



Novel Approaches for Anti-Plague Therapy

Vladimir L. Motin, Ph. D.

Associate Professor

University of Texas Medical Branch, Galveston

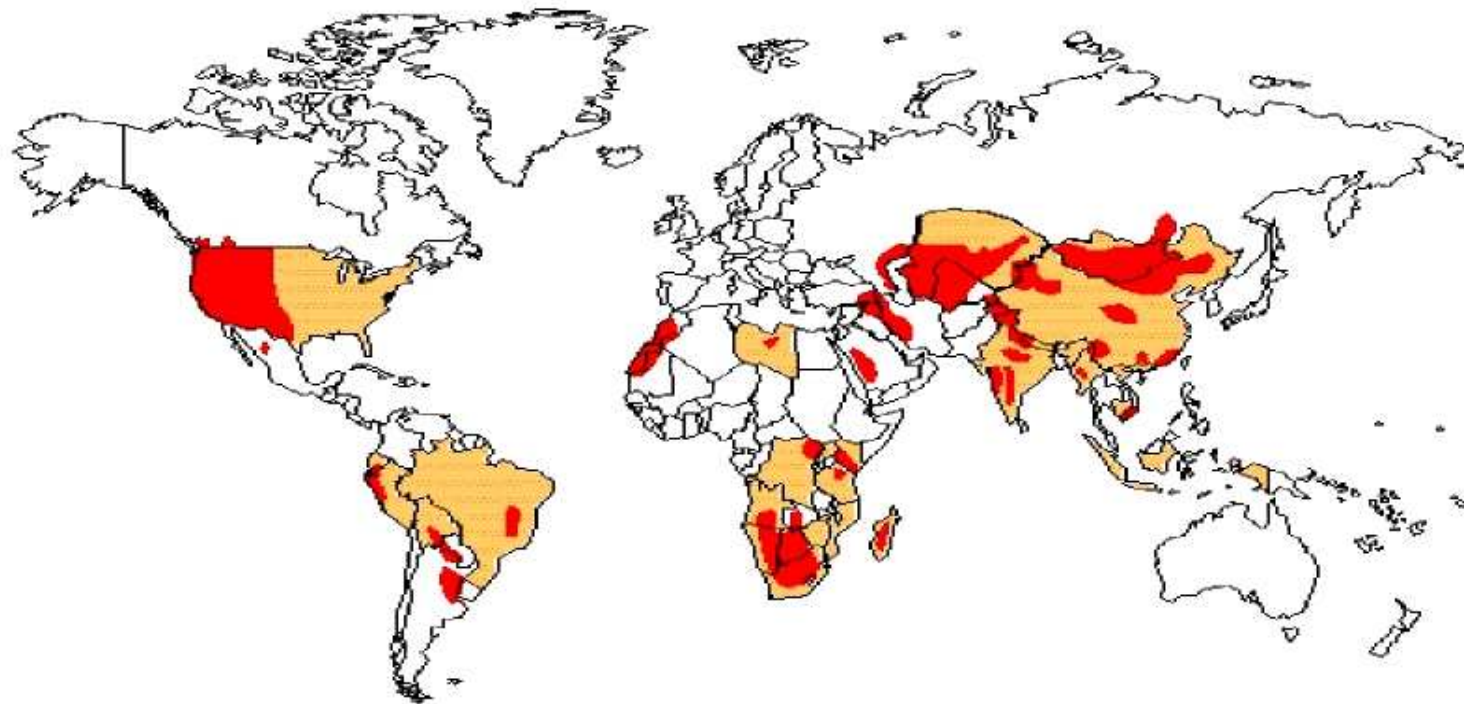
Department of Pathology

Department of Microbiology and Immunology

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World Distribution of Plague, 1998



Yellow Countries reported plague, 1970-1998.

Red Regions where plague occurs in animals.



Signs and Symptoms of *Yersinia pestis* Infection

	From the Bite of Plague-Infected Fleas Common to Rodents			From Human Transmission or Aerosol Release
	Bubonic plague	<i>Y. pestis</i> septicemia or primary septicemic plague	Secondary pneumonic plague	Primary pneumonic plague
Incubation period	2 to 10 days	2 to 10 days	2 to 3 days	1 to 6 days or 2 to 4 days
Disease manifestation from infected fleabite	Most patients develop acutely swollen tender lymph node, or bubo. No person-to-person transmission.	A small minority of patients develop septicemia without a discernible bubo. No person-to-person transmission.	A small minority of patients with bubonic or septicemic plague develops pneumonic plague. Disease may then spread via respiratory droplets.	
Early stages	Malaise, high fever, and one or more tender lymph nodes. Liver and spleen tender and palpable. One-fourth of patients have various types of skin lesions: pustule, vesicle, eschar, or papule at the site of the fleabite.	Malaise and high fever. <i>Y. pestis</i> spreads to the central nervous system, lungs, and further.	Malaise, high fever, chills, headache, myalgia, cough, production of bloody sputum.	Malaise, high fever, chills, headache, myalgia, cough, production of bloody sputum.
Advanced stages	Destruction and necrosis of lymphatic system, bacteremia, septicemia, endotoxic shock, disseminated intravascular coagulation, and coma.	Vasculitis, purpuric skin lesions, necrosis of small vessels, and gangrene in the digits and nose. May lead to thrombosis, disseminated intravascular coagulation, and coma.	Toxemia. Chest X-ray diagnostic of pneumonia, progressing rapidly to dyspnea, stridor, and cyanosis and then finally to respiratory failure, circulatory collapse, and a bleeding diathesis.	Toxemia. Chest X-ray diagnostic of pneumonia, progressing rapidly to dyspnea, stridor, and cyanosis and then finally to respiratory failure, circulatory collapse, and a bleeding diathesis.
Infection control to prevent transmission	Transmitted by infected bodily fluids only. Use standard precautions.	Transmitted by infected bodily fluids only. Use standard precautions.	Can be transmitted via respiratory droplets. Use droplet precautions.	Can be transmitted via respiratory droplets. Use droplet precautions.
Percentage of all cases in U.S. from 1947 to 1996	84%, with 14% observed mortality.	13%, with 22% observed mortality.	2% to 12%, with 57% observed mortality.	

Source: Inglesby TV, et al. Plague as a biological weapon: medical and public health management. *JAMA* 2000;283(17):2281-90.



The Bubo (enlarged and inflamed lymph nodes around arm pits, neck and groin)



The Septicemic Plague (disseminated intravascular coagulation)



Chest radiograph of patient with primary pneumonic plague

Plague Pandemics

- **The First Pandemic**

Justinian's plague, 6th century: Middle East, Mediterranean basin, North Africa, Central and South Asia

~100 million people died

- **The Second Pandemic**

Black Death, 14th century:
Asia, Europe, North Africa

~one quarter of the European population died



BLACK DEATH EUROPEAN TOUR	
DEC 1347	CONSTANTINOPLE
DEC 1347	MARSEILLES
JUNE 1348	NAPLES
JUNE 1348	ROME
DEC 1348	BRISTOL
JUNE 1349	LONDON
JUNE 1349	OXFORD
DEC 1349	DUBLIN
DEC 1349	YORK
JUNE 1350	MAGDEBURG
JUNE 1350	ROSTOCK HAMBURG
DEC 1350	WISBY
DEC 1350	DANZIG

- **The Third Pandemic**

19th century: China, South & North America, South Africa, Madagascar, India, etc.

Current Therapy and Prophylaxis for Plague

Type of therapy	Primary	Alternative (s)	Adjunctive therapy	Investigational prospects
Plague				
All clinical forms	Streptomycin ^a or gentamicin ^b	Doxycycline ^c [32] Ciprofloxacin ^d [34,37] Chloramphenicol for plague meningitis ^e [32]	Supportive therapy for endotoxemia as needed Sympathetic blockade for acral gangrene [37]	Passive immunization with monoclonal antibodies [14]
Pre-exposure prophylaxis	None available in US other than strict isolation of patients with primary or secondary pneumonic plague. Killed cell vaccine possibly available in UK [40]			Recombinant subunit vaccines containing F1 and V antigens [39,43] DNA vaccine coding for derivatives of the F1 antigen [44]
Post-exposure prophylaxis	Presumptive therapy with doxycycline	Ciprofloxacin Chloramphenicol		

- **Post exposure prophylaxis in case of suspected or confirmed exposure to pathogen: 7 days**

Persons who must be present in an area where a plague outbreak occurring can protect themselves for 2 to 3 weeks by taking antibiotics

- **Treatment of suspected or confirmed clinical cases: 10 days**
Early treatment of pneumonic plague is essential. To reduce the chance of death, antibiotics must be given within 24 hours of first symptoms.

Natural Resistance of *Y. pestis* to Antibiotics (Madagascar)

- **1995**

A plague isolate contained a multi-resistant transferable plasmid.

The organism produced TEM-1 β -lactamase, chloramphenicol acetyltransferase, and a streptomycin-modifying enzyme.

- **1996**

A second strain was identified with a plasmid that encoded for the streptomycin-modifying phosphotransferase gene, which resulted in high-level streptomycin resistance.

Targeting Bacterial Virulence

- Search for compounds that affect essential bacterial virulence systems with no or modest effect on bacterial growth
- This chemical attenuation will enable the host to clear the infection

Possible applications/advantages

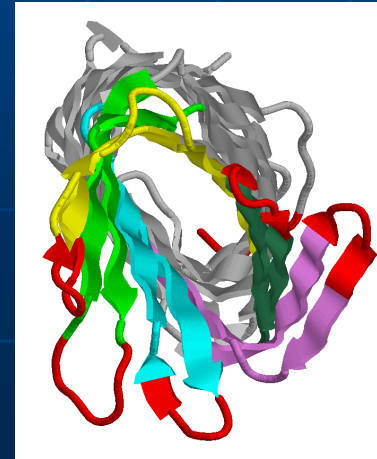
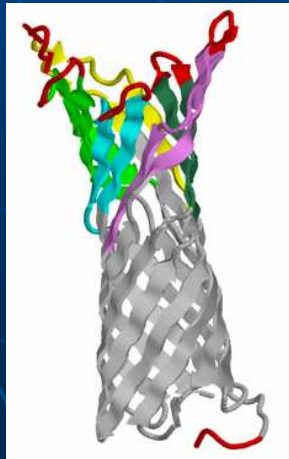
- Treatment against strains resistant to conventional antibiotics and vaccines
- Selective pressure for resistance will be less than traditional drugs designed to kill bacterial pathogen
- Broad antibacterial range (evolutionary conservation of virulence systems)
- Chemical genetics for understanding bacterial virulence

***Y. pestis* Plasminogen Activator (Pla)**

- The *pla* gene is located on small *Y. pestis*- specific plasmid pPCP1
- Pla is an outer membrane protease with significant homology to OmpT of *E.coli* (omptin family of proteases)
- In *Y.pestis* Pla activity appears to be necessary for infection upon administration by peripheral routes (intradermal, subcutaneous, or intranasal)
- Isogenic *pla* mutants of epidemic *Y. pestis* strains KIM and CO92 showed up to 10^6 logs reduced virulence by the subcutaneous route
- Pla allows *Y. pestis* to replicate rapidly in the airways, thus playing the essential role in causing pneumonic plague
- Plasminogen-deficient mice had a 100-fold increase in the LD₅₀ in comparison with those in normal mice
- An Invasive Role of Pla: Clearing Fibrin Deposits That Trap the Pathogen; Dissemination of Bacteria through Host Tissues

Pla of *Y. pestis* as a Therapeutic Target

- The resolved structure of OmpT from *E. coli* (Pla homolog) has a β -barrel topology with 10 transmembrane β -strands and five surface-exposed loops
- Although OmpT (as well as Pla) is widely referred to as being serine protease, the structure contradicts such a classification. Most likely Pla is not a serine, but rather an aspartate protease
- Neither substrate specificity nor inhibitors have been found or predicted for the Pla enzyme
- The Pla molecule is self-cleaved (the autoprocessing site is located within Loop 5)



Potential Substrates Based on Sequence of Loop 5 (site of Pla autoprocessing)

- **A small library of 24 overlapping peptides (beads)**



- **A small library based on chain elongation (beads)**



- **A partial loop 5 peptide**



Potential Substrates Based on Sequence of mammalian plasminogen

Arg⁵⁶⁰-Val⁵⁶¹

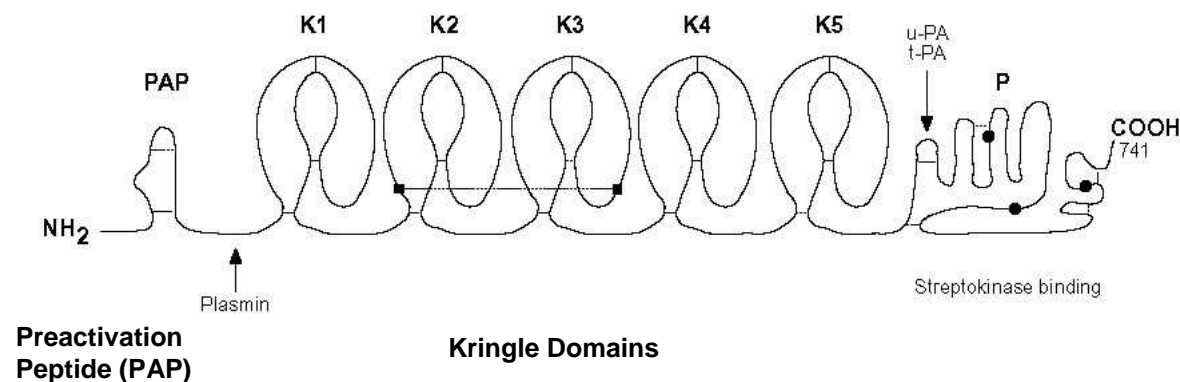
↓ endogenous PA

Native human Plg Lys—Lys—Cys—Pro—Gly—Arg—Val—Val—Gly—Gly—Cys—Val—Ala—His

Bovine Plg Lys—Lys—Cys—Ser—Gly—Arg—Ile—Val—Gly—Gly—Cys—Val—Ser—Lys

Activation of plasminogen into plasmin occurs when plasminogen activators (t-PA, u-PA) cleave a unique bond in the serine protease domain resulting in two polypeptide chains, linked to each other via two disulphide bonds.

Domain structure of the human plasminogen molecule

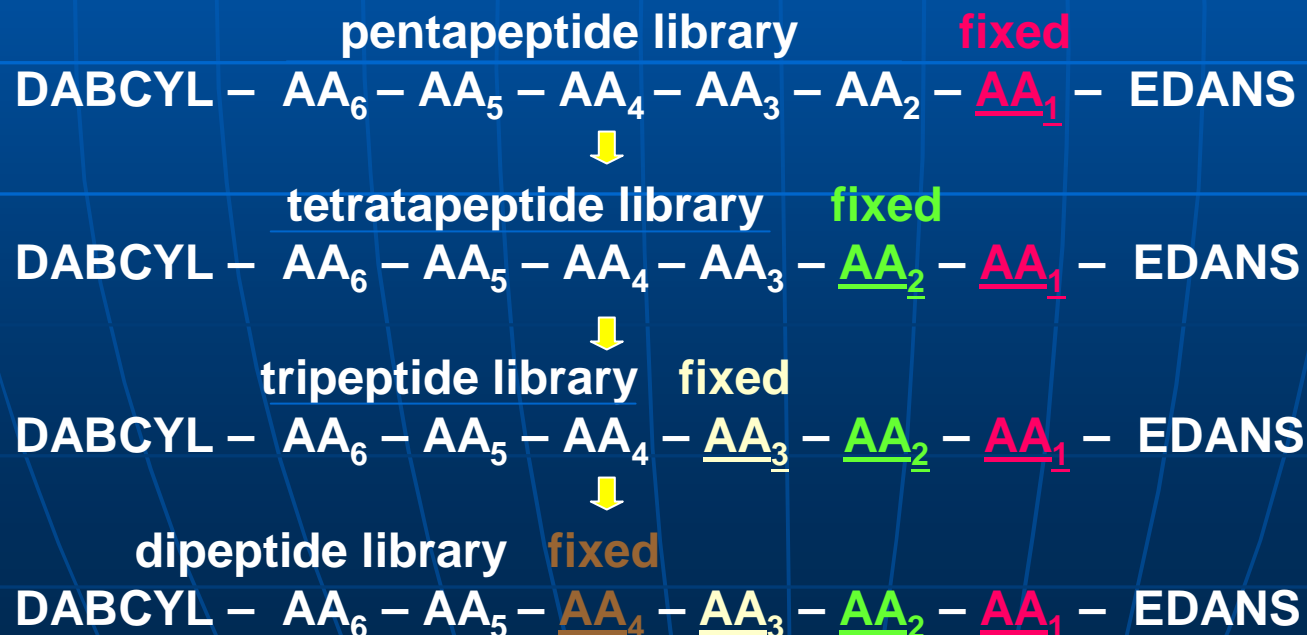


Profiling of Pla Specificity by using Combinatorial Fluorogenic Substrate Libraries

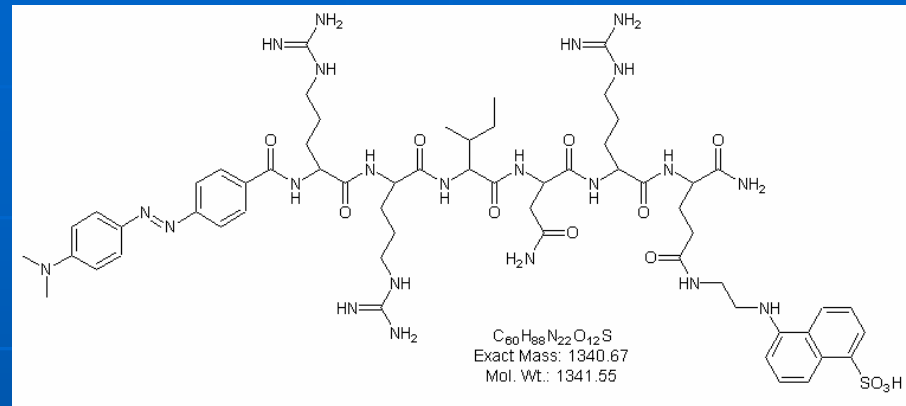
DABCYL – AA₆ – AA₅ – AA₄ – AA₃ – AA₂ – AA₁ – EDANS

Substrate AA-V- 107: all possible combination of 6 a.a. residues.
64 x 10⁶ compounds (hexapeptide library)

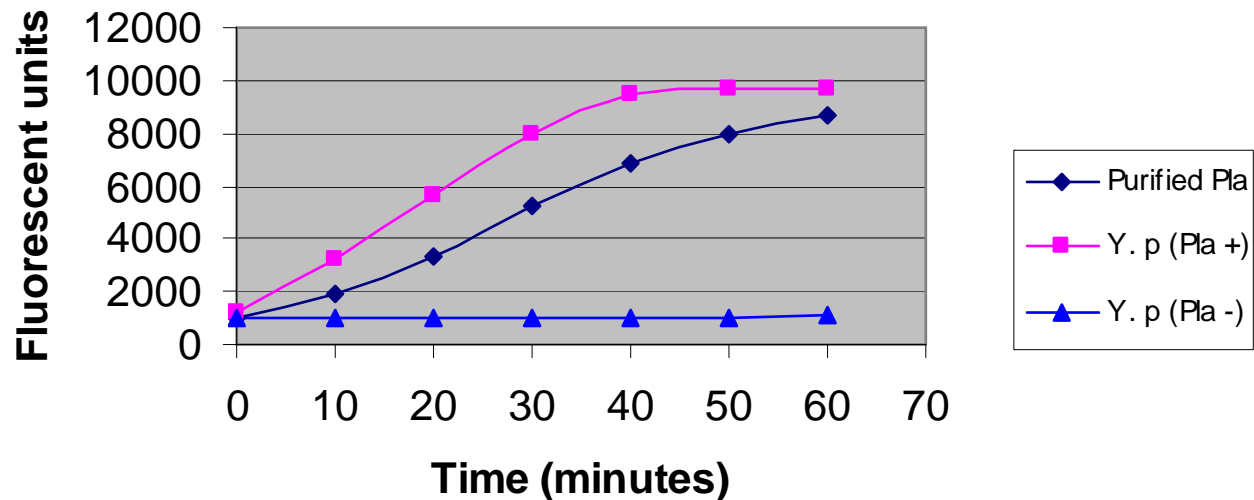
Positional scanning-synthetic combinatorial libraries: libraries in which one position was held constant. Test Pla activity. Select preferred a.a.



Cleavage of the Pla Substrate



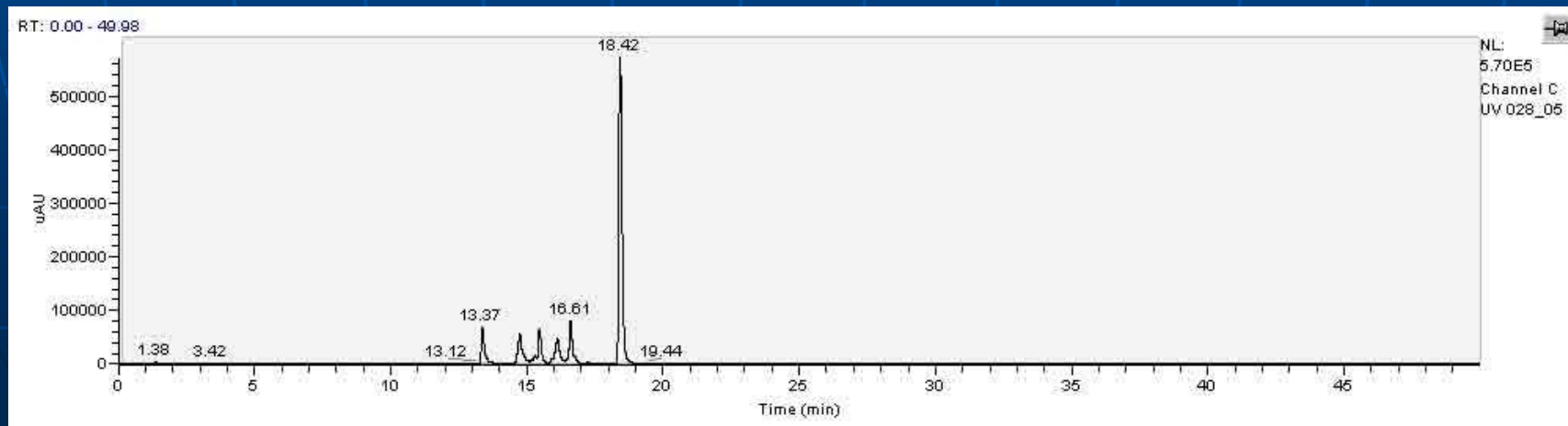
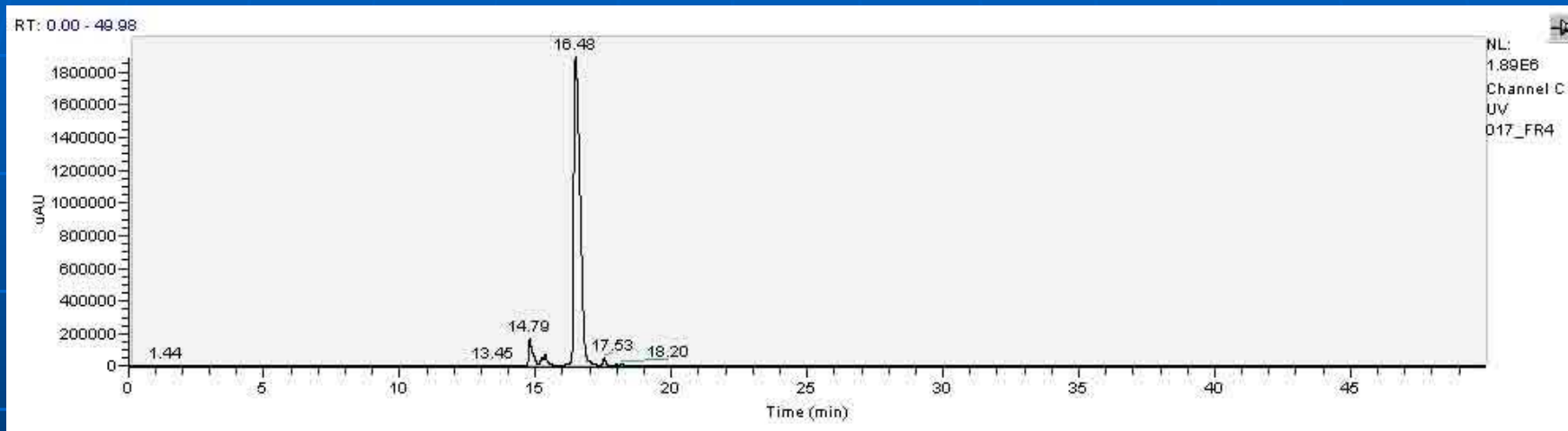
Kinetics of Cleavage of Identified Fluorescent Substrate by Pla



Identification of the Cleavage Site of the Pla Substrate

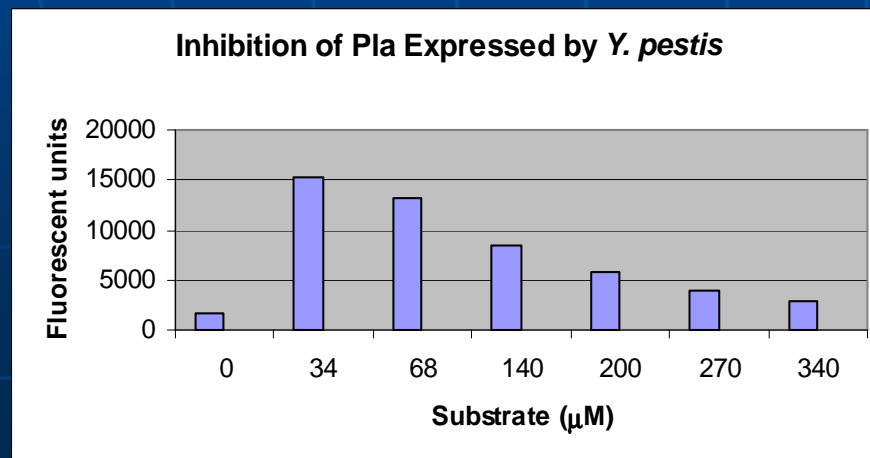
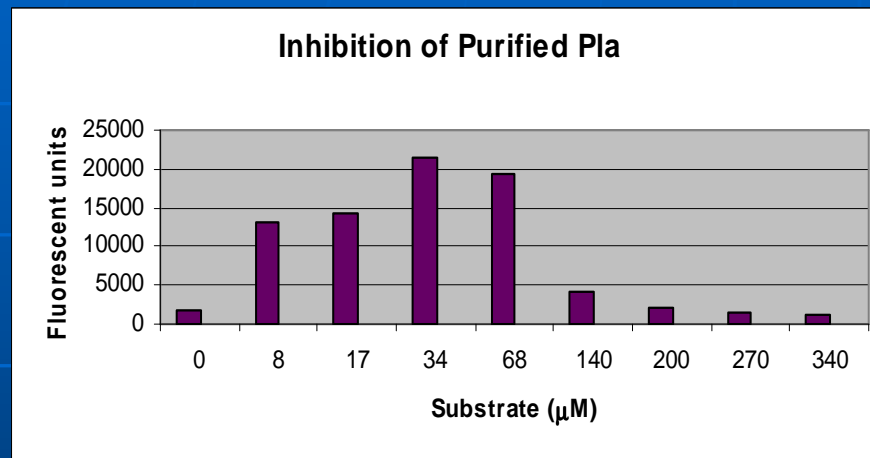
The cleavage site was determined by Liquid Chromatography Mass Spectrometry (LC/MS)

DABCYL – Arg –  Arg – Ile – Asn – Arg – Glu – EDANS

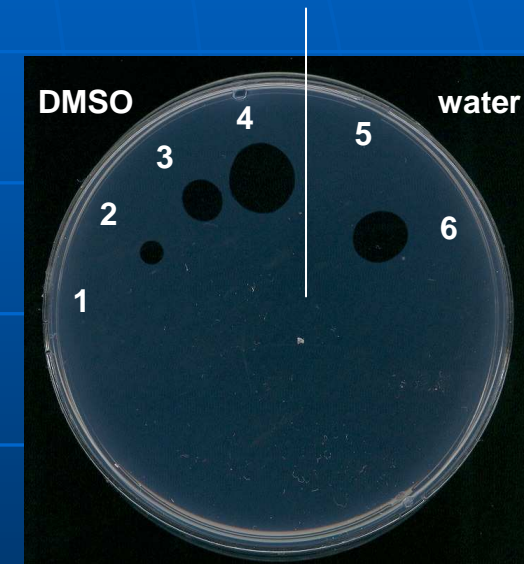


Inhibition of Pla activity by the substrate

Fluorimetric Assay



Functional Assay on Fibrin Plate



High-Throughput Screening of Compound Libraries

The National Screening Laboratory for the Regional Centers of Excellence in Biodefense and Emerging Infectious Diseases (NSRB)

384 well plate: *E. coli* cells expressing Pla + inhibitor (library), incubation.
Add Pla substrate, kinetic measurement of fluorescence

54,100 compounds screened
(mostly small molecules)

124 hits
(at least 50% decrease in the signal intensity)

Currently: completed verification and secondary assays

Next step: Medicinal Chemistry (NSRB resources)

Future Directions

- Optimization of selected substrates and inhibitors that block plasminogen activation *in vitro* (from peptide based substrates to peptide-based inhibitors to peptidomimic inhibitors/drugs and to small-molecule drugs)
- Test identified Pla inhibitors for prophylactic and therapeutic treatments of plague infection (murine model)
- Could be useful in combination with the traditional drugs and vaccines?
- Advantage: Pla is located at the bacterial surface, thereby eliminating the necessity for inhibitors to be permeable into the cell
- The existence of a predicted 3D-model for Pla

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