INTERNALLY QUENCHED FLUOROGENIC SUBSTRATES FOR ANTHRAX LETHAL FACTOR

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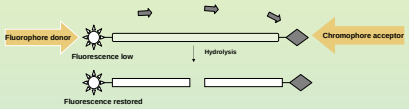
ABSTRACT

Lethal factor (LF) is the enzymatic component of anthrax lethal toxin which specifically cleaves the MAPK-kinase proteins. Molecular mechanisms that link this proteolysis with pathogenesis of the toxin remain unclear, however, LF presents as an ideal target for therapeutic inhibitors. Identification of potential inhibitory drug leads is dependent on a sensitive and rapid method for screening large numbers of compounds. One of the most efficient methods for this type of assay is based on the use of internally quenched fluorescent substrates. These substrates contain a fluorescent group paired with a suitable chromogenic group so that before cleavage the fluorescence is quenched by fluorescence resonance energy transfer (FRET). Fluorescence is restored as cleavage occurs and enzymatic activity can be monitored continuously. A FRET substrate for LF, MAPKKide®, has been designed at LIST laboratories. The Km value is 4.7 µM. This substrate is ideally suited for evaluating the IC(50)s and inhibitor modality for large screens of potential inhibitors. Several metalloprotease inhibitors, including the antibiotic, antinonin, have been examined using MAPKKide®. The IC(50) measured for actinonin with LF is 18 µM. A Dixon plot indicated noncompetitive inhibition. Inhibitor specificity for LF was confirmed using an unrelated enzyme that also cleaved MAPKKide®. One disadvantage associated with FRET substrates is that the fluorophores absorb in the range of aromatic compounds that are potential inhibitors, thus complicating the interpretation of the data. Another FRET substrate for LF, using a fluorophore excited at longer wavelengths, has been designed at LIST laboratories. This FRET substrate can also be detected using an argon ion laser.

INTRODUCTION

The fluorescence of MAPKKide® is internally quenched by fluorescence resonance energy transfer as shown.



MAPKKide® containing either the o-aminobenzoic acid/2,4 dinitrophenol (oAbz/Dnp) FRET pair or the Fluorescein-thiocarbamoyl/DABCYL (FTC/DABCYL) FRET pair have been synthesized. Cleavage of 5 µM of either substrate by LF results in a 6-fold enhancement in fluorescence. LF enzymatic activity can be monitored continuously by recording the increase in fluorescence intensity with time. The fluorescence is a linear function of cleaved MAPKKide® concentration up to 12 µM for the FTC/DABCYL-substrate and up to 30 µM for the oAbz/Dnp-substrate.

In the present study, a series of matrix metalloproteinase inhibitors have been screened for relative LF inhibitory potency. The specificity of inhibition was evaluated using trypsin which also cleaves MAPKKide®.

MATERIALS

The MMP inhibitors, actinonin, CL-82198, and Epigallocatechin Gallate (ECGC) were purchased from BIOMOL, Plymouth Meeting, PA. Tetracycline was obtained from Sigma, St. Louis, MO. The remainder of the inhibitors were purchased from Calbiochem, San Diego, CA. Trypsin, sequencing grade, was from Roche. The purified recombinant anthrax lethal factor (LF) and both MAPKKide® substrates are products of List Biological Laboratories.

METHODS

Fluorimetric assays: Continuous assays were performed on a SPECTRAmax GEMINI XS fluorescence microplate reader (Molecular Devices, Sunnyvale, CA) using Greiner FLUO-TRAC black flat-bottomed plates (E&K Scientific, Campbell, CA). Stock solutions of each test compound were made in DMSO. Final dilutions were made in the assay buffer (20 mM HEPES, pH 8.2) and the pH was checked and adjusted where necessary. In order to eliminate the inhibitory effects of DMSO, the final concentration was less than 1.0% of the total volume (250 µl). Lethal factor was added to dilutions of each inhibitor for a final concentration of 5 nM for oAbz/Dnp-substrate and 10 nM for FTC/DABCYL-substrate. 20 nM trypsin was added to the same dilutions of inhibitor and assays were run in parallel. The plate was incubated at room temperature for 20 min to ensure equilibrium and then at 37oC for 10 min prior to addition of substrate. The reaction was initiated by the addition of 5 µM MAPKKide®. The time-dependent increase in fluorescence intensity was monitored at 37ºC every 40 sec for 10-20 min. The excitation and emission wavelengths were set to 312 nm and 418 nm, respectively, for the oAbz/Dnp-substrate and 490 nm and 523 nm using a cutoff filte of 495 nm for the FTC/DABCYL-substrate.

METHODS (cont.)

Determination of IC50 and mode of inhibition: The IC50 values (inhibitor concentration giving 50% inhibition of LF) were obtained from plots of % inhibition, calculated from ((1-(vi/vo))x100) where v_i is the initial velocity in the presence of the inhibitor and v_o is in the absence of inhibitor, versus concentration of inhibitor.

Dixon plots were used to determine whether the inhibition of selected inhibitors was competitive, noncompetitive or uncompetitive. The inverse of the initial reaction velocities, expressed as relative fluorescence units (RFU) per sec, for a series of inhibitor concentrations at three different substrate concentrations, were plotted as a function of inhibitor concentration.

HPLC: The cleavage of MAPKKide® by the metalloprotease inhibitor, GM6001, and the GM6001, negative control, was monitored using HPLC. The HPLC was run on a Varian ProStar Inert, analytical and prep system using a C-18 reverse phase 4x250 mm column, a flow rate of 1.0 ml/min, and a linear gradient of 0-60% acetonitrile containing 0.1% TFA. The chromatography was monitored at 510 nm, the absorbance wavelength maximum for the FTC substrate fragment, and 440 nm which is the maximum for the DABCYL fragment. For the GM6001 sample, 0.5 mM of the inhibitor was incubated with 10 nM LF for 30 min prior to addition of 5 µM MAPKKide®. Injections alternated between samples with and without GM6001. The GM6001, negative control, was run similarly using 0.1 mM compound.

TABLE I: IC_{50} values for a series of potential LF inhibitors

Compound	oAbz/Dnp Substrate		FITC/DABCYL Substrate	
	IC_{50} (µM) with LF	Inhibition of trypsin	IC_{50} (µM) with LF	Inhibition of trypsin
tetracycline	168	Yes	> 1000	
EGCG	6	Yes	2	Yes
Actinonin	22	No	29	
MMP-3 Inhibitor II			145	No
MMP-8 Inhibitor I			44	No
GM6001			9	No
GM6001, negative control			>500	No
CL82198			156	Yes
In-2-LF			0.021	NA

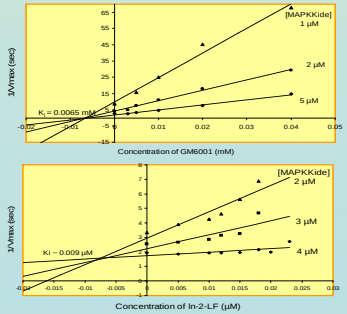


Fig.1:
A Dixon Plot obtained for the matrix metalloproteinase inhibitor, **GM6001**, using 10 nM LF and 5 µM MAPKKide® at 37ºC.

B Dixon Plot obtained for the Anthrax Lethal Factor Protease Inhibitor, **In-2-LF** using 10 nM LF and 5 µM MAPKKide® at 37ºC.

Fig. 2: RP-HPLC of 5 µM MAPKKide® incubated at RT with 10 nM LF for 9 min or 5 hrs. Chromatography was monitored at 440 nm (λmax DABCYL) and 510 nm (λmax FTC).

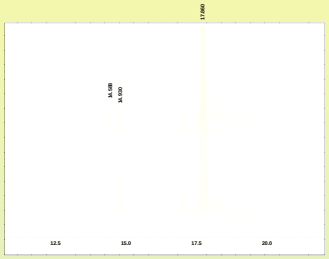
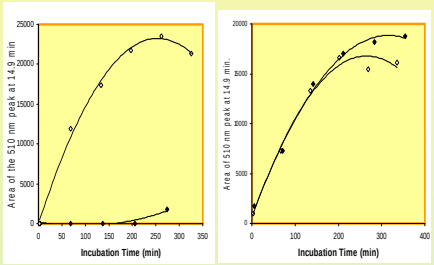


Fig. 3: Cleavage of MAPKKide® in the presence of GM6001 Inhibitor (A) and GM6001, negative control (B).



RESULTS AND DISCUSSION

- IC_{50} values for the compounds evaluated as potential inhibitors of LF are listed in **Table 1**. For several compounds both the oAbz/Dnp and the FTC/DABCYL MAPKKide®were used. Inhibition of trypsin by these compounds is also reported.

  Tetracycline inhibits the hydrolysis of the oAbz/Dnp MAPKKide® . However, the fact that trypsin is also inhibited by tetracycline suggests a false positive. This appears to be confirmed using the FTC/DABCYL substrate since no inhibition of LF is observed for tetracycline using this peptide. Tetracycline absorbs in the UV and can complicate the interpretation of the quenching observed using the oAbz/Dnp MAPKKide® which also absorbs in the UV. This problem is eliminated using the FTC/DABCYL substrate.

  EGCG inhibits both trypsin and LF using both substrates. Again this suggests a false positive. In this case quenching of both substrates was observed in the presence of EGCG alone suggesting that quenching is due to an actual interaction between the EGCG and the substrates.

  This interaction may, in fact, protect these substrates from cleavage by LF which is falsely interpreted as inhibition of LF.

  The antibiotic, actinonin, inhibits the hydrolysis of both substrates. There is no inhibition of trypsin cleavage of the oAbz/Dnp substrate suggesting that actinonin is a specific inhibitor of LF.

- Dixon plots for the MMP inhibitor, GM6001, and the lethal factor protease inhibitor, In-2-LF, are shown in **Fig 1**.

  For **GM6001**, the lines obtained for different substrate concentrations converge at the x-axis indicating that GM6001 behaves in a non-competitive manner. This means that GM6001 has an affinity for both the free and complexed enzyme but it does not compete with the substrate for binding to LF. The value obtained for the dissociation constant, K_i, for the LF-GM6001 is 6.5 µM which is in the range of the IC_{50} value of 9 µM.

  For **In-2-LF**, the plot indicates competitive inhibition. The In-2-LF inhibitor competes with the substrate for binding to LF, as expected [Tonello et al., Nature, 418:386, 2002]. The value obtained for K_i is 9 nM which is also in the range previously reported by Tonello, et al.

- HPLC chromatograms for the hydrolysis of FTC/DABCYL MAPKKide® by LF are shown in **Fig 2**.

  In the 510 nm traces (bottom), the peak at 14.93 min increases in intensity with time and was identified as the peak from the FTC fragment of the MAPKKide® based on the maximum absorbance in a scan of the peak.

  In the 440 nm traces (top), two peaks, one at 14.59 min and the other at 14.93 min increase in intensity with time. The peak at 14.59 was identified as the peak from the DABCYL fragment of the MAPKKide® based on the maximum absorbance (440 nm) in a scan of the peak.

  Since FTC has a high extinction coefficient, it is not surprising that it is also observed in the 440 nm chromatogram.

- The hydrolysis of FTC/DABCYL MAPKKide® in the presence of GM6001 and GM6001, negative control, was monitored using HPLC (**Fig 3**).

  Plots of the area of the 14.9 min peak at 510 nm versus incubation time confirm the fluorescence assay results. GM6001 inhibits cleavage of the MAPKKide® (**Fig 3: A**). In the presence of GM6001, negative control, on the other hand, there is no evidence of inhibition of LF (**Fig 3:B**). Interestingly, these two compounds are mirror images of one another and GM6001 is a possible lead for an LF inhibitor.