BML-KI585-0020, and BML-EI300-9090 to RT to thaw.
2. Dilute inhibitor (GM6001, BML-EI300-9090) 1:32 in assay buffer BML-KI585. For example, add 1µL inhibitor into 31µL assay buffer and mix.
3. Dilute sufficient volume of substrate (BML-P132) to 50 µM in assay buffer (20 µL needed per well).
4. Dilute ADAM17 enzyme to 1.1 U/µL in assay buffer to required total volume (20 µL are needed per well).

5. Pipet assay buffer into each desired well of the 1/2 volume microplate as follows:

   Calibration = 70 µL in 3 wells (see step 11) Control (no inhibitor) = 60 µL

   Inhibitor GM6001 = 40 µL

   Test inhibitor = varies (see Table 1)

   Example of plate:

well#	sample
A1	Calibration
B1	Calibration
C1	Calibration
D1	Control
E1	Control
F1	Inhibitor GM6001
G1	Inhibitor GM6001
H1	Test inhibitor
A2…	Test inhibitor

6. Allow microplate to equilibrate to assay temperature (e.g. 37°C).
7. Add 20 µL ADAM17 (diluted in step 5) to the control, inhibitor GM6001, and test inhibitor wells. Final amount of TACE will be 22 U per well (220 mU/µL).
8. Add 20 µL GM6001 inhibitor to the inhibitor GM6001 wells only. Final inhibitor concentration = 500nM. Note: 500nM GM6001 will inhibit ADAM17 by approximately 75% under these conditions (see Figure 1).
9. Add desired volume of test inhibitor to appropriate wells. See Table 1.
10. Incubate plate for 30-60 minutes at reaction temperature (e.g. 37°C) to allow inhibitor/enzyme interaction.

11. In the meantime, calibrate a fluorescent microplate reader, using Ex/Em ≈ 328/420nm: Prewarm assay buffer to reaction temperature in 3 wells in the microplate, then to each add 20 µL BML-P132 substrate peptide to give the concentration to be used in the assay (e.g., for 10 µM final add 20 µL 50µM) and mix. When the fluorescent signal is constant, use this reading as the zero (Blank) value in relative fluorescence units (RFUs). Using the same wells, with their mixtures of substrate peptide and buffer, add 10 µL diluted calibration standard BML-P127-9090 to give 3 different final molar concentrations ranging between 2 and 10% of the substrate peptide molar concentration (e.g., 200, 500, and 1000nM) and measure their fluorescence. Use these values to build a standard curve relating micromolar BML-P127-9090 concentration (x axis) to RFUs (y axis). The slope of the line is the conversion factor (CF). If multiple concentrations of substrate peptide are used, such as in kinetic determinations, this step should be performed for each substrate concentration, due to absorptive quenching by the substrate peptide. **Note: this calibration can be done at any time.**
12. Start reactions by adding 20 µL BML-P132-9090 substrate (diluted and equilibrated to reaction temperature in step 3). Final substrate concentration = 10 µM.
13. Continuously read plates in a fluorescent microplate reader, using Ex/Em ≈ 328/420nm. For example, record data at 1 minute time intervals for 10 minutes at set reaction temperature (e.g. 37°C).
14. Perform data analysis (see below).

NOTE: Retain microplate for future use of unused wells.

TABLE 1. Example of Samples.

Sample	Assay buffer	TACE (1.1 U/µL)	Inhibitor (2.5 µM)	Substrate (50µM)	Total Volume
Control	60 µL	20 µL	0	20 µL	100 µL
Inhibitor GM6001	40 µL	20 µL	20 µL	20 µL	100 µL
Test inhibitor*	X µL	20 µL	Y µL	20 µL	100 µL

*Test inhibitor is the experimental inhibitor. Dissolve/dilute inhibitor into assay buffer and add to appropriate wells at desired volume “Y”. Adjust volume “X” to bring the total volume to 100 µL.

DATA ANALYSIS

Plotting

15. Plot data as RFUs (minus Blank RFU value determined during calibration, step 11) versus time for each sample.
16. Determine the range of initial time points during which the reaction is linear.
17. Obtain the initial reaction velocity (**V**) in RFUs/min: determine the slope of a line fit to the initial linear portion of the data plot using an appropriate routine.
18. It is best to use a range of inhibitor concentrations, each in duplicate. Average the slopes of duplicate samples.

Data Reduction

To determine inhibitor % remaining activity:

Inhibitor % activity remaining = (**V** inhibitor / **V** control) x 100

See Figure 1 for example of results.

Figure 1. Inhibition of TACE by GM6001

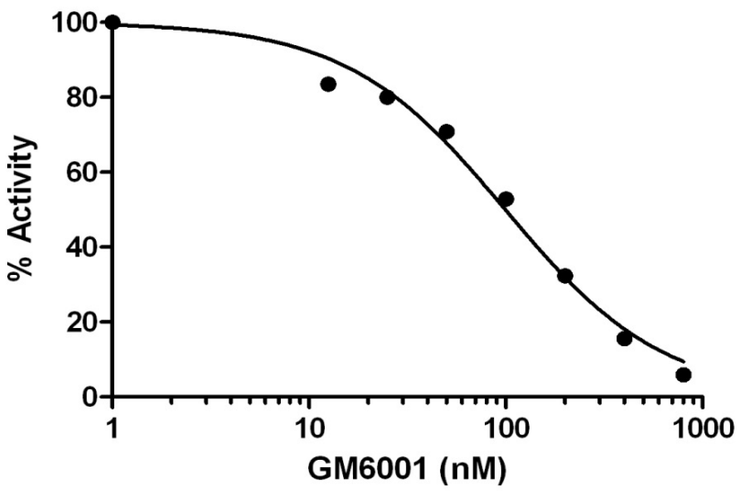

Figure 1. Dose-response determination for GM6001 inhibition of TACE activity. IC_{50} = 100nM GM6001.

To determine the activity of the samples expressed as picomoles substrate hydrolyzed per minute:

X pmoles substrate/min = 1/CF x **V** x vol

Where CF is the conversion factor (micromolar concentration/RFUs, from step 11), **V** is initial reaction velocity (RFUs/min, from step 17), and vol is the reaction volume in microliters (100).

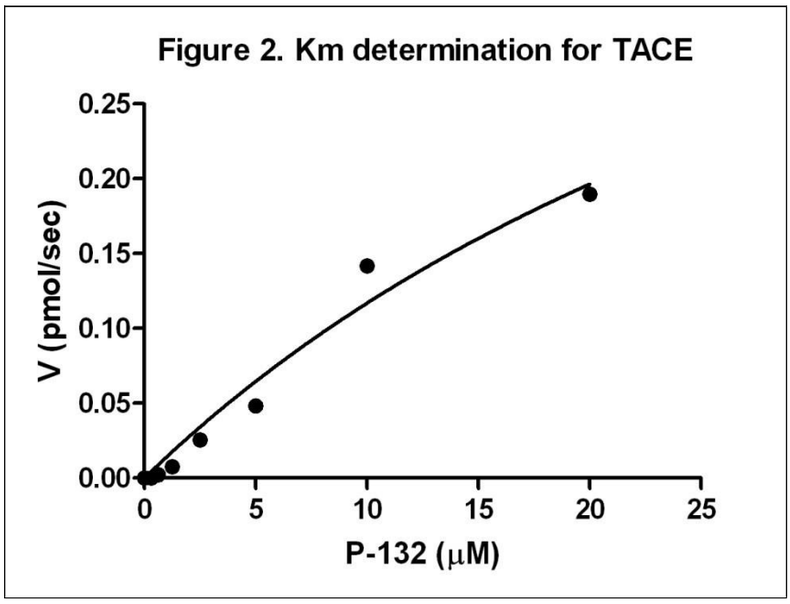

Figure 2. Example graph for k_{cat}/K_m determination for TACE. K_m = ~40μM.

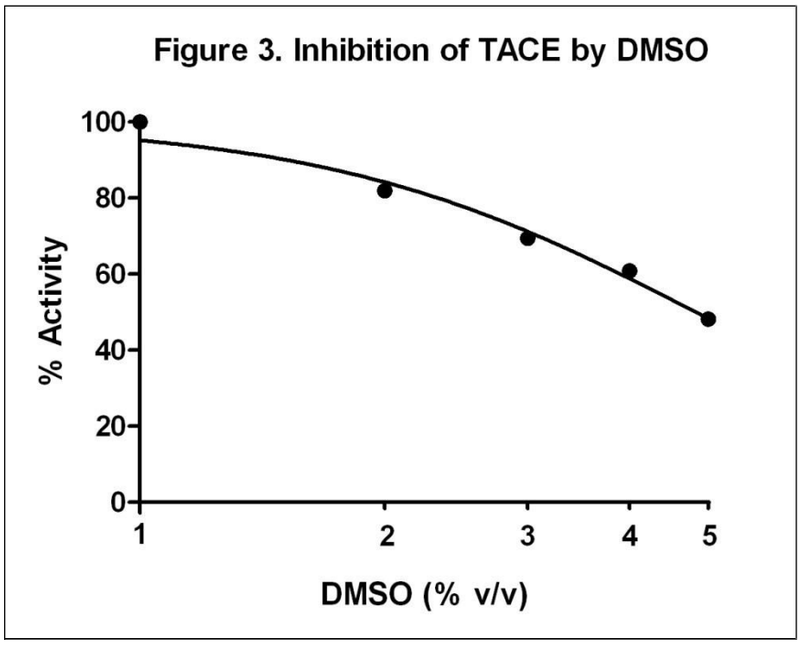

Figure 3. Dose-response determination for inhibition of TACE activity by DMSO. The activity was reduced to 50% of maximal at ~4.8% DMSO (v/v). Final concentrations were 10 μM substrate and 22 U/μL TACE.

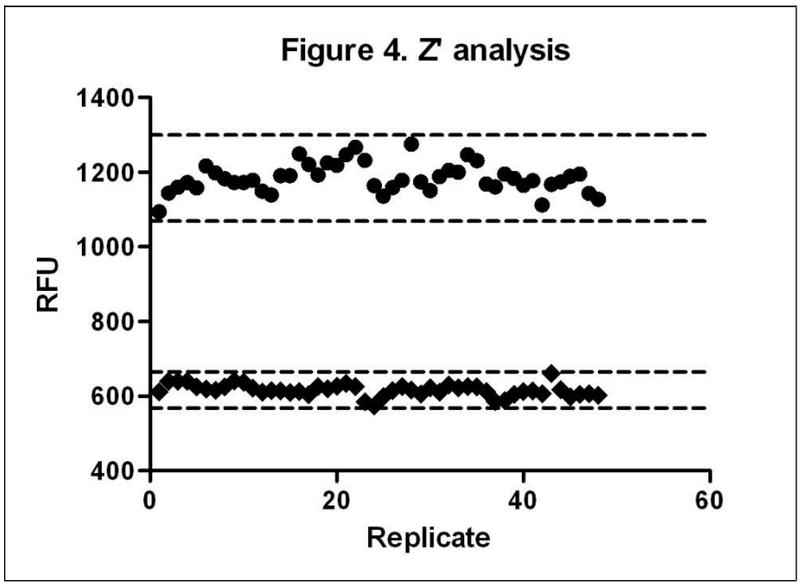

Figure 4. Z' analysis: TACE enzyme was pre-incubated without (●) or with (◆) GM6001 inhibitor for 60 minutes at 37°C before the addition of BML-P132 substrate. After the addition of substrate, the reaction proceeded at 37°C before the fluorescence was measured using an excitation filter of 360/40nm and an emission filter of 460/40nm on a BioTek Synergy2 plate reader. Final concentrations were 10 μM substrate, 22 U/μl TACE, 800nM GM6001, 1.0 % DMSO. The Z' factor for this assay was 0.71, (Z' factor = $1-((3SD^{control} + 3SD^{inhibitor})/(mean^{control} - mean^{inhibitor}))$. Dashed lines indicate 3 standard deviations.

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TRADEMARKS AND PATENTS

Several Enzo Life Sciences products and product applications are covered by US and foreign patents and patents pending.

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GLOBAL HEADQUARTERS
Enzo Life Sciences Inc.
10 Executive Boulevard
Farmingdale, NY 11735
Toll-Free:1.800.942.0430
Phone:631.694.7070
Fax: 631.694.7501
info-usa@enzolifesciences.com

EUROPE/ASIA
Enzo Life Sciences (ELS) AG
Industriestrasse 17
CH-4415 Lausen
Switzerland
Phone:+41/0 61 926 89 89
Fax:+41/0 61 926 89 79
info-ch@enzolifesciences.com

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