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Yeast-hybrid based high-throughput assay for identification of anthrax lethal factor inhibitors

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ABSTRACT

Inhibitors of anthrax lethal factor (LF) are currently being sought as effective therapeutics for the treatment of anthrax. Here we report a novel screening approach for inhibitors of LF, a yeast-hybrid-based assay system in which the expression of reporter genes from a Gal4 promoter is repressed by LF proteolytic activity. Yeast cells were co-transformed with LF and a chimeric transcription factor that contains an LF substrate sequence inserted between the DNA-binding and activation domains of Gal4. In the resulting yeast cells, LF cleaves the substrate, thus inactivating the chimeric Gal4 and resulting in lack of expression of reporter genes. Compounds that inhibit LF cleavage of its substrate are identified by changes in reporter gene activity. Relative to *in vitro* screens for inhibitors of LF proteolytic activity, this screen has the advantage of excluding compounds that are toxic or non-permeable to eukaryotic cells. Additionally, the screen has the advantage of being fast, easy and cheap because exogenous LF and substrate are not needed. An initial chemical library screen with this system has identified four candidate inhibitors which were confirmed to inhibit LF protease activity in an *in vitro* assay. Furthermore, FBS-00831, one of the compounds identified, protects Raw 264.7 macrophages from anthrax lethal toxin and the possible binding site on LF was also evaluated by molecular docking.

1. Introduction

Anthrax is a zoonotic disease whose etiologic agent is a Gram-positive sporulating bacterium, *Bacillus anthracis* [1,2]. Virulence of this bacterium relies on an antiphagocytic capsular antigen, which is unique among bacterial capsules consisting of poly-D-glutamic acid, and the tripartite anthrax toxin (protective antigen, PA; lethal factor, LF; edema factor, EF). LF is a Zn^{2+}-dependent metalloprotease, which specifically cleaves mitogen-activated protein kinase kinase (MAPKK) family members, leading to macrophage lysis of toxin-challenged cells [3; review within, 4]. Current FDA-approved treatments for anthrax infection involve antibiotics that kill the bacteria, but antibiotics cannot neutralize the toxins already released by the bacteria into the body. Therefore, LF is an attractive therapeutic target.

A wide variety of approaches have been used to identify LF inhibitors including library screening and optimization [5,6], MS-based screening [7], scaffold-based NMR screening [8], and using synthetic peptides as LF substrates [9]. Although synthetic peptide LF-substrates are convenient to screen for LF inhibitors, screens utilizing native MEK1 could identify chemicals with different inhibitory mechanisms because other regions distal to the cleavage site also play important roles in LF-mediated proteolysis [10] and in LF-substrate interaction [11]. High-throughput screening (HTS) assays that reveal the ability of a compound to inhibit cleavage of MAPKK1 could indicate the blockage of toxin internalization or the proteolytic activity of LF. In this respect, cell-based assays are an increasingly attractive alternative to *in vitro* biochemical assays for HTS since LF functions in the cytosol. In addition, cell-based screens can, in principle, eliminate many compounds with undesirable toxicity, biological instability, or poor availability.

Here we report a cell-based assay in *Saccharomyces cerevisiae* (yeast) that is applicable to monitor LF protease activity and to screen for potent inhibitors in a high-throughput format. We screened a compound collection to evaluate this assay and identified putative LF inhibitors followed by molecular docking to identify the LF binding site. The resulting molecular scaffolds can be further used to design therapeutically novel inhibitors of LF.

Abbreviations: LF, lethal factor; PA, protective antigen; LeTx, lethal toxin consisting of PA and LF; DNA-BD, DNA-binding domain; AD, transcription activation domain; cGAL4-LFS, minimal Gal4 transcription factor containing an optimized LF peptide substrate between the core Gal4 DNA-BD and AD; cGAL4-MEK1, minimal Gal4 chimera containing the full-length native LF substrate, MEK1.

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2. Materials and methods

2.1. Construction of LF and LF-substrate expression plasmids

The pGBKT vector was used as a bait expression vector in a yeast-hybrid-based screen. This pGBKT ΔBD vector encodes the *Trp1* gene, which acts as a selectable marker gene in tryptophan deficient medium. The LF gene was then subcloned into this vector (pGBKT ΔBD/LF). To prepare LF-substrate expression plasmids, the Gal4 DNA-BD was first subcloned into a pGADT 7 (LEU2, a selection marker on leucine deficient medium) vector at the C-terminus of the Gal4 transactivation domain. The genes encoding LF substrates, either peptide substrate LFS or full-length native substrate MEK1, were added between the Gal4 transcription activation domain (AD) and the Gal4 DNA-BD. The peptide substrate LFS consists of 11 amino acids, RRKKVYPYPME, which is an optimized LF cleavable sequence with a cleavage rate approximately 100-fold higher than the native substrate (MEK2) [5]. This peptide is linked with the Gal4 transactivation domain and DNA-BD using glycine linkers. Another LF-substrate expression vector contains full-length rat MEK1, of which Lys97 was replaced with Arg to inactive kinase activity.

2.2. Yeast strains and manipulation

Yeast strain AH109, consists of the following genotype: *MAT*a, two selectable markers (deletion of trp1-901 and the double mutation of leu2-3, 112), and four reporter genes ($GAL1_{UAS}$-$GAL1_{TATA}$-HIS3, $GAL2_{UAS}$-$GAL2_{TATA}$-ADE2, $MEL1_{UAS}$-$MEL1_{TATA}$-lacZ, and $MEL1_{UAS}$-$MEL1_{TATA}$-MEL1). Yeast cells were maintained on YPDA medium containing 1% yeast extract, 2% peptone, 2% glucose, and 0.003% adenine hemisulfate. Synthetic dropout (SD) media are designated by the missing nutrient components (e.g., SD/-Trp, -Leu, -His, and -Ade medium that lacks tryptophan, leucine, histidine, and adenine, respectively). Growth and transformation of yeast were accompanied by standard yeast experimental techniques [12].

2.3. Establishment of yeast cell lines co-expressing LF and its substrate

The plasmid pGBKT ΔBD/LF was transformed into yeast cells, and cells harbouring this plasmid were selected on SD/-trp agar plates. Cells were then transformed with either LF-substrate expression vectors (pGADT 7/cGal4-LFS or pGADT 7/cGal4-MEK1) or control vectors (minimal cGal4 transcription factor consisting of the Gal4 AD and DNA-BD, pGADT 7/cGal4, or wild-type full-length Gal4 transcription factor-expression vector, pCL1 [13]). Transformants were maintained on SD/-Trp or SD/-Leu medium.

2.4. Chemical library screening

Screening plates were prepared as follows. Sterile, molten SD medium (SD agar/-Trp, -Leu, -His, and -Ade) cooled to 55 °C was added to 96-well plates containing a unique chemical compound in each well (~6500 represented scaffolds, Korea Research Institute of Chemical Technology) or an equal volume of DMSO to a total volume of 300 μl. Plates were incubated at room temperature with gentle shaking for 30 min. Then, cells co-expressing LF and cGal4/MEK1 were inoculated at a density of 3×10^4 cells in a chemical library plate and incubated at 37 °C for 48 h in a humidified incubator. Cells grown in the nutrient deficient medium containing chemical compounds were selected by comparing with cells expressing LF/cGal4-wt (LF/minimal cGal4 transcription factor consisting DNA-BD and AD) and LF/cGal4-MEK1 in the same minimal nutrient medium without chemical compounds. Selected cells were transferred onto a filter membrane and further analyzed for β-galactosidase activity to confirm the inhibition of LF-mediated cleavage of cGal4-MEK1. Briefly, filter membranes were frozen and thawed several times to lyse the cells and the membrane was placed on the filter paper containing the β-galactosidase reaction mixture (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, pH 7.0, 10 mM KCl, 1 mM $MgSO_4$, 3.8 mM β-ME, and 0.82 mM X-gal [5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside]). To quantify β-galactosidase activity, cells were inoculated in the minimal nutrient medium containing the same chemical compound and grown for 40 h. The cells were harvested by centrifugation and lysed by repeated freeze–thaw cycles. Cell free extract was added into the β-galactosidase reaction mixture and incubated at 30 °C for 3 h. The activity was then measured by recording optical density at 620 nm. The inhibition potency of hit compounds on cultured macrophage, Raw 264.7 was determined as reported previously [14].

2.5. In vitro assay of LF activity

The expression, purification and *in vitro* assay of LF was carried out as reported previously [14,15]. See Supplementary material for details.

2.6. Molecular docking

The hit compound, FBS-00831 was docked onto LF by using AutoDock 4.0 [16,17]. See Supplementary material for details.

3. Results

3.1. A cell-based protease assay to monitor LF activity

A cell-based LF activity assay system was developed in high-throughput format (Fig. 1) that exploits a yeast-hybrid-based assay [18]. In this assay, the proteolytic cleavage of LFS or MEK1 by LF cleaves the Gal4 transcription factor chimera to dissociate two functional domains, resulting in the inactivation of cGal4. Inactivation of cGal4 inhibits the expression of four reporter genes which have been placed under the control of the chimeric transcription factor in the genetically modified yeast strain AH109 (Fig. 1). The expression of this set of reporter genes produces a visible readout of cell growth on minimal medium (*HIS3* and *ADE2*) and of color development on X-gal reagent (*LacZ* and *MEL1*). As a control, the co-expression of LF and cGal4-LF substrate (LF/cGal4-LF substrate i.e. LF/cGal4-LFS and LF/cGal4-MEK1) in yeast cells significantly reduced cell growth in minimal medium (SD/-Trp, -Leu, -His, -Ade) (Fig. 2A). However, LF/cGal4-wt expressing cells grow normally in the same minimal medium and cells reached stationary phase following 42 h of incubation. The full-length substrate chimera (cGal4-MEK1) and a short peptide-substrate chimera (cGal4-LFS) led to reduced yeast growth. However, after 40 h of incubation, cells expressing cGal4-LFS and cGal4-MEK1 grow to approximately 50% and 20% of cGal4-wt cells, respectively.

cGal4-MEK1 was cleaved more efficiently by LF than cGal4-LFS within cells (Fig 2B) which suggests that MEK1 forms a more stable enzyme–substrate complex compared to LFS. All further experiments were therefore carried using cells co-expressing LF/cGal4-MEK1 as an assay model system. LF/cGal4-MEK1 expressing cells showed growth inhibition on solid culture medium as well as decreased β-galactosidase activity (Fig. 2C). These results indicate that the cGal4-MEK1 based assay system is suitable to monitor LF activity in yeast strain AH109. The reliability of the assay method was validated by measuring assay variation for cell growth and β-galactosidase activity. Cell growth and β-galactosidase activity in LF/cGal4-MEK1 expressing cells were significantly reduced com-

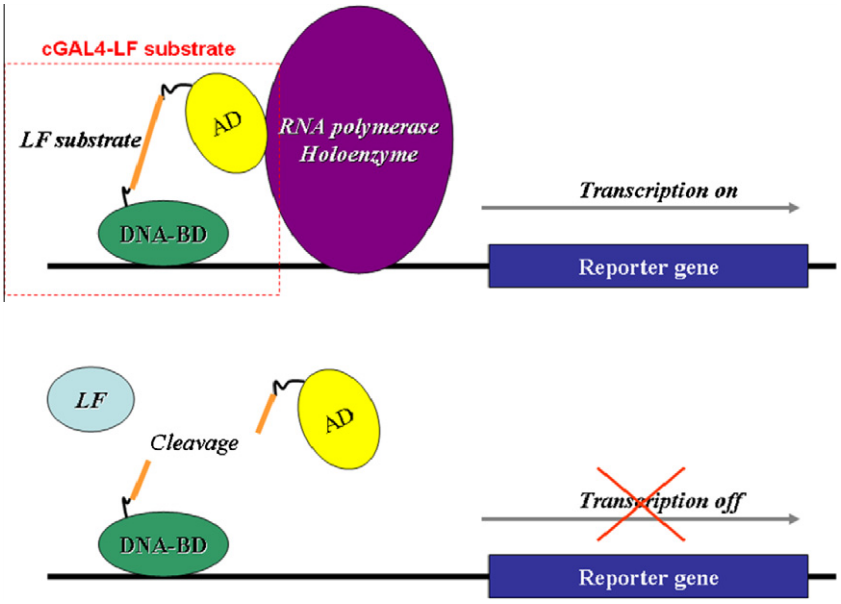

Fig. 1. Schematic diagram of the LF activity assay system that relies on the yeast-hybrid assay. LF activity is monitored by a cleavage of the LF-substrate chimera (cGal4). This cleavage turns off the reporter gene expression, which inhibits yeast cell growth under minimal nutrient conditions.

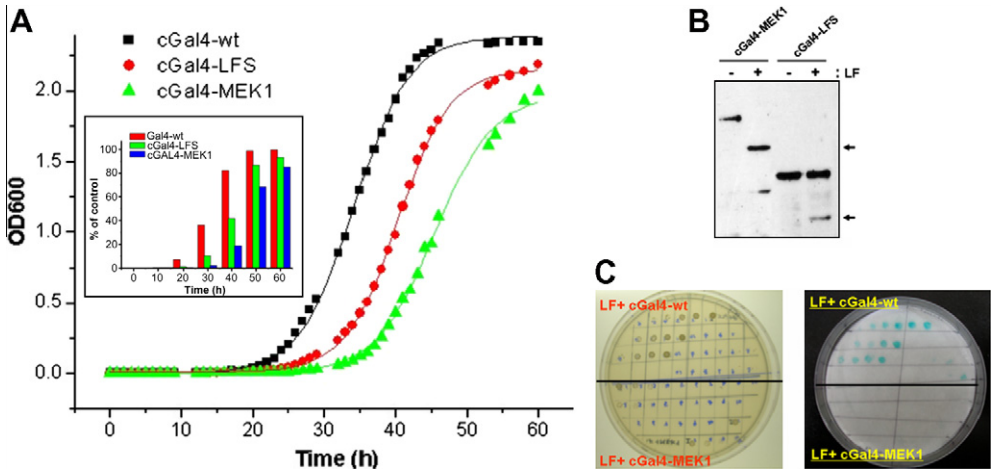

Fig. 2. A cell-based protease assay to monitor anthrax lethal factor activity. (A) The growth curves of yeast cells expressing LF and cGal4-wt or cGal4-LF-substrate chimeras. Inset picture shows the growth measured at various time intervals in cGal4-LF-substrate chimeras expressing cells normalized with respect to the growth measured in cGal4-wt expressing cells. (B) Cleavage of LF substrate in yeast cells co-expressing LF/cGal4-LF substrate. Cells were harvested using culture medium without leucine (cGal4-LFS and cGal4-MEK1) or tryptophan and leucine (cells co-expressing LF/cGal4-LF substrate) for 48 h. Yeast protein extracts were prepared in the cracking buffer containing 8 M urea, and 5% (w/v) SDS. The cleavage of the cGal4 chimera containing LF-substrate was determined by immunoblotting using an anti-Gal4 DNA-BD antibody. LF cleaves the very N-terminus (Ile8-Pro9) of the MEK1 protein, therefore, cleavage of cGal4-MEK1 (80.0 kDa) generates a Gal4-AD free protein (61.2 kDa) consisting of N-terminal eight amino acid missing MEK1 and Gal4 DNA-BD. The cleavage of cGal4-LFS (38.8 kDa) results in 20 kDa fragment including some remaining peptide substrate and the Gal4 DNA-BD. The arrows indicate the cleavage products. (C) Growth (left) and β-galactosidase activity (right) of cells expressing LF with cGal4-MEK1 or cGal4-wt on solid assay culture medium (SD agar/-Trp, -Leu, -His, -Ade) after 40 h. 4×10^4 cells (2 μl of cultures with 2×10^7 cells/ml cell density) were placed on the first spot and the serial five-fold dilutions were placed on the next spot. The data are representative of at least three-independent experiments.

pared to LF/cGal4-wt expressing cells (Table 1). Thirty independent cultures for each condition were used to determine the Z′-factor, an indication of assay robustness. Z′-factors for β-galactosidase activity in cGal4-LF substrate assay system was 0.773. See the Supplementary material for Z′-factor calculation details.

3.2. Inhibitor screening

LF/cGal4-MEK1 expressing cells displayed reduced cell growth inhibition in the presence of LF protease inhibitors (Fig. 3A). Initially, various known LF-inhibitors (In-2-LF [19], GM6001 [5] or *O*-phenanthroline) were tested for their ability to restore cell growth on minimal nutrient medium. Cell growth was reduced by only 24% and 21% in the presence of In-2-LF (25 μg/ml) and GM6001 (125 μM), respectively, when compared to cell growth in the absence of any LF inhibitor (72%). However, 10 mM *O*-phenanthroline reduced growth of both LF/cGal4-wt and -MEK1 expressing cells by >95% even though this concentration was previously reported to inhibit LF activity [20]. Interestingly, DMSO (5%) alone reduced cell growth by 20–30% in both types of chimera expressing cells. Therefore, the negative effect of DMSO on cell growth was considered during the chemical library screening as

Table 1
Robustness of cGal4-LF substrate transcription factor cleavage assay in LF expressing cells.

Cell line	Cell growth on the selection medium (OD_{600})	β-galactosidase activity[a] (Units)
LF and cGal4-wt	1.608 ± 0.088	0.895 ± 0.062
LF and cGal4-MEK1	0.033 ± 0.018	0.010 ± 0.005

[a] Data were analyzed by unpaired *t*-test. The two-tailed *P* value is <0.0001.

most of the inhibitors were dissolved in it. During inhibitor screening, the final concentration of each chemical was <5% (v/v) of the culture medium.

Following validation, a chemical library that represents 6500 scaffold molecules was subsequently used to screen to discover LF inhibitors. Four hit compounds (CBB-0348, CBT-0052, FBS-00831, and FBS-08879) were identified as LF inhibitors with novel chemical backbones (Fig. 3B). All hit compounds inhibited LF protease activity towards GST-MEK1 *in vitro* (Table 2) and did not inhibit other protease activities including thrombin (a serine protease) and botulinum neurotoxin (a Zn^{2+} dependent protease) (data not shown). Interestingly, the LF inhibition potential of FBS-00831 is comparable to the previously described LF inhibitor GM6001 [21] whereas other three hits showed ~6–9 folds higher inhibition potential (Table 2). Furthermore, FBS-00831 protected Raw 264.7 cells by LeTx challenge [21] without any significant cytotoxicity (*Supplementary* Fig. 1) however; the IC_{50} of FBS-00831 in the cell protection assay is 10-fold higher than that of GM6001 (Table 2).

3.3. Molecular docking of FBS-00831

FBS-00831 was selected for docking analysis due to its high LF inhibition potential. Fig. 4 depicts the clustering of the bound conformations and the best clustered positions of FBS-00831 with the X-ray crystal structure of LF (PDB ID: 1J7N (4)). The amplitude of the histogram reflects the number of conformations in a particular cluster, while the dissociation constant (K_D) reflects the highest-affinity binding mode in a given cluster. The histogram of FBS-00831 suggests the two best conformational clusters with a calculated K_D of 6 μM and 13 μM (Fig. 4A). The positions of FBS-00831 highest affinity and best clustered binding modes are shown in the LF crystal structure (Fig. 4B). Some additional weak binding conformations were found that showed relatively poor clustering. Molecular docking analysis showed that the FBS-00831-1 cluster (one in Fig. 4B) binds to LF domain II and lies near the PA-binding domain I. However, the FBS-00831-2 cluster (two in Fig. 4B) binds to LF domain III, which is close to the substrate-binding site.

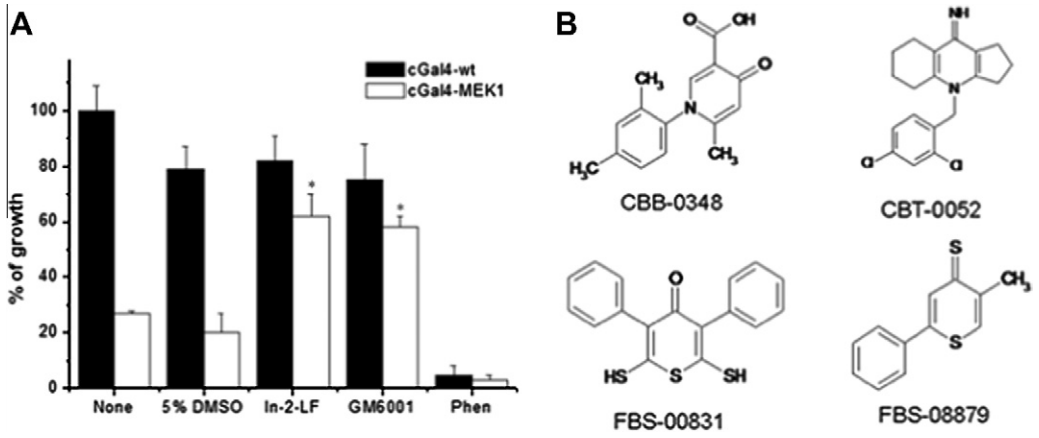

Fig. 3. Yeast growth validation and identification of new hit compounds. (A) Yeast growth in minimal nutrient medium containing LF-inhibitors. Percentage of growth is normalized using cells expressing LF and cGal4-wt as control in a medium without inhibitor. The data (mean ± SD) are obtained from five-independent experiments for each condition. $^{*}P < 0.001$. (B) Structures of the hit compounds identified in the screening.

Table 2
LF inhibitor compounds identified from high-throughput screening.

Chemical ID#[a]	β-galactosidase activity[b] (%)	IC_{50} for *in vitro* assay[c] (mM)	IC_{50} for LeTx[d] (mM)
GM6001[e]	100	0.04 ± 0.008	0.06 ± 0.004
CBB-0348	68 ± 8*	0.28 ± 0.068*	N.D.
CBT-0052	58 ± 4**	0.34 ± 0.071**	N.D.
FBS-00831	85 ± 3	0.1 ± 0.012[f]	0.6 ± 0.009
FBS-08879	73 ± 7	0.25 ± 0.083$	N.D.

[a] Chemical ID is shown as original chemical library indications of KRICT.
[b] β-galactosidase activity of yeast cells grown with GM6001 was used as control (100%) to compare the inhibition potency of the hit compounds. The cells expressing LF/cGal4-MEK1 were grown in the nutrient deficient medium in the presence of 50 μM GM6001, or 100 μM of the indicated chemical compound for 40 h.
[c] IC_{50} values were determined using an *in vitro* MEK cleavage assay. Assay method is described in "Supplementary material". Each value is obtained using 50% cleavage of GST-MEK1 on a densitometric analysis.
[d] IC_{50} value indicates 50% of inhibitor concentration required to protect macrophage-like cell line (Raw 264.7) from LeTx (200 ng/ml of each PA and LF) treatment. Before the toxin challenge, inhibitor was added to the culture medium. Cell viability was measured the following day using the MTT method.
[e] The β-galactosidase activity is significantly lower and IC_{50} value is significantly higher than in the presence of FBS-00831 by $^{*}P < 0.05$ and $^{**}P < 0.01$. $^{\$}IC_{50}$ value is significantly higher than in the presence of GM6001 by $P < 0.01$.
[f] The *in vitro* IC_{50} of FBS-00831 was obtained from the previous reports [21]. Data (mean ± SD) shown in this table are obtained from three-independent experiments and analyzed by one-way analysis of variance (ANOVA) with a Tukey–Kramer multiple comparisons test. N.D. not determined.

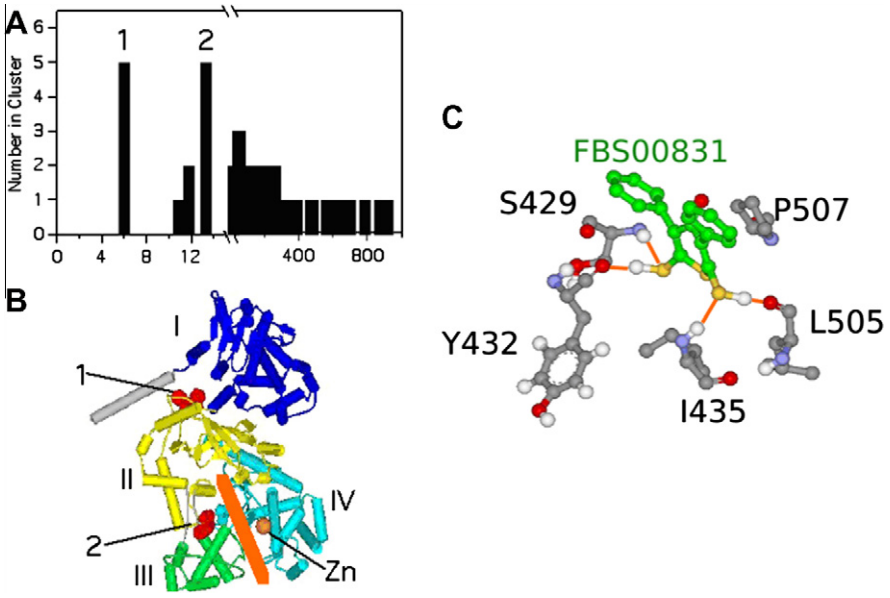

Fig. 4. Clustering of bound states and tightest bound state of LF-FBS-00831. (A) A histogram of clustered conformations of FBS-00831 with the predominant binding modes denoted with arabic numerals. (B) LF structure in the presence of the most predominant binding modes for FBS-00831 with their corresponding arabic numerals. The domains are colored and denoted with roman numerals (I-IV). Zn^{2+} and MEK2 binding sites are shown as a pink sphere and orange line, respectively. (C) Highest affinity conformational binding mode of FBS-00831 with the surrounding residues. The carbon atoms of protein residues and FBS-00831 are colored gray and green, respectively. The oxygen, nitrogen, sulfur, and hydrogen atoms are colored red, blue, yellow, and white, respectively. Hydrogen bonds are colored orange, while potential hydrogen bonds or electrostatic interactions are shown as dotted lines. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper).

The binding mode was analyzed using the residues of the highest-affinity binding conformations of LF-FBS-00831 (Fig. 4C). FBS-00831 interacts with S429, Y432, I435, L505, and P507 of LF. The residues S429, Y432, and I435 from the 2α1-2β1 loop region form one side of the inhibitor-binding pocket while the other side is formed by L505 and P507 of the 2β4-2β5 loop region. Thiols of FBS-00831 are anchored to the binding site by hydrogen bonds formed with four residues of LF. The backbone carbonyl of Y432 and L505 form hydrogen bonds with the thiol proton with distances of 2.1 Å and 2.0 Å, respectively. Additional hydrogen bonds are observed between the thiol sulfurs and the backbone amide protons of S429 and I435 with distances of 2.6 Å and 2.5 Å, respectively. Furthermore, P507 stacks up against the thionone parent functional group.

4. Discussion

The yeast-hybrid-based assay reported here relies on a site-specific cleavage of the chimeric yeast Gal4 transcription factor (cGal4) that contains an LF-substrate (LFS, an optimized LF-substrate peptide [5] or MEK1, a cellular substrate of LF [22]) inserted between the transactivation domain (AD) and the DNA binding domain (DNA-BD) of Gal4. The 3D-structure of the LF active site-peptide complex [11] and an LF domain analysis study [10] proposed that the LF-interacting region (LFIR) in the C-terminal kinase domain of MEK1 is important for LF-mediated proteolysis of MEK1. In addition, basic residues clustered on the LF-substrate are believed to play an important role in LF-substrate interaction. In this study, the low cleavage efficiency of the peptide-substrate chimera may be a result of the strong positive charge on the Gal4 DNA-BD near the short peptide substrate fragment which can disturb the charges and proximal interactions between LF and substrate (LF/cGal4-LFS, see cleavage products in Fig. 2B).

The Z′ factor ranges from one (perfect assay) to zero and generally displays a value of 0.75 or higher for high performance assays [23]. The Z′ factor reported here (0.773) is comparable to other established assays [7]. Notably, the β-galactosidase activity was more stable than the cell growth assay. However, this difference can be explained by the promoter activities of cGal4 controlled-reporter genes. In yeast AH109 cells, cGal4-controlled-reporter genes have different promoter activities in the following order: GAL1 UAS/TATA (HIS3) > GAL2 UAS/TATA (ADE2) > MEL1 UAS/TATA (LacZ and MEL1). Therefore, incomplete proteolysis of cGal4-MEK1 can induce HIS3 and ADE2 gene expression for cell growth on the selection medium to some extent. However, this small amount of non-cleaved cGal4-MEK1 product is not sufficient to drive LacZ gene expression because of its weak promoter activity (a high K_D value for cGal4-MEK1).

The inhibition of both LF/cGal4-wt and -MEK1 expressing cells in the presence of *O*-phenanthroline indicates that this yeast hybrid-based assay system could eliminate highly toxic compounds from the chemical library screen, which is not surprising considering the cytotoxicity of subjecting cells to a high concentration of this metal chelator. Four hit compounds were identified in this screen which inhibited LF activity and allowed yeast cells to grow in the nutrient deficient medium, but this inhibitory effect is not manifested in mammalian cells. While 100 μM FBS-00831 blocked LF activity in yeast cells as determined by β-galactosidase activity, its IC_{50} value for the mammalian cell protection assay against LeTx is 0.6 mM (Table 2). Mammalian cell protection assay results suggest that the differences between yeast and mammalian cells, such as permeability, metabolism, and detoxification, requires additional efforts to ensure identified chemical hits function as LF inhibitors within mammalian cells. Overall, these results suggest that FBS-00831 can interfere with LF-PA interactions to inhibit toxin formation as well as interfere with LF-substrate interaction. Considering that most of the current LF inhibitors interact with the LF active site (domain III and IV) to inhibit LF proteolytic activity [24], and reviewed in [25], FBS-00831 has a potential advantage over other inhibitors due to its dual function. The flanking phenyl groups of FBS-00831 appear to essentially stick out into the bulk aqueous solvent toward LF domain I. These residues might interfere with PA binding by interacting with LF domain I, while the thiol groups serve as anchors to the LF protein.

In summary, this study describes a novel LF inhibitor screening system in a high-throughput format. Despite these concerns like

differences between yeast and mammalian cells, the screening system reported here has an advantage over existing animal cell-based screens [26,27] in that the hits are specific for LF activity inhibition rather than blocking entry or altering host response to toxin action. Therefore, target identification appears to be straight forward to obtain LF inhibitors. Although library screening has identified a novel hit compound with comparable *in vitro* inhibition potency to GM6001, this compound requires further modification to use as an effective LF inhibitor in humans.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.12.015.

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