Enzyme kinetic measurements for a combinatorial library of inhibitors of *Pseudomonas* elastase

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- ***Pseudomonas*** **elastase activity monitored using Abz-Nba internally quenched substrate**
- **K_m and V_{max} calculated using MARS Data Analysis Software**
- **Michaelis Menten kinetics determined for a library of 160 elastase inhibitors**

Introduction

Pseudomonas elastase (pseudolysin, LasB) is a metalloprotease virulence factor secreted by the opportunistic pathogen *Pseudomonas aeruginosa*. As one of the main virulence factors of this bacterium, it contributes to chronic and intractable infection in various disease states from the cystic fibrosis lung, to chronic ulcers of the skin.
The central role of LasB makes it a key drug target in this process, and so a library of inhibitor candidates was developed for screening against this enzyme. Assays were performed using a filter-based microplate reader from BMG LABTECH, which allowed highly adaptable data capture, and screening of multiple compounds in parallel. Data was analysed directly within the MARS software, which allowed extraction of subsets of data post-assay.

Assay Principle

The assay principle is shown in figure 1.

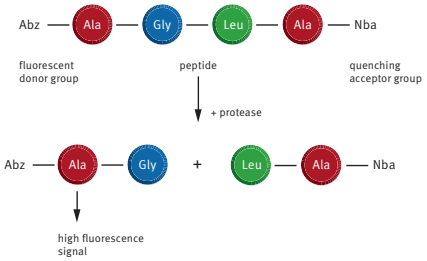

Fig. 1: Assay principle for the determination of LasB activity.

The internally quenched protease substrate Abz-peptide-Nba (2-aminobenzoyl-Ala-Gly-Leu-Ala-4-nitro-benzylamide) gives only a low fluorescence signal. After cleavage of the peptide bound by LasB the fluorescent donor group cannot transfer the energy to the quenching acceptor group resulting in a high fluorescence signal which is directly related to the enzymatic activity.

Materials & Methods

- Abz-Ala-Gly-Leu-Ala-Nba
  (Peptides International, US)
- Library of LasB inhibitors, synthesised at The School of Pharmacy, Queens' University, Belfast

Kinetic measurement
LasB was prepared at 1 in 1000 dilution from 100 µg/mL stock, and used at 10 µL per well, giving a working concentration of 1 ng of LasB per well.
The K_m of the substrate was first calculated by assay of a series of concentrations of substrate from 20 µM to 1000 µM, against a fixed concentration of LasB.

Inhibitor studies
Stock solutions of inhibitors were prepared in DMF at 10 mM, and further diluted when required. Instrument settings employed were as follows:
No. of flashes per well: 10
Target temperature: 37 °C
Ex filter: 310/10 nm and Em filter: 460/10 nm

All assays were performed in buffer containing 0.05 M TRIS HCL, 2.5 mM $CaCl_2$, 1 % DMF, pH 7.2, across a range of concentrations of inhibitor.

Results & Discussion

The results can be seen in figure 2, followed by graphical display of the rate of hydrolysis vs substrate concentration (figure 3), and a double reciprocal or Lineweaver-Burk plot, figure 4.

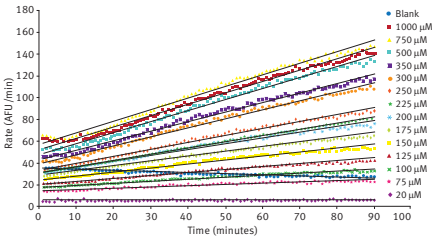

Fig. 2: K_m determination for the LasB substrate Abz-Ala-Gly-Leu-Ala-Nba.

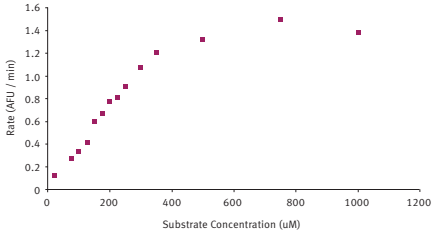

Fig. 3: Rate of substrate hydrolysis by LasB vs substrate concentration.

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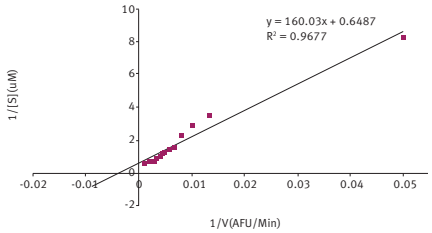


Fig. 4: K_m determined by Lineweaver-Burk plot. The double reciprocal of the data from figure 3 is used to linearise the data.

The slope of the line on the Lineweaver-Burk plot gives K_m / V_{max}, while the X- intercept gives - 1 / K_m, and the Y- intercept, 1 / V_{max}. The data from figure 4 can therefore be used to calculate K_m by solving the equation of the line Y = mX + c, where m = slope.

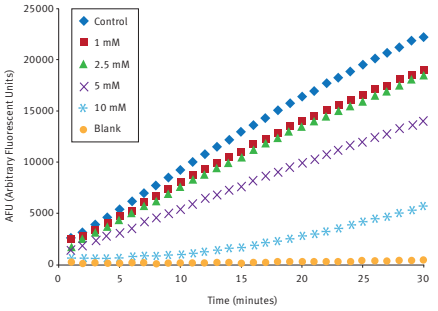


Fig. 5: Progress Curves for hydrolysis of substrate by LasB in the presence of a range of concentrations of a typical LasB inhibitor.

Linear transformation provides a value for the slope of the line, according to the equation y = mx + c. The K_i could be determined for each inhibitor in turn, via the Michael Menten equation (figure 6 and table 1).

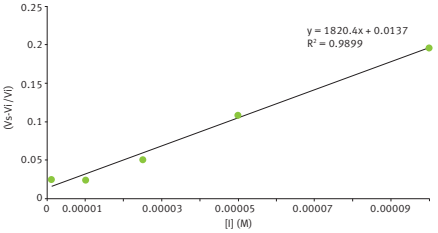


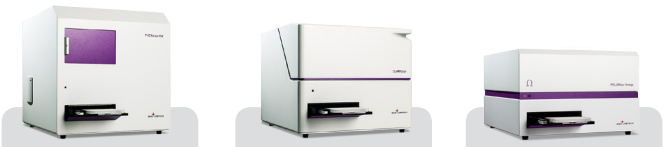
Fig. 6: Linear transformation of progress curves for a typical LasB inhibitor.

Table 1: K_i Values (µM) for inhibitor library. 'NI' (No Inhibition) has been stated for values over 1000 µM. Values in grey identify a general trend for low K_i values in inhibitors containing P'1 Trp and Tyr residues.

Ki (µM)								
	Basic		Aromatic		Large Aliphatic		Acidic	
P'$_2$	Lys	Arg	Phe	Trp	Val	Leu	Asp	Glu
P'$_1$								
His	332	(NI)	21	18	47	306	(NI)	(NI)
Arg	135	(NI)	224	125	(NI)	(NI)	650	(NI)
Lys	433	(NI)	126	(NI)	555	123	971	(NI)
Ile	190	(NI)	(NI)	366	1.8	1.3	142	(NI)
Phe	76	(NI)	146	206	11	645	(NI)	(NI)
Leu	14	623	113	300	(NI)	53	587	(NI)
Trp	10	25	1.1	49	4.1	3.7	38	91
Ala	153	115	(NI)	395	51	21	316	(NI)
Met	3.9	6.6	867	204	98	(NI)	7.0	(NI)
Pro	766	56	(NI)	562	157	246	(NI)	(NI)
Cys	274	646	131	108	161	(NI)	(NI)	(NI)
Asn	289	280	37	70	180	508	(NI)	(NI)
Val	22	69	72	(NI)	10	69	(NI)	(NI)
Gly	451	641	51	122	457	138	(NI)	(NI)
Ser	(NI)	444	75	(NI)	229	510	(NI)	(NI)
Gln	380	217	(NI)	91	937	540	(NI)	(NI)
Tyr	8.5	3.0	6.5	14	0.77	33	5.5	27

Conclusion

The microplate readers from BMG LABTECH offer convenient calculation of K_m, adaptable assay optimization, parallel assay of multiple inhibitors, and isolation of subsets of data post-assay.



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