nature biotechnology

Chemical screening by mass spectrometry to identify inhibitors of anthrax lethal factor

Dal-Hee Min[1], Wei-Jen Tang[2] & Milan Mrksich[1]

Mass spectrometry (MS) analysis is applicable to a broad range of biological analytes and has the important advantage that it does not require analytes to be labeled. A drawback of MS methods, however, is the need for chromatographic steps to prepare the analyte, precluding MS from being used in chemical screening and rapid analysis. Here, we report that surfaces that are chemically tailored for characterization by matrix-assisted laser-desorption ionization time-of-flight MS eliminate the need for sample processing and make this technique adaptable to parallel screening experiments. The tailored substrates are based on self-assembled monolayers that present ligands that interact with target proteins and enzymes. We apply this method to screen a chemical library against protease activity of anthrax lethal factor, and report a compound that inhibits lethal factor activity with a K_i of 1.1 M and blocks the cleavage of MEK1 in 293 cells.

This paper describes a strategy for using matrix-assisted laser-desorption ionization (MALDI) time-of-flight (TOF) MS to evaluate large libraries of low molecular weight compounds in enzyme activity assays. This rapid testing of thousands of molecules, commonly referred to as chemical screening, is an important activity in drug discovery where it is used to identify compounds that inhibit biological activities and that can therefore serve as lead compounds in medicinal chemistry programs[1,2]. More recently, chemical screening has become an important technology in basic research laboratories, where it is used to identify small molecules that serve as reagents to study the roles of proteins in cellular processes[3–6]. Many of the assays used in chemical screening rely on fluorescent strategies to report on enzymatic activities, including the use of fluorescence resonance energy transfer (FRET) in protease assays[7], fluorescence polarization with labeled antibodies in kinase assays[8–10] and environmentally sensitive fluorophores in activity assays[11]. Notwithstanding the importance of these methods, the need for labels can be a detriment, in part because the label can compromise the activity of the substrate and in part because some enzymatic activities are not easily adapted to fluorescent labels. Further, the fluorescence properties of small molecules in the libraries that are tested can lead to false positive signals.

MS methods avoid the need for labels in analyzing the products of enzymatic reactions because they report on the mass of the substrate, an intrinsic property of every molecule. Hence, MS methods may offer wide generality in serving as a detection technology in chemical screening. To realize this benefit, however, it is important to simplify the preparation of analytes for MS analysis, which currently requires a chromatographic purification to enrich the analyte and remove spectator molecules and excess salt. The number of sample preparations required in chemical screening make such treatments both expensive and slow, and consequently have prevented the use of MS in these applications. Here, we describe a class of surfaces that both simplifies sample preparation and gives clear and easily interpretable peaks in MS. This strategy combines self-assembled monolayers (SAMs) that are engineered to measure enzymatic activities with MALDI-TOF mass spectrometry to detect those activities in a method we term SAMDI (self-assembled monolayers for MALDI).

To demonstrate the utility of the SAMDI approach for chemical screening, we designed an assay to identify lead molecules that could act against anthrax, a disease caused by the Gram-positive bacterium *Bacillus anthracis*. Anthrax bacteria secrete three major virulence factors: protective antigen, edema factor and lethal factor[12,13]. Protective antigen binds to a TEM-8 cell-surface receptor of the host cell and mediates delivery of edema factor and lethal factor into the cytosol[14]. Edema factor is an adenylate cyclase that causes edema, and lethal factor is a zinc-dependent protease that cleaves the N terminus of mitogen-activated protein kinase kinase (MAPKK or MEK1), resulting in an inactivation of mitogen-activated protein kinase (MAPK)[15–21]. For these reasons, lethal factor and edema factor have been identified as targets for antianthrax drug discovery programs[22,23]. In this work, we demonstrate a screening assay to identify inhibitors of the lethal factor toxin.

RESULTS

SAMDI assay of lethal factor activity

The assay for lethal factor activity uses a SAM that presents a peptide against a background of tri(ethylene glycol) groups (**Fig. 1a**)[24,25]. The peptide is a substrate for lethal factor and is cleaved at the proline residue[26]. The glycol groups that surround the peptide serve to prevent nonspecific adsorption of protein to the surface and ensure that all the immobilized peptide remains available for interaction with the

[1]Department of Chemistry, Institute for Biophysical Dynamics, The University of Chicago, 5735 S. Ellis Avenue, Chicago, Illinois 60637, USA. [2]Ben-May Institute for Cancer Research, The University of Chicago, 924 East 57th Street, Chicago, Illinois 60637, USA. Correspondence should be addressed to M.M. (mmrksich@uchicago.edu).

Published online 16 May 2004; doi:10.1038/nbt973

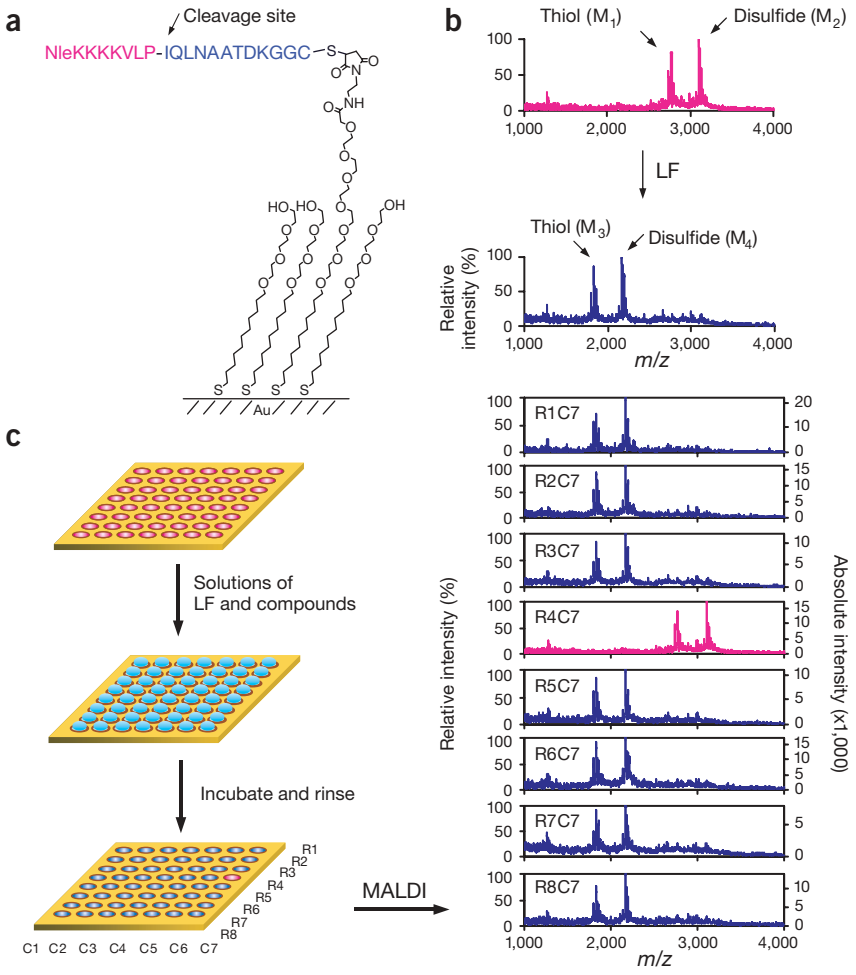

Figure 1 SAMDI-based screen for lethal factor toxin. (**a**) Structure of the SAM presenting a peptide substrate for lethal factor. The arrow indicates the site at which lethal factor cleaves the peptide[26]. (**b**) MALDI-TOF mass spectra of this monolayer before and after treatment with lethal factor. The mass spectra show two peaks, corresponding to the peptide-terminated alkanethiol and the mixed disulfide derived from a glycol-terminated alkanethiol. Lethal factor–mediated proteolysis of the immobilized peptide gave two new peaks corresponding to loss of the terminal peptide fragment. (**c**) Scheme for the screening of a chemical library to identify inhibitors of lethal factor. Solutions containing lethal factor and a cocktail of compounds from the library were arrayed onto SAMs and incubated for 10 min at 37 °C. The slides were rinsed, treated with matrix and scanned by MALDI-TOF MS to record a spectrum for each spot, thereby identifying compounds that blocked lethal factor activity. Representative spectra are shown for eight different spots. Approximately 0.1% of a 10,000 library compound showed partial or complete inhibition. In the spectra shown, R4C7 spot contained a compound that completely blocks lethal factor by showing only peaks corresponding to the uncleaved peptide-terminated disulfide and thiol.

enzyme[27,28]. Analysis of the substrate with a commercial instrument for MALDI-TOF showed mass to charge peaks corresponding to the peptide-terminated alkanethiolate (M_1, molecular formula = $C_{125}H_{226}N_{30}O_{35}S_2$, $[M_1 + H]^+$ = 2,773, $[M_1 + Na]^+$ = 2,795) and the disulfide substituted with one peptide and one glycol group (M_2, molecular formula = $C_{142}H_{260}N_{30}O_{39}S_3$, $[M_2 + H]^+$ = 3,107, $[M_2 + Na]^+$ = 3,129) (**Fig. 1b**)[29,30]. The well-defined surface chemistry and the minimal fragmentation of ionized molecules are both important for giving clear and easily interpreted spectra. When this substrate was treated with lethal factor and rinsed, MALDI-TOF MS revealed that these peaks were absent and gave rise to new peaks corresponding to proteolysis of the peptide (thiol; M_3, molecular formula = $C_{79}H_{140}N_{18}O_{27}S_2$, $[M_3 + H]^+$ = 1,838, $[M_3 + Na]^+$ = 1,860, disulfide; M_4, molecular formula = $C_{96}H_{174}N_{18}O_{31}S_3$, $[M_4 + H]^+$ = 2,172, $[M_4 + Na]^+$ = 2,194; see **Supplementary Figs. 1** and **2** online). The quality of the spectra for these experiments is lower than is common in MALDI-TOF MS, including the use of monolayer surfaces[30]. The lower quality spectra result from the use of an oversized plate to accommodate a large number of spots in the array. The plate does not optimally fit in the commercial mass spectrometer, resulting in a nonoptimal distance of the plate and the source, with a loss of resolution. The loss in resolution does not compromise the assay because the peaks for the full-length peptide and cleaved peptide are well resolved. This example illustrates that the combination of SAMs and MALDI-TOF MS provides a straightforward and label-less method for measuring lethal factor proteolytic activity.

Chemical screening

We applied the SAMDI assay in a chemical screen of 10,000 molecules to identify inhibitors of lethal factor (**Fig. 1c**). We first prepared cocktail solutions containing lethal factor (200 nM) and eight compounds from the library (~10 M each) in the assay buffer. To prepare the SAMDI plate, we first machined a 10 by 10 array of circular grooves (2 mm in diameter) onto a glass plate, then deposited a gold film on the plate, and assembled a SAM presenting the peptide on the substrate. The cocktails containing lethal factor and compounds were arrayed onto the plate within the circular grooves—which served to control the spreading of the drop to a constant area—and then incubated for 10 min at 37 °C in a humidified chamber. The substrates were rinsed with distilled water, dilute hydrochloric acid (1 M), distilled water and absolute ethanol. The substrates were then treated with matrix (5% 2,4,6-trihydroxyacetophenone in methanol) and analyzed by MALDI under linear mode to obtain a mass spectrum for each circular region. We obtained satisfactory MS spectra from our system under linear mode, which is more sensitive than reflector mode. **Figure 1c** shows representative MS data for the screen. The majority of spots show complete cleavage of the immobilized peptides. Eleven of the spots (approximately 1%) show no or partial proteolysis of the peptide, indicating the presence of an inhibitor in the cocktail. We repeated the assay with each of the eighty-eight compounds present in these eleven samples and found one compound, DS-998, that completely blocked lethal factor activity at 10 M concentration.

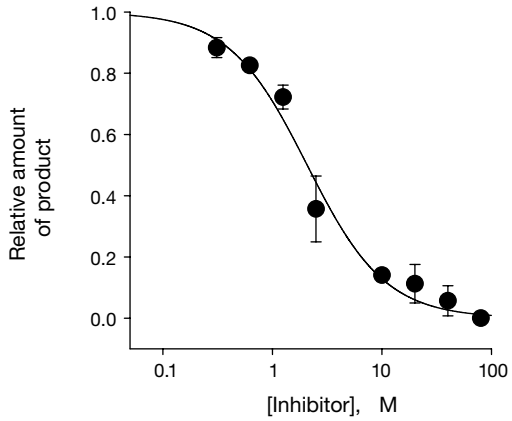

Figure 2 Dose-response curve from the SAMDI assay. Mixtures of lethal factor and various concentrations of DS-998 were spotted on the SAMs presenting substrate peptides and the extent of enzyme reaction was determined by MALDI-TOF MS as described in methods. Relative peak intensities were plotted versus concentrations of DS-998 at log scale.

Quantitative evaluation of DS-998 by SAMDI

We next used the SAMDI format assay to characterize the concentration-dependent inhibition of lethal factor by the candidate DS-998. We prepared several solutions containing lethal factor (100 nM) and DS-998 at concentrations ranging from 0.31 to 80 M. These solutions (0.8 l each) were applied onto a SAMDI plate presenting the peptide substrate as described above and incubated for 10 min at 20 °C. The SAMDI plates were rinsed to stop the reactions and analyzed as described above. A plot relating the extent of peptide proteolysis to the concentration of inhibitor showed that enzyme activity decreased with increasing concentrations of DS-998. This experiment was done under conditions that gave ~40% proteolysis of the peptide, and therefore the relative amount of peptide cleavage is an approximate measure of the relative activity of the lethal factor enzyme. Therefore, determination of the relative activity of the enzyme in this assay indicates the fraction of enzyme that is bound by DS-998. Consistent with this interpretation, the activity data were fit well by a 1:1 inhibition model, and showed that DS-998 inhibits 50% of lethal factor activity (IC_{50} value) at a concentration of ~2 M (**Fig. 2**). In the next section, we show that the SAMDI analysis of the IC_{50} value is in good agreement with an analysis using a conventional *in vitro* assay.

Kinetic characterization of DS-998

We characterized the activity of DS-998 in a solution-phase assay to confirm the inhibition and to determine the dissociation constant for binding of the molecule to lethal factor. The assay used the peptide Ac-NleKKKKVLP-*p*NA as the substrate for lethal factor, and contains a C-terminal *p*-nitroanilide (*p*NA) residue at the cleavage site (**Fig. 3a**)[23,26]. The rate of the enzymatic reaction was monitored spectrophotometrically, by measuring the time-dependent concentration of the *p*-nitroaniline that is released in the proteolysis. The initial rates showed a sigmoidal dependence on the concentration of DS-998 and provided an inhibition constant (K_i) of ~1 M (**Fig. 3b**). Further characterization showed that DS-998 is a noncompetitive inhibitor (**Fig. 3c**). To confirm that DS-998 acts as a specific inhibitor of lethal factor, we investigated its ability to inhibit other peptidases—including trypsin, β-lactamase and carboxypeptidase A—and found it had no effect on these enzymes at concentrations up to 100 M (data not shown).

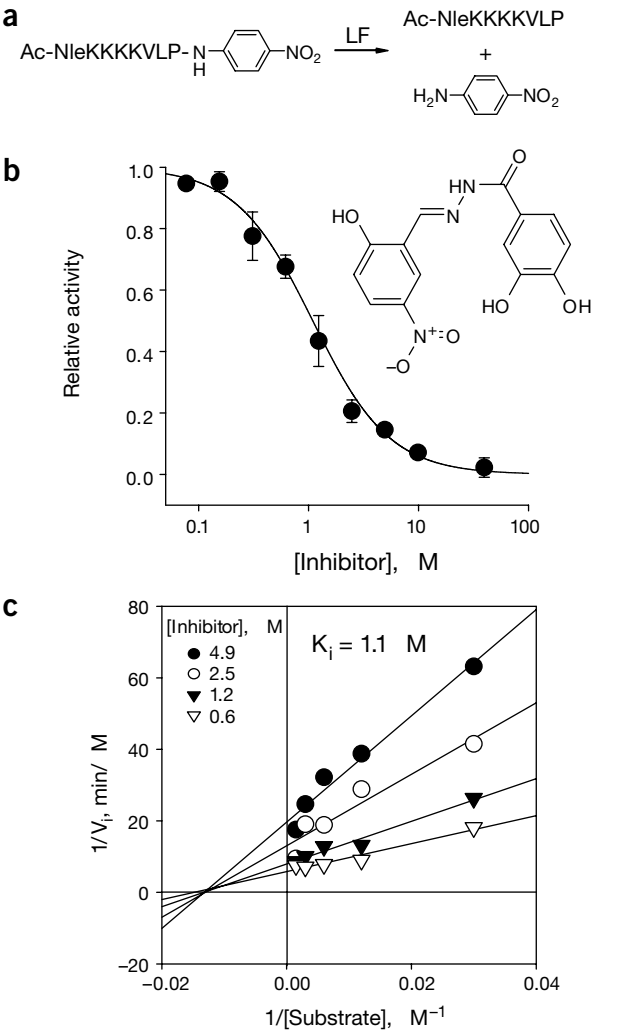

Figure 3 Kinetic characterization of DS-998. (**a**) Structure of a *p*-nitroanilide -terminated peptide that was used for solution-phase assays of the enzyme activity. The enzymatic reaction is monitored by spectroscopic measurement of the *p*-nitroaniline (405 nm) that is released by the enzyme action. (**b**) Measurement of the initial rates for enzyme activity in the presence of DS-998 at concentrations ranging from 0.04 to 80 M provided an inhibition constant of 1.1 M. (**c**) A plot that relates the inverse of initial velocities and the inverse of concentrations of substrate for several concentrations of the inhibitor confirms that the inhibitor acts noncompetitively.

Cell-based assays of DS-998

We next characterized the activity of DS-998 in a cellular assay to establish that it acts selectively on lethal factor and does not have cytotoxic effects. We used established assays in human melanoma UACC-257 cells[31]. As expected, treatment of the cells with lethal toxin (a mixture of protective antigen (250 ng/ml) and lethal factor (15 ng/ml)) induced differentiation and melanogenesis of these cells based on characteristic changes in the morphology and pigmentation (**Fig. 4a,b**). Cells that were first incubated with the inhibitor (20 M) and then treated with lethal factor showed no visible changes, as did cells treated with control vehicle or inhibitor alone (**Fig. 4**). This result indicates that DS-998 can effectively block the lethal factor–induced differentiation and melanogenesis of melanoma cells. We next tested whether the inhibitor can block the proteolysis of

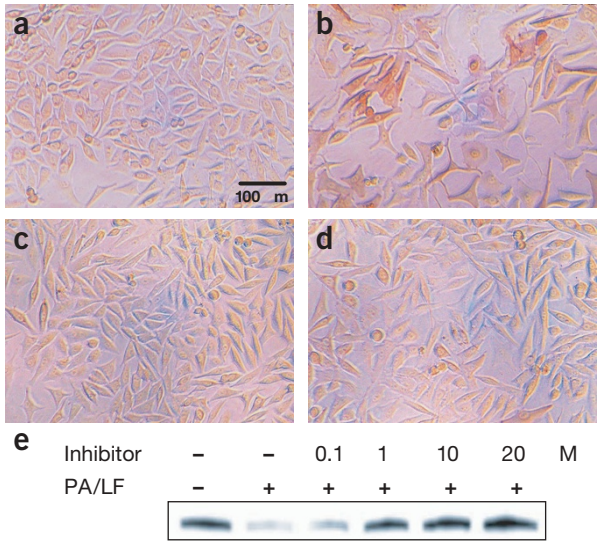

Figure 4 Activity of DS-998 in cellular assays. UACC-257 cells were used to establish that the inhibitor is active against lethal factor in cultured cells at concentrations that pose no cytotoxicity[31]. Cells were cultured for 72 to 96 h and examined for morphologies and pigmentation that are characteristic of lethal factor–treated melanoma cells. (**a**) Melanoma UACC-257 cells without any treatment showed polygonal shapes and light yellowish color. (**b**) Cells treated with protective antigen (250 ng/ml) and lethal factor (15 ng/ml) displayed characteristic changes in both cellular morphology and apparent color[31]. (**c**) Cells treated with inhibitor alone (20 M) did not show any distinguishable cytotoxicity over as many as 4 d in culture. (**d**) The inhibitor (20 M) blocked lethal factor–induced morphological change and pigmentation. (**e**) Western blot showed that cleavage of HA-MEK1 catalyzed by lethal factor was blocked by DS-998 in HEK 293 cells. First lane: HA-MEK1 overexpressed in HEK 293 cells. Second lane: decreased amount of uncleaved HA-MEK1 due to cleavage of N-terminal residues and HA-tag by lethal factor. Third lane to sixth lane: the amount of uncleaved HA-MEK1 was dependent on the concentration of added DS-998 (0.1, 1, 10, 20 M).

MEK1 (MAPKK) by lethal factor in human embryonic kidney 293 (HEK 293) cells. We monitored the quantity of the N-terminal influenza hemagglutinin-epitope-tagged (HA)-MEK1 in HEK 293 cells that were transiently transfected with plasmid encoding HA-MEK1. We found that DS-998 had a dose-dependent effect on protecting N-terminal degradation of HA-MEK1, and showed complete inhibition of lethal factor activity at concentrations between 1 and 10 M (**Fig. 4**). These studies establish that DS-998 can specifically block the proteolytic activity of lethal factor in tissue culture cells.

Activity in macrophage cells

We further characterized DS-998 using J774 cells to determine whether the small molecule protects macrophages from lethal factor–mediated cell death. We used the established MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide or thiazolyl blue) method to quantify cell viability[32]. In this method, the yellow tetrazolium MTT undergoes cleavage within metabolically active cells to form a purple formazan dye. The resulting intracellular formazan precipitate can be solubilized in detergent solution and quantified spectrophotometrically at 595 nm. Using this method, we first measured the cytotoxicity of DS-998 by incubating J774 cells in the presence of DS-998 at concentrations ranging from 1.6 to 200 M for 4 h. The compound DS-998 did not show substantial toxicity for concentrations below 10 M (**Fig. 5a**). To determine the concentration of lethal factor required to induce apoptosis, we incubated J774 cells with the toxin at concentrations ranging from 0.16 to 100 ng/ml with lipopolysaccharide (LPS) (100 ng/ml) and protective antigen (200 ng/ml) for 4 h (**Fig. 5b**). As expected, lethal factor–induced cell death in a concentration dependent manner, with 50% of cells undergoing death at about 20 ng/ml of lethal factor. We next evaluated the ability of DS-998 to block lethal factor–induced macrophage death. J774 cells were first incubated with DS-998 at concentrations ranging from 0 to 5 M for 30 min followed by an exchange of the media to introduce LPS (100 ng/ml), protective antigen (200 ng/ml), and lethal factor (40 ng/ml) DS-998 (at the original concentration). Cell viability was measured using the MTT method after a 4-h culture. The extent of cell death was expressed relative to a control containing LPS, protective antigen and lethal factor. We found that DS-998 increased cell viability in a dose-dependent manner (**Fig. 5c**). For example, DS-998 at a concentration of 5 M decreased lethal factor–mediated cell death to 60% of the level observed in the absence of the small molecule.

Determination of Z′-factor

It is important to have a basis to compare the SAMDI method with current methods used in chemical screening. A widely used method for assessing the robustness of screening assays—in terms of distinguishing positive signals from background—is determination of the Z′- factor[33]. The Z′-factor ranges from 1 (perfect assay) to 0 and generally displays a value of 0.75 or higher for high performance assays. To determine the Z′-factor, a plate or chip containing both positive and negative controls are assayed and used to determine the mean and standard deviation for signals from both the positive control and negative control points. These values are used to determine the Z′-factor according to equation (1). The term $|\ _{c+} - \ _{c-}|$ denotes the difference between the mean of the positive control and the mean of the negative control and, σ_{c+} and σ_{c-} represent standard deviations for the positive and negative controls respectively.

$$Z'\text{-factor} = 1 - \frac{(3\sigma_{c^+} + 3\sigma_{c^-})}{|\ _{c^+} - \ _{c^-}|} \quad (1)$$

To measure this value for the SAMDI method, we acquired mass spectra of positive controls (24 spectra) and negative controls (24 spectra). For positive controls, 24 spots on the monolayer were incubated with lethal factor (200 nM in the assay buffer) under conditions that give complete cleavage of the peptide. For the negative controls, 24 spots were incubated in the assay buffer that did not contain lethal factor. The absolute peak intensities at *m/z* 3,107 ($[M_2+H]^+$) and at *m/z* 2,172 ($[M_4+H]^+$) for the positive and negative controls were normalized using equation (2).

$$I' = \frac{I_{M_4}}{(I_{M_4} + I_{M_2})} \quad (2)$$

The Z′-factor was calculated from normalized peak intensities (I') and gave a value of 0.83 (**Fig. 6**). This value compares very favorably with established assays, including those based on FRET, and establishes that the SAMDI method does have the characteristics required to make it valuable in chemical screening applications.

DISCUSSION

This example illustrates the characteristics of SAMDI that will make it an important technique in chemical screening. Most importantly, the method does not require the substrate—in this case the peptide that is cleaved by lethal factor —to be labeled with isotopic or fluorescent reporter groups. Consequently, less synthetic effort is

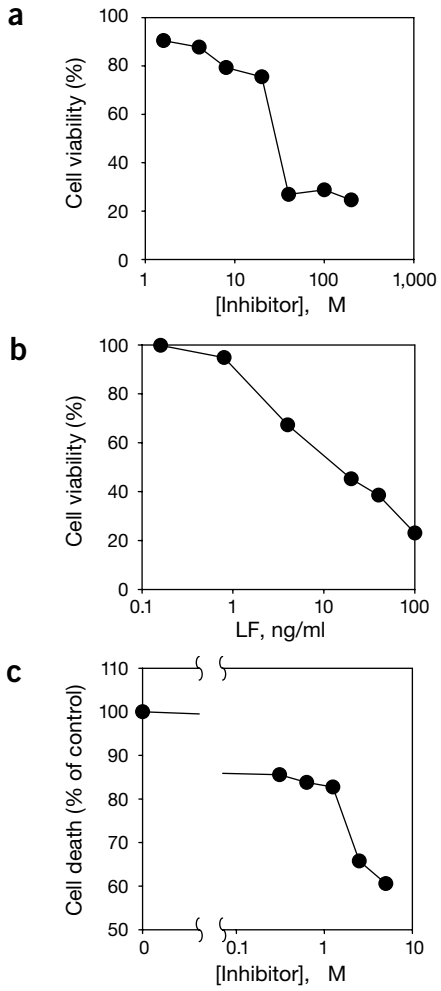

Figure 5 Inhibition of lethal factor–induced macrophage death by DS-998. (**a**) Cytotoxicity of DS-998 towards macrophage J774. Cells were treated with DS-998 and, after 30 min, medium was replaced with fresh medium containing DS-998 at various concentrations. MTT assay was performed 4 h later to determine cell viability. (**b**) Apoptosis of J774 cells was dependent on lethal factor concentration. Cells were treated with mixtures of lethal factor (various concentrations), protective antigen (200 ng/ml) and LPS (100 ng/ml). Cell viabilities were evaluated using MTT method 4 h later. (**c**) DS-998 inhibited apoptosis induced by lethal factor dose dependently. DS-998 was added at indicated concentrations 30 min before addition of second dose of DS-998, lethal factor (40 ng/ml), protective antigen (200 ng/ml) and LPS (100 ng/ml). Cell viabilities were evaluated using MTT method 4 h after addition of DS-998, lethal factor, protective antigen and LPS.

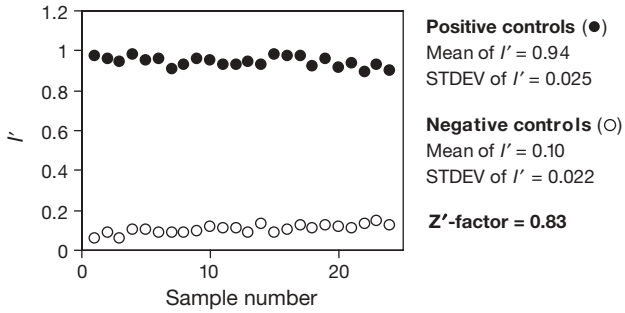

Figure 6 Z′-factor for the SAMDI assay. Normalized peak intensities of positive controls (24 samples) and negative controls (24 samples) were plotted versus sample numbers. Mean ($_{c+}$ for positive and $_{c-}$ for negative controls) and standard deviation (σ_{c+} for positive controls and σ_{c-} for negative controls) of normalized intensities (I') were shown for both positive and negative controls. Calculated Z′-factor was 0.83.

required in formatting an assay—an especially important consideration in assays that use arrays of peptides—and there is less risk that the introduction of a reporter group can compromise the activity of the ligand[10]. This feature will be especially important in assays of activities that are not easily monitored with labels (for example, carbohydrate modifying enzymes[34]). A second benefit of SAMDI stems from the mass-resolved signals in MS measurements. In the common fluorescence-based screens, it can be difficult to determine the threshold levels of fluorescence that are indicative of a hit (because of nonuniform activities of immobilized substrates and high background signals[5]). Additionally, in spectroscopic methods done in multiwell plates, the candidate compounds can often interfere with the signal of interest, resulting in false positive signals. SAMDI, by contrast, resolves the signals for the substrate and product of an enzymatic assay, leading to a straightforward identification of active compounds with fewer false positive and false negative signals. A third benefit owes to the use of surface chemistries that prevent the nonspecific adsorption of protein, thereby eliminating many species in the reaction mixture that contribute to background and ensuring that all of the immobilized ligands remain active towards the enzyme. SAMDI also avoids the chromatographic steps that are required to desalt and enrich samples before MS analysis[35]. Finally, the availability of commercial instruments for MALDI-TOF MS and for preparing arrays makes this technique straightforward to apply to other enzyme assays.

This work demonstrates an effective strategy for using tailored substrates to simplify the chip-based analysis of biological activities with mass spectrometry, and for making this technique applicable to parallel screening in drug discovery. By combining an array format assay with MS, we have demonstrated accurate evaluation of enzyme activities on a surface without isotopic or fluorescent labeling. In applying this method to the anthrax lethal factor, we have found a low molecular weight chemical that may facilitate basic research on anthrax and the development of antianthrax pharmaceutical agents.

METHODS

Reagents. Lethal factor and protective antigen were purchased from List Biological Laboratories and stored as recommended. We purchased 2,4,6-trihydroxyacetophenone from Aldrich Chemical Co. Reagents for peptide synthesis were purchased from AnaSpec. Thiazolyl blue (MTT) was purchased from Sigma. A chemical library of 10,000 molecules was purchased from Chembridge. The substrates were analyzed on a Voyager-DE Biospectrometry mass spectrometer. The peptide, NleKKKKVLPIQLNAATDKGGC, was prepared on 9-fluorenyl methyloxy-carbonyl (Fmoc)-Rink amide 4-methylbenzhydrylamine resin using an ABI 430A peptide synthesizer in the protein-peptide core facility at the University of Chicago. The assay buffer for enzyme reactions was 25 mM HEPES at pH 7.0 containing 10 mM NaCl, 5 mM $MgCl_2$, 50 M $CaCl_2$ and 50 M $ZnCl_2$.

Preparation of SAMs. Titanium (5 nm) and gold (15 nm) were evaporated onto glass coverslips. Gold-coated coverslips were immersed in an ethanolic solution containing a maleimide-terminated disulfide and a tri(ethylene glycol)-terminated disulfide to generate maleimide functionalized SAMs (ref. 25). A cysteine-terminated peptide substrate for lethal factor was immobilized by spotting the peptide solution (1 mM in pH 7.0 Tris) on the monolayer for 30 min at 37 °C in a humidified chamber.

Evaluation of LF-mediated cleavage on SAMs. The peptide-immobilized substrate was first thoroughly rinsed with deionized water and ethanol, and then dried under a stream of nitrogen. LF-mediated cleavage was done by applying a solution of lethal factor (200 nM) in the assay buffer onto the substrate, and after incubation for 10 min at 37 °C, the substrate was rinsed with distilled water, dilute hydrochloric acid (1 M), distilled water absolute ethanol. The substrate was then treated with matrix (5% 2,4,6-trihydroxyacetophenone in methanol). Mass analysis was carried out under linear mode in positive ions with a 337 nm nitrogen laser.

Chemical screening. Cocktail solutions containing lethal factor (200 nM) and eight compounds from the library (~10 M each) were prepared in the assay buffer containing 0.5% DMSO. To prepare SAMDI plate, a 10 by 10 array of circular grooves (2 mm in diameter) was initially engraved onto a glass plate. Titanium (5 nm) and gold (15 nm) were then evaporated onto the plate. The substrate peptide-presented SAMs were formed as described above. The cocktail solutions (0.8 l for each solution) were spotted into each of the circular regions of the substrate and then incubated for 10 minutes at 37 °C in the humidified chamber. The substrates were rinsed with distilled water, dilute hydrochloric acid (1 M), distilled water and absolute ethanol. The matrix solution was applied to the substrate and mass analyses were carried out for each circular region.

Evaluation of dose-dependent inhibition by SAMDI. Mixtures of lethal factor (100 nM final concentration) and various concentrations of DS-998 were prepared in the assay buffer containing 0.5% DMSO, and 0.8 l of each mixture was applied onto the peptide-presented SAMs. After incubation for 10 min at 20 °C, the substrate was rinsed and treated with the matrix. Mass analysis was done in reflector mode. The relative amount of the cleaved peptide was calculated by measuring the relative intensities of peaks corresponding to disulfides ($[M_2 + H]^+$ and $[M_4 + H]^+$).

Synthesis of Ac-NleKKKKVLP-*p*NA. The peptide sequence from leucine to norleucine was synthesized by a standard Fmoc-solid peptide synthesis methodology using 2-chlorotrityl chloride resin. The fully protected peptide was cleaved from the resin by addition of a 1:2:7 mixture of acetic acid, trifluoroethanol and dichloromethane. The peptide was coupled to the C-terminal amino acid, proline *p*-nitroanilide using 1,3-diisopropylcarbodiimide, 1-hydroxybenzotriazole, N,N-diisopropylethylamine and 4-dimethylaminopyridine in N,N-dimethylformamide. Protecting groups were removed in a cocktail of trifluoroacetic acid (10 ml), water (0.5 ml), diethanedithiol (0.25 ml), thioanisol (0.5 ml) and phenol (0.75 g). The peptide was characterized by NMR and MALDI- TOF MS.

Inhibition kinetics. Lethal factor (5 nM final concentration) was premixed with various concentrations of the inhibitor in the assay buffer containing 0.5% DMSO and the reaction was initiated by addition of *p*-nitroanilide peptide substrate (final concentration was 250 M) to measure initial velocities.

Cell culture. Human melanoma UACC-257 cells were obtained from the National Institutes of Health and cultured in F12K medium supplemented with fetal calf serum (FCS), horse serum (HS), glutamine, penicillin and streptomycin. Cells were treated with purified protective antigen (250 ng/ml) and lethal factor (15 ng/ml) in growth medium for 4 d. Blocking of morphological change and pigmentation was observed by addition of the inhibitor (20 M) to the culture medium 30 min before the addition of protective antigen and lethal factor. Controls were treated with equal volumes of DMSO and with protective antigen in the presence or absence of DS-998.

HEK 293 cells were maintained in DMEM containing FCS, glutamine, penicillin and streptomycin. A plasmid encoding HA-MEK1, pCEP4-HA-Mek1, was obtained from Melanie Cobb, Department of Pharmacology, University of Texas Southwestern Medical Center. Transfections were done with Fugene purchased from Roche Applied Science. Transfected cells were further incubated for overexpression of HA-MEK1 for 2 d. Cell cultures were treated with variable concentrations of DS-998 (0–20 M) for 1.5 h and then, protective antigen (200 ng/ml) and lethal factor (200 ng/ml) were added. The cells were lysed after 2 h, and the lysate was denatured before separation on a gel.

J774 cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 containing FCS, glutamine, penicillin and streptomycin. To test the cytotoxicity of DS-998, cells were treated with DS-998 and, after 30 min, the media were replaced with fresh media containing DS-998 at various concentrations. The concentrations of lethal factor required to induce apoptosis were determined by adding mixtures of various concentrations of lethal factor, protective antigen (200 ng/ml) and LPS (100 ng/ml). Dose-dependent inhibition of lethal factor–induced cell death was observed by treating cells with DS-998 at indicated concentrations for 30 min and then, adding second dose of DS-998, lethal factor (40 ng/ml), protective antigen (200 ng/ml) and LPS (100 ng/ml). Cell viabilities were evaluated 4 h after the addition of DS-998 and/or lethal factor, protective antigen and LPS using MTT method for all the above experiments[32]. To determine cell viabilities, 10 l of MTT solution (5 mg/ml, in phosphate buffered saline) was added to 100 l of medium in each well of the 96-well plate. The plate was placed in a cell culture incubator until purple precipitates were clearly visible (approximately 1 h) and then, 100 l of solubilization solution (10% SDS in 0.01 M HCl) was added. The plate was further incubated in the dark overnight at 20 °C and the absorbance in each well was measured at 595 nm in a microtiter plate reader.

Western blot. Proteins from whole cell lysates were separated using 4%–15% Tris-HCl PAGE. The full length HA-MEK1 protein was determined by western blot analysis with an anti-HA antibody. For immunoblotting, proteins were transferred onto a nitrocellulose membrane. Horse radish peroxidase (HRP)-conjugated anti-HA antibody was purchased from Roche and used as recommended.

Z′- factor. Mass spectra of positive controls (24 spectra) and negative controls (24 spectra) were acquired, and for each spectrum, the absolute peak intensity at the *m/z* 2,172 corresponding to cleaved peptide ($[M_4 + H]^+$) was normalized relative to the peak intensity of uncleaved peptide (*m/z* 3107, $[M_2 + H]^+$) using the equation (2). Where peaks cannot be defined, such as a peak of cleaved peptide in negative controls, the baseline intensity was used for normalization. Data processes including calculation of peak intensities and baseline intensities were done using Data Explorer software (version 4.0, Applied Biosystems). The normalized intensities were plotted versus sample numbers (**Fig. 6**) and Z′-factor was calculated using equation (1).

Note: Supplementary information is available on the Nature Biotechnology website.

ACKNOWLEDGMENTS

This work was supported by the National Science Foundation, the Ludwig Fund for Cancer Research and a Burroughs Wellcome Graduate Fellowship to D.-H.M.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Received 29 January; accepted 17 March 2004
Published online at http://www.nature.com/naturebiotechnology/

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