Nature Vol 418, 25 July 2002
Brief communication – Pharmacology

Screening inhibitors of Anthrax lethal factor

Pre-final version:

A simple high throughput assay and inhibitors of the *Bacillus anthracis* lethal factor metalloprotease

Anthrax is an infectious disease caused by the gram-positive, spore-forming bacterium *Bacillus anthracis*[1]. The clinical presentation and outcome depend on the route of entry. Cutaneous anthrax is the commonest form which appears as black painless pustules and it is rarely fatal, if appropriately treated. The rare systemic anthrax usually follows inhalation of spores which are phagocytosed and transported to lymph nodes where the bacteria germinate. Thereafter, bacteria disseminates and reach the blood flow, rapidly cause overwhelming disease.

Anthrax has recently emerged as a terrorist bioweapon and this calls strongly for novel and specific therapeutics[2].

A major role in host killing is played by the anthrax lethal toxin (LeTx), consisting of PA (protective antigen), which mediates the binding and entry of LF (lethal factor) into the cytosol of macrophages and other cells[3]. The injection of LF + PA into animals causes a shock-like syndrome closely mimicking the final stages of anthrax. LeTx has specific cytopathic effects on macrophages[4], and there is evidence that TeTx-induced macrophage cytotoxicity plays a primary role in anthrax [3,4].

LF is a metalloprotease specific for the MAPK-kinase family of proteins, which are cleaved within their N-terminals[5,6,7,8]. We have recently identified the consensus motif present at the cleavage site[7] (table 1, A). On this basis we

have synthesized several possible substrates and inhibitors of the metalloproteolytic activity of LF. Table 1 reports the sequence of some p-nitroanilide substrates which are endowed with very favourable kinetics and detection characteristics (see the right part of table 1) and high sensitivity (1-2 nanograms can be detected in the 200 μl of buffer present in each well of the 96 well plate). Also coumarin derivatives are good substrates and they allow the detection of minute amounts of LF (>20 times lower than those detectable with p-nitroanilides). With these substrates, the assay of LF metalloproteolytic activity can be performed on plate readers with visible or fluorescence detectors available virtually in any hospital or research laboratory. Moreover, these novel synthetic substrates are very appropriate for high throughput screenings of chemical libraries for the identification of specific inhibitors of LF metalloproteolytic activity.

The conversion of peptide substrates of metalloproteases into hydroxylamine derivatives generates competitive inhibitors[9]. Following this indication, we have synthesized the corresponding hydroxylamine derivatives of the peptides listed in table 1. These peptide hydroxamates give *in vitro* nanomolar inhibition constants (table 1): in particular In-2-LF is the most powerful *in vitro* inhibitor.

Since LF acts in the cell cytosol[1,2], it is essential that an LF inhibitor is capable of entering cells. In-1- and In-2-LF include a strongly cationic stretch which resemble those of peptides capable of entering cells directly from the plasma membrane[10]. These two peptides inhibited LeTx cytotoxicity both in RAW264.7 and J774.A1 macrophage cell lines which are commonly in the assay of LF. In-2-LF was again found to be more effective. The inhibitor entry in the cells was directly observed using a fluoresceine derivative (not shown). Fig. 1 shows that In-2-LF is active inside cells as it substantially inhibits both the cleavage of MEK-3 (used here as a paradigm of MAPKK cleavage in general) (panel c) and the killing of macrophages (panels a and b), an effect

which follows LF entry into the cytosol[1,2]. The present findings provide the materials and the information necessary to develop large scale searches for inhibitors that will provide molecules to be considered as therapeutics for both cutaneous and systemic anthrax, as recently discussed[11].

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Footnote: To promote research in this area, we have not applied for patents. Moreover, LF substrates and inhibitors are made available upon request from the University of Padova (fiorella.tonello@unipd.it).

Table 1 Top panel: sequences at and around the LF cleavage sites (vertical red line) of the various MAPK-kinases. The highlighted sequence of MEK2 was used as template for the design of the first substrate. Lower panel: sequences of the peptide substrates of LF (left) and corresponding kinetic constants of substrates and inhibitors (right).

Peptides from leucine to the N-terminal amino acid were synthesized by Fmoc (9-fluorenyl methyloxy-carbonyl)-solid phase peptide synthesis. the C-terminal amino acid, derivatized with p-nitroaniline or with hydroxylamine (Sigma) were attached to the peptides by a condensation reaction in solution with HATU (O-(7-azabenzotriasol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate) and sym.-collidine. The peptides were purified by reverse phase HPLC C18.

Substrate concentration were determined by measuring the absorption of the peptide-p-nitroanilide at 342 nm (ε_{342} = 8270 M^{-1} cm^{-1}). The enzymatic reactions were performed in 25 mM Na_2HPO_4, 15 mM Na Cl, pH 7.4, 25°C, with an enzyme concentration of 1 nM and substrate concentration ranging from 1 to 5 times the K_m. The release of p-nitroaniline was monitored by recording the absorption at 405 nm with a spectrophotometer Perkin Elmer λ 5 (ε_{405} = 9920 M^{-1} cm^{-1}). The inhibitors concentrations were deduced from their absorbance at 280 nm using extinction coefficients obtained from their amino acid composition. The inhibition constants were obtained using the corresponding pNA peptides as LF substrates.

Figure 1 In-2-LF inhibition on RAW264.7 macrophage cell line. **a**, Transmission light microscopy of control cells (left), cells treated with Letx (200ng/ml LF, 400ng/ml PA) in the absence (middle) or presence of 40μM In-2-LF (right) for 1 h. Letx at the concentrations used, causes cell death (middle) whilst of In-2-LF reduces cell death (right). **b**, Protection of RAW264.7 macrophage death by In-2-LF. Cells were plated in 96-well plates at a density of $2x10^4$/well in Dulbecco's modified Eagle's medium supplemented with heat-inactivated foetal calf serum 1 day before the experiment. Cells were treated with Letx (100ng/ml LF and 400ng/ml PA in serum free medium) and In-2-LF peptide was added at the indicated concentrations 5min after the addition of Letx. After 2 h, cells were washed with PBS and cell death was assayed with MTS tetrazolium (Promega cat.#G3580). Cell death is expressed as percentage of the control treated with Letx only. The average of three independent experiments is reported. **c**, Cleavage of MKK3b in RAW264.7 cells treated with Letx (800ng/ml) determined by western blotting with anti-MEK3 antibodies (Santa Cruz, USA).

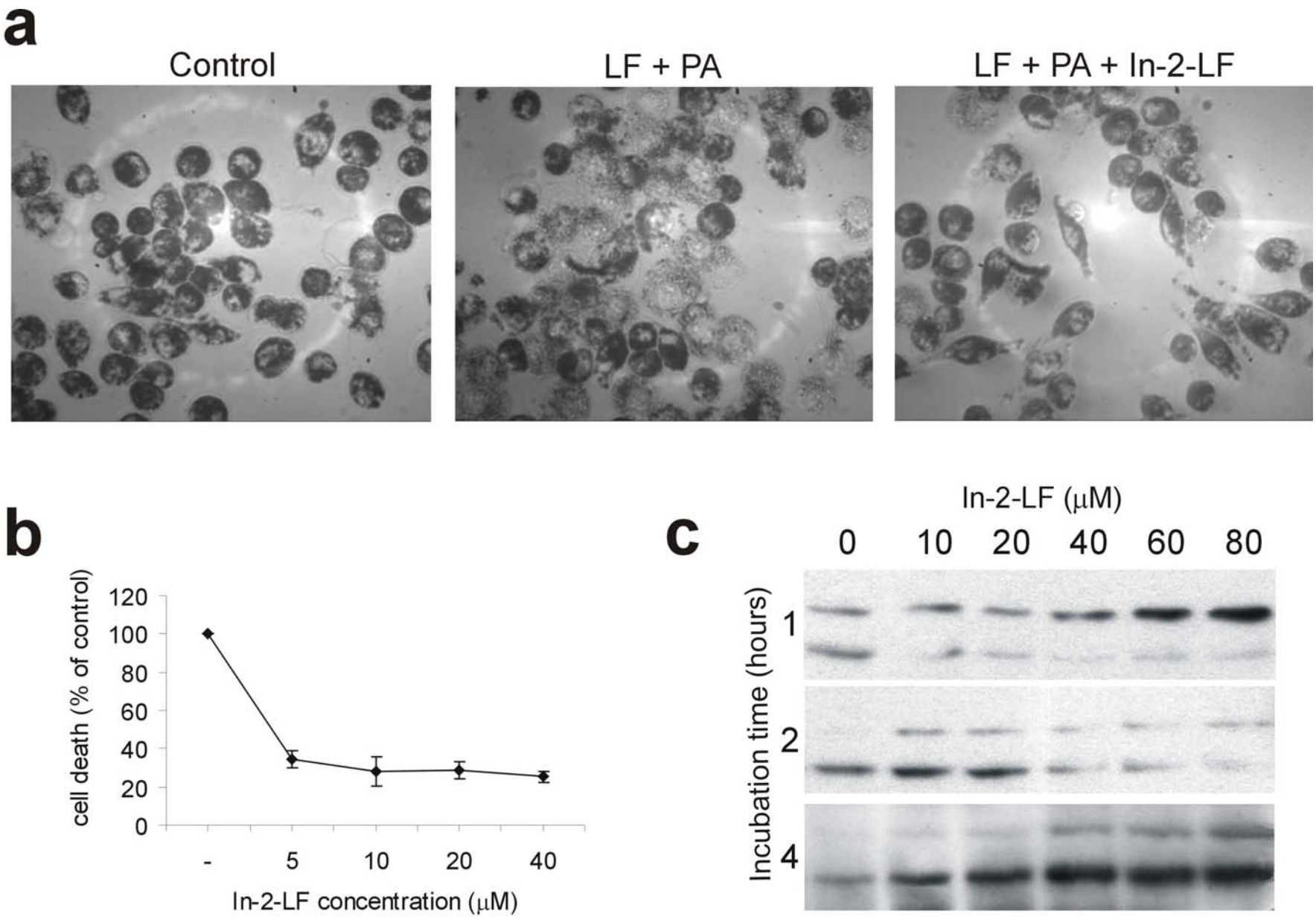
The definitive version is available at www.nature.com

The authors acknowledge the Nature publishing group.

Substrate site			P_8	P_7	P_6	P_5	P_4	P_3	P_2	P_1	P_1'	P_2'	P_3'	P_4'	P_5'	P_6'	P_7'	P_8'
MEK1	(P^8-I^9)		M	P	K	K	K	P	T	P	I	Q	L	N	P	A	P	D
MEK2	(P^{10}-A^{11})	M L	A	R	R	K	P	V	L	P	A	L	T	I	N	P	T	I
MKK3b	(R^{26}-I^{27})		S	K	R	K	K	D	L	R	I	S	C	M	S	K	P	P
MKK6b	(K^{14}-I^{15})		K	K	R	N	P	G	L	K	I	P	K	E	A	F	E	Q
MKK4	(K^{45}-L^{46})		Q	G	K	R	K	A	L	K	L	N	F	A	N	P	P	F
MKK4	(R^{58}-F^{59})		P	P	F	K	S	T	A	R	F	T	L	N	P	N	P	T
MKK7	(Q^{44}-L^{45})		Q	R	P	R	P	T	L	Q	L	P	L	A	N	D	G	G
MKK7	(G^{76}-L^{77})		A	R	P	R	H	M	L	G	L	P	S	T	L	F	T	P
Consensus				+	+	+	+		h		h							

Peptide substrates:

													k_m (μM)	V_{max} (nM/sec)	Inhibitors (hydroxamates)	Ki (nM)
				Ac-M	L	A	R	R	R	P	V	L	P	pNA	30 ± 2	4.5 ± 0.4	-	-
				Ac-M	L	A	R	R	R	P	V	L	R	pNA	12.6 ± 0.5	3.9 ± 0.1	In-0-LF	< 1000
Ac-G	Y	βA	R	R	R	A	R	R	R	R	V	L	R	pNA	1.8 ± 0.2	3.7 ± 0.1	In-1-LF	44 ± 2
Ac-G	Y	βA	R	R	R	R	R	R	R	R	V	L	R	pNA	1.5 ± 0.2	6.5 ± 0.5	In-2-LF	1 ± 0.2


a
Control
LF + PA
LF + PA + In-2-LF
b
cell death (% of control)
120
100
80
60
40
20
0
-
5
10
20
40
In-2-LF concentration (μM)
c
In-2-LF (μM)
0
10
20
40
60
80
Incubation time (hours)
1
2
4