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Discovery of a Small Molecule that Inhibits the Interaction of Anthrax Edema Factor with Its Cellular Activator, Calmodulin

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Summary

The catalytic efficiency of adenylyl cyclase activity of edema factor (EF) from *Bacillus anthracis* is enhanced by approximately 1000-fold upon its binding to mammalian protein calmodulin (CaM). A tandem cell-based and protein binding-based screen of a 10,000 member library identified a molecule that inhibits the EF-CaM interaction and therefore the adenylyl cyclase activity. A combination of fluorescence spectroscopy and photolabeling studies showed that the molecule targets the CaM binding region of EF. A series of related compounds were synthesized and evaluated to identify one compound, 4-[4-(4-nitrophenyl)-thiazolylamino]-benzenesulfonamide, that maintained activity against EF but showed minimal toxicity to two cultured cell lines. This compound represents an important reagent to study the role of EF in anthrax pathology and may represent a drug lead against anthrax infection.

Introduction

The association of proteins plays a role in all cellular processes. Protein-protein interactions serve to organize and maintain the cytoskeletal structure in the cell, modulate enzymatic activities, and localize enzymes and substrates at discrete positions in the cell. For these reasons, the discovery of small molecules that inhibit protein-protein interactions is a goal of many research programs that seek inhibitors for use as reagents in cell biology and as therapeutics in medicine. This paper reports a strategy that combines cell-based screening with protein interaction screening to identify a small molecule that inhibits the activation of edema factor by calmodulin in cells.

The identification, or design, of small molecules that inhibit protein-protein interactions is substantially more difficult than is the development of inhibitors targeting enzyme active sites or ligand binding pockets [1]. For the latter case, the availability of natural ligands and substrates provides a good starting point for developing inhibitors. Furthermore, active sites of enzymes and ligand binding pockets of receptors are intrinsically matched for binding of small molecules, which can fill these binding pockets and make extensive contacts to the protein. The development of small molecules that inhibit protein-protein complexes, by contrast, does not benefit from previously characterized "lead" compounds. Further, the relatively flat surface of a protein does not provide the three-dimensional pockets that are believed to be important for high affinity and selective binding of small molecules. In practice, the small number of inhibitors of protein-protein interactions (IPPIs) have been discovered through screening of diverse chemical libraries. Recent examples of IPPIs have served as important reagents for dissecting complex cellular processes [2–6].

The interaction between *Bacillus anthracis* EF and CaM provides an appealing target for the development of an IPPI. *B. anthracis*, or anthrax, secretes several proteins relevant to the anthrax pathology. EF is a secreted protein that possesses Ca^{2+}/CaM-dependent adenylyl cyclase activity. Lethal factor (LF) is a metalloprotease, and protective antigen (PA) helps the delivery of EF and LF into target cells. According to currently accepted models (for a review, see [7]), secreted PA first binds to a cellular anthrax receptor and undergoes proteolysis by furin [8]. The proteolyzed product of PA oligomerizes on the cell surface, providing binding sites for EF and LF. Following receptor-mediated endocytosis, conformational changes of PA induce the release of EF and LF into the cytosol, where EF forms a complex with cellular CaM. This EF-CaM complex catalyzes the conversion of ATP to a second messenger, cAMP. The complex of EF-CaM is approximately 1000-fold more active than is EF alone [9, 10], providing a strategy to activate the adenylyl cyclase activity in the host cell. Therefore, by interrupting the EF-CaM interaction with a small molecule, the pathological effects of EF may be reduced significantly.

The recently solved crystal structure of the EF-CaM complex reveals that the protein-protein interaction is quite distinct from other CaM binding proteins [9, 11]. The structures of several complexes of CaM with CaM binding proteins reveal that the central α helix of CaM is kinked and wrapped around the α-helical CaM binding domains [11]. However, in the structure of the EF-CaM complex, the central α helix of CaM adopts an extended conformation surrounded by two domains of EF [9, 10]. For this reason, a small molecule that binds to the CaM binding region of EF may not interfere with the many other important roles of CaM in cells.

To identify a small molecule that inhibits the EF-CaM interaction, we screened a library containing 10,000 diverse structures using a combination of a cell-based assay and a surface plasmon resonance (SPR) spectroscopy-based protein-protein binding assay. This screen identified one compound as an inhibitor of the EF-CaM complex. In vitro assays showed that the compound targets EF and inhibits the catalytic activity of EF by disrupting the EF-CaM interaction. This initial hit was toxic to cultured mammalian cells at concentrations above the IC_{50}. We therefore synthesized a series of

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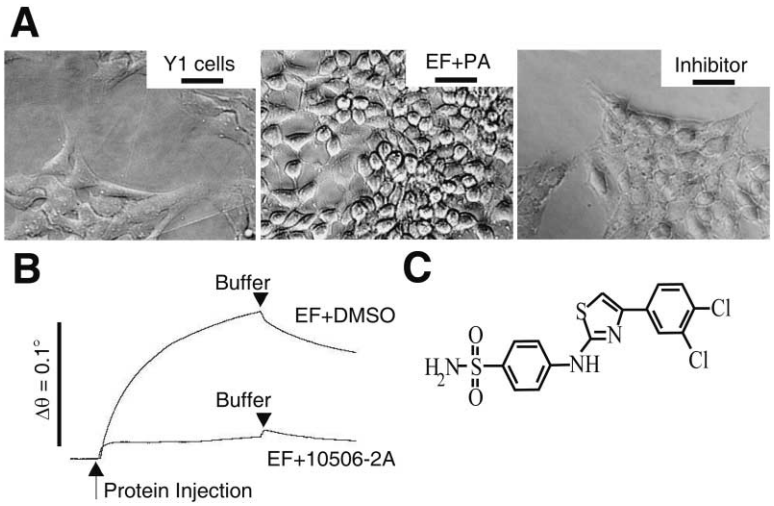

Figure 1. Identification of an Inhibitor that Blocks the Interaction of EF and CaM

(A) A cell-based assay reports on ET-induced morphological changes in Y1 cells. Cells were treated with control medium (left), ET (EF [5 ng/ml]), and PA (25 ng/ml) (middle) or ET+10506-2A (EF [5 ng/ml]), PA (25 ng/ml) and 10506-2A (10 μM) (right).

(B) SPR assays were used to identify a compound, 10506-2A, which acts by inhibiting the binding of EF and CaM. EF (0.8 μM) was mixed with 10506-2A (1% DMSO) and allowed to interact with cutinase-CaM immobilized on the surface for 15 min.

(C) Chemical structure of 10506-2A.

structurally related compounds and identified one that retains inhibitory activity, but with a reduced toxicity to cultured mammalian cell lines.

Results

Screening Strategy

To identify candidate inhibitors of EF, we first screened a library of molecules in a cell-based assay. We started with this assay because our ultimate goal was to identify compounds that were active in cell culture. The assay reports on a cAMP-triggered morphological change in Y1 cells, a cultured line of murine adrenocortical cells (Figure 1A) [12]. The addition of edema toxin (ET; a combination of EF and PA) to Y1 cells leads to an increase in the level of intracellular cAMP, with downstream effects that include the dephosphorylation of paxillin and a loss of focal adhesions [13], leading to a distinct morphological change in cells from flat/spread to round (Figure 1A). Compounds that blocked the EF-induced morphology changes in Y1 cells were then evaluated in a surface plasmon resonance (SPR) spectroscopy-based assay to identify the subset of compounds that act by preventing the association of EF and CaM [14, 15].

Identification of an Inhibitor of the EF-CaM Interaction

We first employed the Y1 cell-based assay to screen the 10,000 compound library in pools of eight in duplicate. We found that approximately 20% of the pools showed precipitation, visible cell death, or unrelated morphological changes in the absence of toxins: compounds in these pools were not analyzed in subsequent experiments. These pools may contain a few potentially active compounds, but because we identified enough active compounds as described below, we decided not to retest compounds in the precipitated pools. We found that approximately 30 pools of compounds inhibited the morphological changes induced by ET. We individually screened each of the 240 molecules in the active pools and identified 27 compounds as initial hits (see Supplemental Data). It has been reported that some compounds identified as IPPIs in cell-based assays act non-specifically by interacting with large numbers of proteins [16]. To exclude compounds that inhibit EF by nonspecific mechanisms, we tested each of these compounds for inhibition of two unrelated enzymes: β-lactamase and α-chymotrypsin. Three of the twenty-seven compounds inhibited these unrelated enzymes at a low micromolar range and were excluded from further study.

The remaining 24 compounds were then individually tested using an SPR-based assay to identify molecules that act by blocking the association of EF with immobilized CaM (Figure 1B). We have previously developed a cutinase-directed method for covalent immobilization of functional proteins on self-assembled monolayers [15] and showed that this strategy is useful for quantitative analysis of the EF-CaM interaction [14]. This method allows immobilization of the CaM protein with a defined density and orientation, and provides a higher activity of the protein than do other immobilization methods that result in random attachment and denaturation of proteins [17]. Furthermore, the monolayers present oligo (ethylene glycol) groups that reduce the nonspecific adsorption of EF [18]. Of the 24 compounds identified above, we found that 1 compound (4-[4-(3,4-dichlorophenyl)-thiazolylamino]-benzenesulfonamide) (10506-2A; Figure 1C) efficiently inhibited the binding of EF to immobilized CaM in a dose-dependent manner with an IC_{50} of 10 μM (Figure 2A).

We next confirmed that 10506-2A inhibits the adenylyl cyclase activity of EF3, a recombinant protein containing the catalytic domain of EF, in the presence of CaM (0.5 μM). A dose-dependence study revealed that 10506-2A inhibits the activity of EF with an IC_{50} of 10–15 μM. This value agrees with the activity found in the SPR assays of protein-protein binding (Figure 2B). Finally, dose-dependent assays with cultured Y1 cells showed that 10506-2A inhibits the ET-induced morphological change at concentrations of 15 μM or higher (Figure 2C). Taken together, these results establish that the catalytic activity of EF can be inhibited by blocking its interaction with CaM.

10506-2A Binds EF to Prevent the Activation of EF by CaM

To gain insight into the mechanism by which 10506-2A disrupts the interaction of EF and CaM, we examined

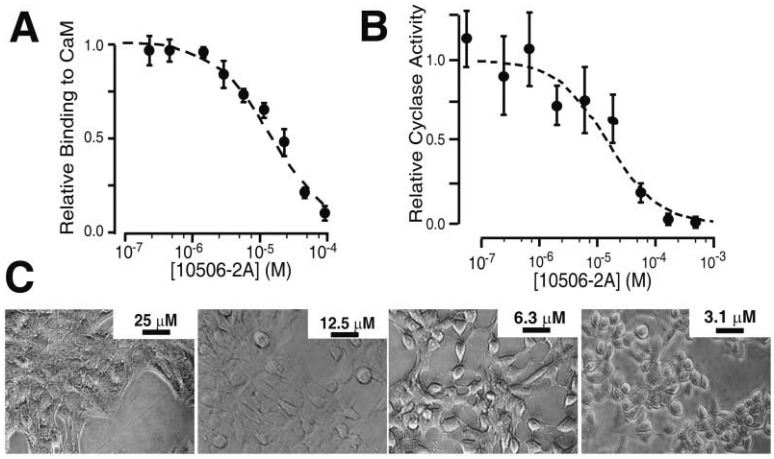

Figure 2. Quantitative Analysis of EF Inhibition by 10506-2A

(A) Dose-dependent inhibition of the binding of EF to immobilized CaM was determined by SPR experiments. The amount of EF that bound to the immobilized CaM is plotted for several concentrations of 10506-2A, where each measurement is the average of three determinations and the standard deviation is given by the error bar.

(B) Dose-dependent inhibition of adenylyl cyclase activity of EF was determined in a cAMP assay. Each data point represents an average of ten independent experiments.

(C) Morphology changes in Y1 cells treated with EF and PA in the presence of 10506-2A are shown. Bars represent 30 μm.

the enzymatic activity of EF at several concentrations of CaM and 10506-2A (Figure 3A). The concentration of CaM that gave one-half the maximum activity of EF (the EC_{50}) was approximately 100 nM. This value is slightly higher than the reported dissociation constant (K_d) of EF and CaM in the absence of DMSO [14]. In the presence of 10506-2A, however, the EC_{50} for CaM increases. For an experiment having 10506-2A present at a concentration of 50 μM, the EC_{50} of CaM increased to 10 μM. This finding is consistent with a model wherein 10506-2A and CaM compete for binding to EF, further supporting the interpretation that 10506-2A is an IPPI.

To obtain direct physical evidence for an interaction of 10506-2A with EF, we employed fluorescence experiments to probe the binding of the molecule with EF and CaM. The inhibitor has an excitation resonance at 328 nm with an associated emission at 405 nm. We acquired fluorescence spectra of the compound in solutions containing EF in concentrations ranging from 0.1 to 100 μM. The emission band showed increasing intensity and a hypsochromic shift with increasing concentrations of EF [19]. Identical experiments with addition of CaM to 10506-2A, by contrast, did not produce significant changes in the fluorescence spectrum of 10506-2A. This experiment provides further support for the interpretation that 10506-2A binds to EF and not to CaM (Figure 3B).

We next performed a photolabeling experiment with an azidophenyl analog of 10506-2A (Figure 4A) to identify the binding site of the molecule on EF. A mixture of the photolabeling analog (50 μM) and EF (10 μM) was irradiated with UV light (254 nm, 300 $\mu W/cm^2$) for 5 min. A MALDI-TOF mass spectrum of the EF protein showed substantial broadening after the irradiation, with an increase in the apparent average mass of 200–400 Da (Figure 4A). Analogous experiments with CaM, by contrast, showed no significant mass changes on irradiation of the protein. Addition of excess 10506-2A prevented the photocoupling of EF by the azidophenyl analog (data not shown). We also used SPR experiments to verify that the azido-photolabeling analog also inhibits the binding of EF to immobilized CaM with a comparable IC_{50} to the parent inhibitor (data not shown).

To define the binding site, we purified the photolabeled EF adduct with HPLC and subjected identical pools of the adduct to protease treatments with trypsin, α-chymotrypsin, or Arg-C. The resulting proteolytic fragments were analyzed by MALDI-TOF mass spectrometry (Figure 4B). In each of the enzymatic digestions, 70%–80% of the primary sequence of EF was represented in the peptide fragments. By using three different digestions, 100% of the primary sequence was represented. We observed a correlation between several peaks that were present at lower intensity in the digests of photolabeled protein and new peaks showing an increase of mass of 344 ± 2 or 366 ± 2 Da. These mass differences can be attributed to coupling of the analog and its sodium adduct, respectively, to EF. Analysis of the exact masses of the peptide fragments revealed the sites of photolabeling of the protein with the small molecule. For tryptic digestions, peptides 631–651, 614–630, 635–651, 654–672, 673–690, and 740–755 formed photoadducts. For ArgC digestions, peptides 631–672 and 631–671 formed adducts, and for α-chymotryptic digestions, peptides 751–777 and 752–773 formed adducts. When these peptides were overlaid on the three-dimensional structures of EF alone and of the EF-CaM complex, all of the identified peptides mapped to the C-terminal helical domain and the interface between the catalytic core of EF and the helical domain (Figure 4C). Each of the peptides was sufficiently proximal to the CaM binding site to perturb CaM binding to EF. In addition to the peptides noted above, several peptides (aa 66–88, 106–137, 215–237, and 171–192) corresponding to the PA binding domain of EF also gave photoadducts with the small molecule.

Specificity of 10506-2A

To ensure that 10506-2A does not inhibit other CaM-interacting proteins, we tested the effects of 10506-2A on two mammalian CaM-dependent proteins and on a secreted bacterial protein. Calcineurin, a mammalian Ser/Thr phosphatase [20], is regulated by binding to CaM. We found that this interaction was not inhibited by 10506-2A in the SPR assays (Figure 5A). Mammalian adenylyl cyclase 1 (mACI) is a transmembrane enzyme regulated by CaM [21]. Adenylyl cyclase assays done in the presence of 10506-2A showed that the inhibitor did not have a significant effect on mACI (Figure 5B). However, CyaA, a CaM-activated adenylyl cyclase se-

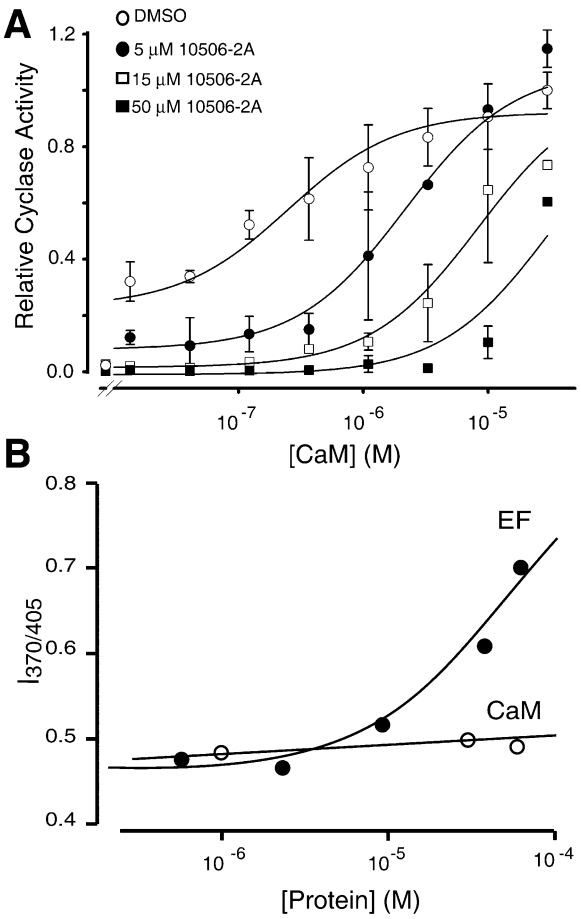

Figure 3. Mechanism of Inhibition

(A) Activation of EF by CaM is inhibited by 10506-2A. EF (1 nM) was mixed with variable amounts of 10506-2A (1% DMSO) and CaM as noted in the figure. The formation of cAMP in vitro was monitored as described in the Experimental Procedures. Each data point represents an average of at least three independent experiments.

(B) Fluorescence titration of 10506-2A in the presence of EF3 or CaM. To determine the molecular target of 10506-2A, fluorescence emission spectral changes of 10506-2A in the presence of EF or CaM were monitored. Intrinsic fluorescence emission spectra of 10506-2A shifts from 370 to 405 nm in the presence of EF, but not in the presence of CaM. Each data point represents an average of at least three independent experiments.

creted by *B. pertussis*, was inhibited by 10506-2A. CyaA has a catalytic domain similar to EF and was reported to interact with CaM in a similar manner [22].

Structure-Function Relationships Identify an Inhibitor with Minimal Toxicity

To define minimal features required for the inhibition of EF, we synthesized several analogs of 10506-2A and evaluated the activities in the SPR binding assay (Figure 5C). The first compound, [(4-phenyl-2-thiazolyl)-4-amino]-benzenesulfonamide, lacks the two chlorides in the right-handed phenyl ring and inhibited the binding of EF to CaM with an IC_{50} value similar to 10506-2A. This compound also inhibited the adenylyl cyclase activity of EF (data not shown). Compounds lacking the sulfonamide moiety of the original hit, 4-(3,4-dichlorophenyl)-N-phenylthiazol-2-amine, or the phenyl-thiazole moiety, 4-aminobenzenesulfonamide, were not active inhibitors. An analog lacking the two chlorides and the sulfonamide groups, N,4-diphenylthiazol-2-amine, was also inactive. Taken together, these results indicate that both the benzenesulfonamide and the phenylthiazole moieties are required for the inhibitory properties of 10506-2A.

To be useful for studies in cell culture, the inhibitor must be nontoxic to uninfected mammalian cells. When we evaluated the toxicity of 10506-2A in Chinese hamster ovary (CHO) and mouse Y1 cells using lactate dehydrogenase (LDH) assays, which is a stable cytosolic protein released into the culture media on cell death, we found that 10506-2A showed significant toxicity at 30–50 μM concentrations (Figure 6A).

To obtain an inhibitor with less cytotoxicity, we synthesized a series of molecules that presented different functional groups on the phenylthiazolyl domain of 10506-2A. One of these analogs, 4-[[4-(4-nitrophenyl)-2-thiazolyl] amino]-benzenesulfonamide (nitro10506-2A), was nontoxic to both CHO and Y1 cell lines (Figure 6A) while the inhibitory effects of nitro10506-2A were similar to those of 10506-2A in both enzyme activity and CaM binding assays (Figures 6B and 6C). The Y1 cell-based assay (Figure 6D) also showed that nitro10506-2A inhibited morphological changes at 12 μM. Finally, we monitored the effect of nitro 10506-2A on the intracellular cAMP level in CHO cells (Figure 6E). We found that nitro 10506-2A did prevent the increase of intracellular cAMP induced by edema toxin (EF+PA) without altering the basal cAMP level (PA alone). Nitro10506-2A represents the best inhibitor found in the current study.

Discussion

This study reports a strategy to screen for inhibitors of protein-protein interactions (IPPIs) that are active in cell culture. Two considerations prompted us to begin the screen with a cell-based assay. First, the Y1 cellular assay, which reports a readily identifiable phenotypic response, is simple and readily adapted to a high-throughput format. The protein-protein binding assay that we use is based on SPR and, in contrast, is not compatible with a high-throughput format. Second, our goal in this work was to identify compounds for use in cell culture. The cell-based assays are required for this purpose because many compounds found in screens of protein-protein binding interactions have undesirable transport, stability, or cytotoxicity to be effective in cell culture. Therefore, in cases where the molecular assay—that is, the assay that measures protein-protein binding interactions—is not suited to rapid and parallel screening, it can be preferable to begin with a simple cellular assay to give a small number of candidate inhibitors. These compounds can then be characterized in molecular assays and further analyzed to understand the mechanism of action.

A common and very powerful format for screening molecules in cell-based assays relies on the yeast two-hybrid method [23]. This method has been particularly important in screens of molecules that disrupt protein-protein interactions. In this case, however, substitution

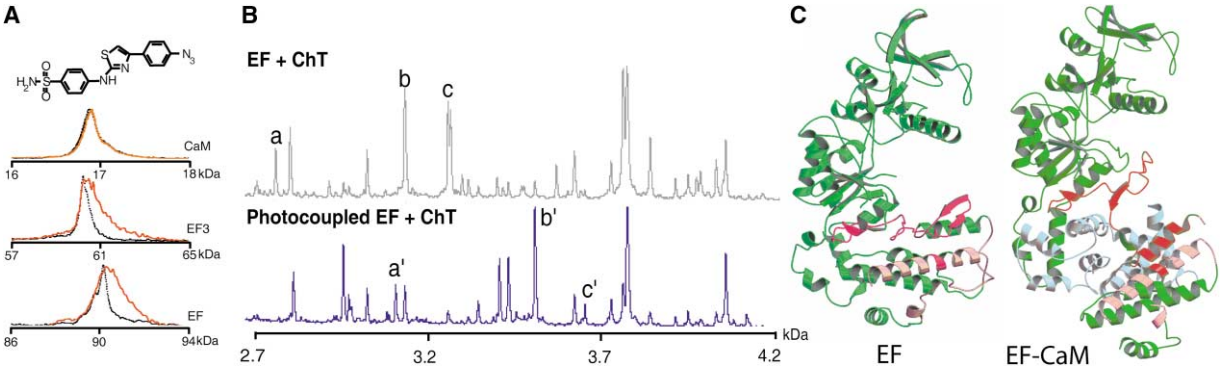

Figure 4. Characterization of the Binding of 10506-2A to EF by Photocrosslinking

(A) Mass spectrometric analysis of CaM, EF3, and EF with and without photocrosslinking of 10506-2A. Each protein (1 mg/ml) was mixed with azido10506-2A (100 μM, 1% DMSO; shown in [A]) and irradiated under UV light. The molecular weight changes of the proteins were measured with MALDI-TOF mass spectrometry to determine the molecular target of 10506-2A. Mass spectrometry of proteins with and without azido10506-2 are shown in red and black, respectively.

(B) A representative result of proteolytic analysis of photocrosslinked EF. Following photocrosslinking with azido10506-2A, EF was digested with α-chymotrypsin, and the fragments were analyzed by MALDI-TOF mass spectrometry. The top panel shows the digestion pattern of untreated EF, and the bottom panel shows the digestion pattern of photocrosslinked EF. Three peaks with reduced intensity after photocrosslinking are labeled as a, b, and c while the corresponding peaks that increased in molecular weight by 344 or 366 Da (the size of azido10506-2A or its sodium adduct, respectively) are labeled a′, b′, and c′. Identity of each peptide was determined by comparison of computer-generated EF-digestion pattern.

(C) The map of azido10506-2A-coupled peptides on the structure of EF3 with and without CaM. EF and CaM are shown in green and blue, respectively. Peptides near 10506-2A binding site were determined as in Figure 4B, and their locations were noted in published EF structures. Peptides appearing in more than one digestion are shown in red, while peptides appearing in only one digestion experiment are shown in pink.

of the EF-CaM protein-protein complex in the transcriptional machinery would result in an enzymatic activity that is toxic to host cells. Using yeast two-hybrid screens in such a system can be complicated. In addition, the cell-based screening method described in this manuscript uses a more pathologically relevant environment without employing fusion proteins or nuclear localization.

As an inhibitor of a CaM binding protein, 10506-2A is somewhat unusual. Numerous CaM antagonists have been developed that bind to the hydrophobic surfaces of CaM that are exposed upon Ca^{2+} binding [24]. However,

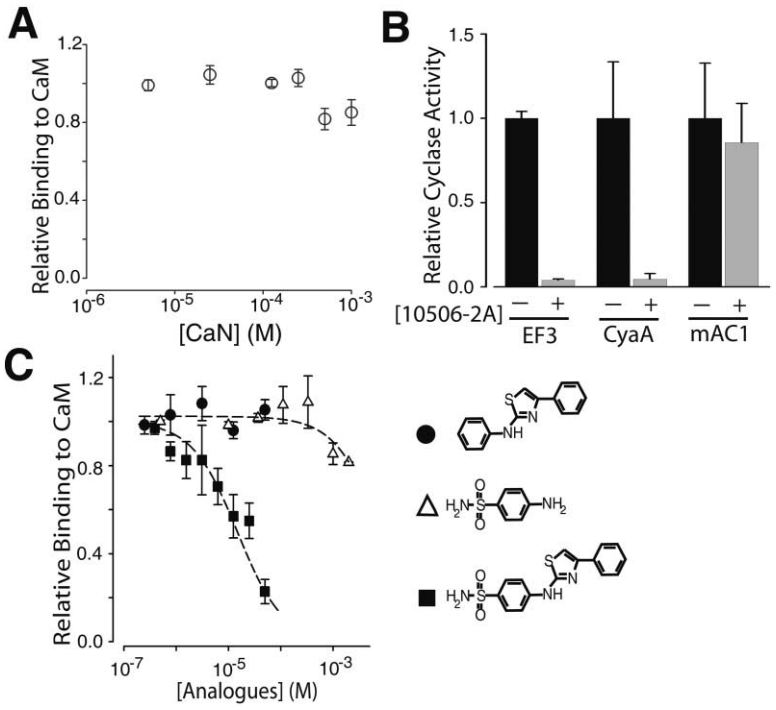

Figure 5. The Specificity of 10506-2A and Its Analogs

(A) The effect of 10506-2A on the binding of calcineurin to immobilized CaM was monitored using SPR spectroscopy as in Figure 2A. Binding was measured with 0.3 μM calcineurin and varying amounts of 10506-2A. Each data point represents an average of three independent experiments.

(B) Effect of 10506-2A on the catalytic activity of bacterial and mammalian adenylyl cyclases. Adenylyl cyclase activities of EF3, CyaA, and mammalian adenylyl cyclase 1 (mAC1) in the presence of 50 μM 10506-2A (gray bars) were measured as in Figure 2B. The relative adenylyl cyclase activities were compared with the activity of each enzyme in the presence of DMSO (black bars). Each bar represents an average of three independent experiments.

(C) Effects of small molecules structurally related to 10506-2A on the binding of EF3 to CaM were monitored using SPR spectroscopy. Binding of EF3 to immobilized CaM was measured in the presence of analogs and 1% DMSO as in Figure 2A. Each data point represents an average of three independent experiments.

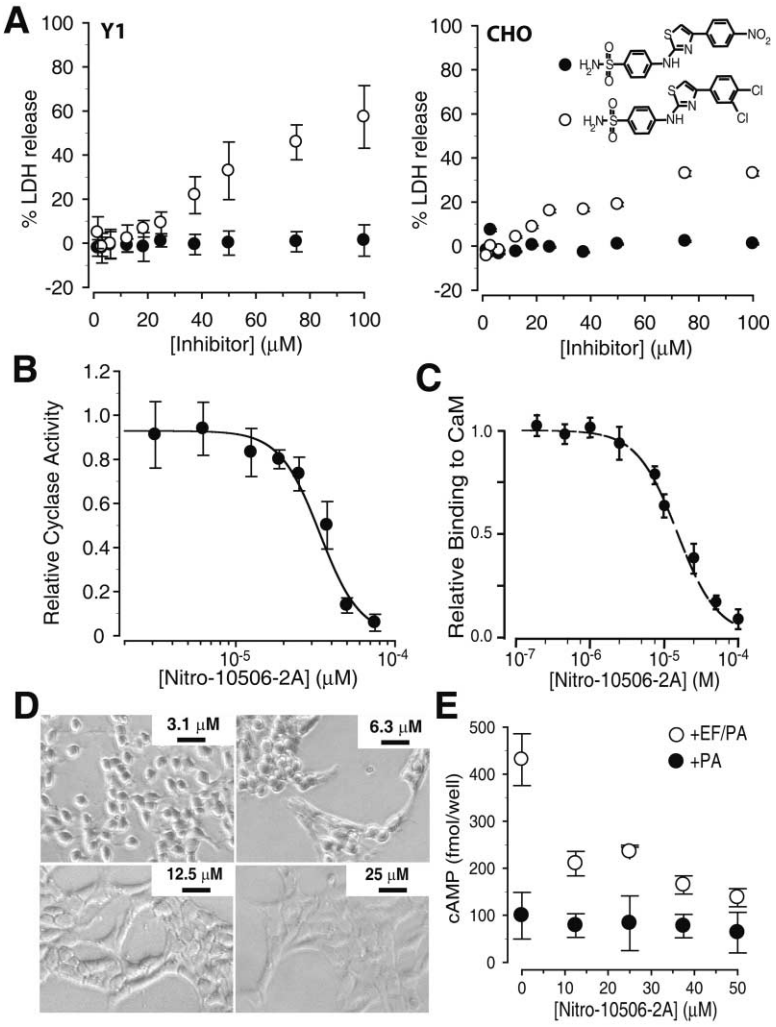

Figure 6. An Inhibitor for the EF-CaM Interaction with Minimal Cytotoxicity

(A) Nitro10506-2A (closed circles) is less toxic than is 10506-2A (open circles) in Y1 (left) and CHO (right) cells. Toxicity of each compound to cultured cells in the absence of EF and PA was monitored using LDH assays as described in the Experimental Procedures.

(B) Inhibition of EF-catalytic activity by nitro10506-2A. Each data point represents an average of three independent experiments.

(C) Inhibition of EF binding to immobilized CaM by nitro10506-2A was monitored using SPR spectroscopy as in Figure 2A. Each data point represents an average of three independent experiments.

(D) Inhibition of ET-induced cell morphological change by nitro10506-2A was monitored as in Figure 2C. Bars represent 30 μm.

(E) Inhibition of ET-induced increases in cellular cAMP by nitro10506-2A was monitored using cAMP-specific antibodies. A mixture of EF (5 ng/ml) and PA (25 ng/ml) (open circles) or PA alone (closed circles) was added to CHO cells that had been preincubated with nitro10506-2A. The levels of cAMP were measured following lysis as described in the Experimental section. Each data point represents an average of three independent experiments.

these compounds have the unwanted effect that they inhibit the interactions of CaM with a large number of target proteins. In one counter example, KN-62, a common inhibitor of calmodulin-dependent protein kinase II (CaMK II), has been shown to act by binding directly to CaMK II [25]. Unlike CaM antagonists, inhibitors such as KN-62 or 10506-2A demonstrate considerable specificity for their target protein over other CaM-activated processes. In our study, we illustrate how three different assays can be used to establish the target of inhibitors identified in screens. Kinetic studies can reveal competitive binding of the small molecule for one of the protein targets. Fluorescence studies, for small molecules that have a strong fluorescence mode, may reveal the protein that is bound by the inhibitor. Finally, photolabeling studies provide the strongest evidence for identifying the site of action of a small molecule IPPI.

Our work used several assays to establish that the 10506-2A inhibitor of the EF-CaM interaction has good specificity. However, we found that 10506-2A is also effective at inhibiting CyaA. Despite the significant homology between CyaA and EF, this finding was unexpected because the CaM binding domains of these two proteins share only limited homology. This result may suggest that CyaA and EF share structural features in the CaM binding regions that are not apparent by analyzing the primary sequences.

In common with many reports of chemical screening, we found that the inhibitor identified in the primary screen ultimately suffered from significant cytotoxic effects on cells. We therefore established the minimal structure-activity relationship and from this information were able to identify an analog that retained inhibitory activity but was tolerated by cells. Our finding that a minor structural change gave a significant reduction in cytotoxicity suggests that the inhibitors may be further enhanced with minimal modifications.

Significance

In this paper, we have reported a screening strategy to identify a small molecule inhibitor of the EF-CaM protein-protein complex from a diverse chemical library. Using a cell-based assay to first screen 10,000 compounds resulted in the identification of several molecules. Further characterization of these candidates in a SPR protein-protein binding assay revealed that only one of these compounds acted through spe-

cific inhibition of the EF-CaM complex. Therefore, this result represents an important addition to the modest number of IPPIs reported to date. We believe that the screening strategy described here—cell-based assays to narrow the number of candidates followed by protein binding assays to identify IPPIs—will find wider use for identifying IPPIs of other protein-protein interactions, provided that a convenient cell-based assay is available. The small molecule inhibitor of the EF-CaM interaction will complement active site targeting inhibitors of EF [26, 27] and will be a useful tool in cellular studies of anthrax toxins and a valuable lead toward anthrax therapeutics. (For recent examples of small molecules that target the active site of EF-CaM, see [26, 27]).

Experimental Procedures

Chemical Library

The chemical library of 10,000 compounds (Library No. ET 350-1) was purchased from ChemBridge (San Diego, CA). Averages and standard deviations of clogP and molecular weight are 3.7 ± 1.5 and 340 ± 71, respectively.

Cell Cultures and Morphological Assay in Y1 Cells

Y1 cells were maintained at 37°C with 5% CO_2 in Dulbecco's modified Eagles medium (DMEM) supplemented with 2.5% (v/v) fetal bovine serum (FBS) and 12.5% (v/v) horse serum. CHO cells were grown in DMEM/F-12 with 10% heat-inactivated FBS. Plates and flasks were coated with 1% gelatin before cells were plated to facilitate cell attachment and spreading. Y1 cells were plated in 96-well plates at 200 μl/well and used when they reached 50%–80% confluence. The chemical library was assayed by preparing solutions containing eight compounds in DMSO (5 mg/ml each) and adding 2 μl of each solution to two wells of Y1 cells. After 1 hr incubation, EF and PA were added (5 and 25 ng/ml final concentrations, respectively) and the morphology of Y1 cells was examined 1, 4 hr, and overnight after the addition of toxin. Effective pools were identified and compounds in these pools were individually screened so that the active compound could be identified. Active compounds were then examined at final concentrations ranging from 1.56 to 50–100 μM.

Surface Plasmon Resonance Spectroscopy

Surface plasmon resonance (SPR) was used to monitor the binding of EF to CaM and to characterize small molecule inhibitors as described previously [14, 15, 27]. In brief, 0.8 μM EF in a solution containing 10 mM Tris-HCl, pH 7.0, 1.0 mM EGTA, 10 mM $MgCl_2$, 100 mM KCl, and 0.96 mM $CaCl_2$ was mixed with inhibitors in DMSO (1% v/v). The mixture was then flowed over the immobilized CaM [14, 15] at a flow rate of 3 μl/min for 15 min. The amount of bound EF was determined from the response change following 10 min washing with the protein-free buffer. The surface was regenerated by washing the surface with 10 mM EGTA for 10 min. The effects of inhibitors on calcineurin were also determined as above using 0.3 μM calcineurin.

Enzymatic Assays

Adenylyl cyclase activities were measured in the presence of 20 mM HEPES (pH 7.2), 5 mM ATP with trace amounts of [γ^{32}P]-ATP, 1 mM EDTA, 10 mM $MgCl_2$, and 1 μM $CaCl_2$ for 10 min at 30°C unless otherwise indicated. CaM titrations were performed in the presence of 10 mM free Mg^{2+}, 2 μM free Ca^{2+}, and 10 mM EGTA. Concentrations of free ions were calculated using the MAXC program (http://www.stanford.edu/~cpatton/max.html). ATP and cAMP were separated by Dowex and alumina columns [21]. Sf9 cell membrane containing type I adenylyl cyclase was prepared and assays were performed as described [28]. DMSO was limited to 1% v/v to avoid inhibition of cyclases. Kinetic measurements of β-lactamase were performed as described [16]. Effects of 10506-2A on the catalysis of α-chymotrypsin were measured using N-benzoyl-L-tyrosine-*p*-nitroanilide as a substrate. 0.8 μM α-chymotrypsin was incubated with 10506-2A for 5 min at 25°C in the same buffer used for SPR experiments and 1.0% (v/v) DMSO. To this mixture, N-benzoyl-L-tyrosine-*p*-nitroanilide in DMSO was added to a final concentration of 250 μM. Final DMSO concentrations in assays were 5% (v/v). The hydrolysis reaction was followed at 25°C for 5 min at 405 nm with a Beckman DU-640 spectrophotometer.

Quantitation of Toxicity and Cellular cAMP Concentrations

Toxicity of 10506-2A was evaluated using the CytoTox 96 kit from Promega Corp. (Madison, WI) as described in manufacturer's instruction. In brief, Y1 cells were plated at 1.5×10^5 or 3×10^5 cells/ml and 10506-2A was applied the following day as described above and incubated for 1 hr. Membrane breakdown was assessed by the amount of tetrazolium salt converted to a formazan product by lactate dehydrogenase, detected by reading the absorbance at 490 nm.

Production of cAMP was determined by using Direct Cyclic AMP Enzyme Immunosassy Kit from Assay Designs, Inc. (Ann Arbor, MI). CHO cells were seeded in a 48-well plate at 1.5×10^5 cells/well and incubated for 24 hr. Nitro10506-2A compound was applied with final concentrations ranging from 3.1 to 100 μM and incubated for 1 hr at 37°C. EF (5 ng/ml) and PA (25 ng/ml) mix was added followed by 2 hr incubation at 37°C. Following lysis with 0.1 N HCl. Concentrations of cAMP were assessed at 405 nm as described in manufacturer's instruction.

Fluorescence Spectroscopy

Fluorescence emission spectra were obtained using Fluoromax 3 with a temperature controller at the Biophysics Core Facilities (University of Chicago). All experiments were performed at 25°C. 0.5 μM 10506-2A in the same buffer used in SPR experiments, 1% (v/v) DMSO, and EF3 or CaM was excited at 328 nm (slit width 5 nm), and emission spectra were collected. The ratios of intensities at 370 and 405 nm were plotted against protein concentrations.

Synthesis of 4-[4-(4-Nitrophenyl)-Thiazolylamino]-Benzenesulfonamide and Related Compounds

Syntheses of 4-[4-(4-nitrophenyl)-thiazolylamino]-benzenesulfonamide was performed according to a reported route for an analogous compound [29]. Briefly, an aqueous solution (100 ml) of 4-thioureidobenzenesulfonamide (1.20 g, 4 mmol) was combined with 4-nitrophenacyl bromide (1.1 equivalents) in ethanol (10 ml). The mixture was refluxed for 2 hr, allowed to cool, and filtered. The product was isolated from a recrystallization in ethanol. ^{1}H-NMR (400 MHz; DMSO-d^6): 7.99(d, J = 8.5 Hz, 2H), 7.82 (m, 4H), 7.43 (s, 1H), 7.19 (d, J = 8.5 Hz, 2H); m/z (APCI, negative ion mode detection): 375.0 $[M\text{-}H]^-$ and 410.9 $[M{\cdot}Cl]^-$. Other analogs were synthesized in the same manner using appropriate 2-bromoacetophenone derivatives.

Photocrosslinking and Mass Spectrometry

4-[[4-(4-azidophenyl)-2-thiazolyl]amino]-benzenesulfonamide (azido10506-2A) was synthesized from 4-thioureido-benzenesulfonamide and 4-azido-2′-bromoacetophenone as described above. Labeling experiments were performed with 1 mg/ml EF or CaM in 50 μl of buffer (10 mM HEPES, pH 7.5, 1% DMSO) containing azido10506-2A at a concentration of 100 μM. The mixture was irradiated with a hand-held UV lamp (254 nm, 300 μW/cm^2) for 5 min. Insoluble particles were formed and removed by centrifugation. Typical labeling yields were 10%–30% when analyzed using analytical HPLC. Proteolysis reactions were done by mixing the samples with ArgC (0.5 μg/ml), TLCK-treated α-chymotrypsin (10 μg/ml), or TPCK-treated trypsin (0.1 μg/ml) at 37°C for 3 hr. Digested peptides were then mixed with an equal volume of sinapinic acid or 2,5-dihydroxybenzoic acid (10 mg/ml in 50% acetonitrile, 0.2% trifluoroacetic acid) and analyzed using matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry. Peaks were assigned using Peptidemass (http://us.expasy.org/tools/peptidemass.html) allowing for one to two missed cuts.

Supplemental Data

Table S1 includes a list of compounds identified in the original screening, including details of their preliminary characterization, and can be found at http://www.chembiol.com/cgi/content/full/11/8/1139/DC1.

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References

1. Pawson, T., and Nash, P. (2003). Assembly of cell regulatory systems through protein interaction domains. Science *300*, 445–452.
2. Toogood, P.L. (2002). Inhibition of protein-protein association by small molecules: approaches and progress. J. Med. Chem. *45*, 1543–1558.
3. Ambroise, Y., Yaspan, B., Ginsberg, M.H., and Boger, D.L. (2002). Inhibitors of cell migration that inhibit intracellular paxillin/alpha4 binding: a well-documented use of positional scanning libraries. Chem. Biol. *9*, 1219–1226.
4. Berg, T., Cohen, S.B., Desharnais, J., Sonderegger, C., Maslyar, D.J., Goldberg, J., Boger, D.L., and Vogt, P.K. (2002). Small-molecule antagonists of Myc/Max dimerization inhibit Myc-induced transformation of chicken embryo fibroblasts. Proc. Natl. Acad. Sci. USA *99*, 3830–3835.
5. Vassilev, L.T., Vu, B.T., Graves, B., Carvajal, D., Podlaski, F., Filipovic, Z., Kong, N., Kammlott, U., Lukas, C., Klein, C., et al. (2004). In vivo activation of p53 pathway by small-molecule antagonists of MDM2. Science *303*, 844–848.
6. Turkson, J., Kim, J.S., Zhang, S., Yuan, J., Huang, M., Glenn, M., Haura, E., Sebti, S., Hamilton, A.D., and Jove, R. (2004). Novel peptidomimetic inhibitors of signal transducer and activator of transcription 3 dimerization and biological activity. Mol. Cancer Ther. *3*, 261–269.
7. Collier, R.J., and Young, J.A. (2003). Anthrax toxin. Annu. Rev. Cell Dev. Biol. *19*, 45–70.
8. Molloy, S.S., Bresnahan, P.A., Leppla, S.H., Klimpel, K.R., and Thomas, G. (1992). Human furin is a calcium-dependent serine endoprotease that recognizes the sequence Arg-X-X-Arg and efficiently cleaves anthrax toxin protective antigen. J. Biol. Chem. *267*, 16396–16402.
9. Drum, C.L., Yan, S.Z., Bard, J., Shen, Y.Q., Lu, D., Soelaiman, S., Grabarek, Z., Bohm, A., and Tang, W.J. (2002). Structural basis for the activation of anthrax adenylyl cyclase exotoxin by calmodulin. Nature *415*, 396–402.
10. Drum, C.L., Yan, S.Z., Sarac, R., Mabuchi, Y., Beckingham, K., Bohm, A., Grabarek, Z., and Tang, W.J. (2000). An extended conformation of calmodulin induces interactions between the structural domains of adenylyl cyclase from Bacillus anthracis to promote catalysis. J. Biol. Chem. *275*, 36334–36340.
11. Hoeflich, K.P., and Ikura, M. (2002). Calmodulin in action: diversity in target recognition and activation mechanisms. Cell *108*, 739–742.
12. Schimmer, B.P. (1979). Adrenocortical Y1 cells. Methods Enzymol. *58*, 570–574.
13. Han, J.D., and Rubin, C.S. (1996). Regulation of cytoskeleton organization and paxillin dephosphorylation by cAMP: studies on murine Y1 adrenal cells. J. Biol. Chem. *271*, 29211–29215.
14. Shen, Y., Lee, Y.S., Soelaiman, S., Bergson, P., Lu, D., Chen, A., Beckingham, K., Grabarek, Z., Mrksich, M., and Tang, W.J. (2002). Physiological calcium concentrations regulate calmodulin binding and catalysis of adenylyl cyclase exotoxins. EMBO J. *21*, 6721–6732.
15. Hodneland, C.D., Lee, Y.S., Min, D.H., and Mrksich, M. (2002). Selective immobilization of proteins to self-assembled monolayers presenting active site-directed capture ligands. Proc. Natl. Acad. Sci. USA *99*, 5048–5052.
16. McGovern, S.L., Caselli, E., Grigorieff, N., and Shoichet, B.K. (2002). A common mechanism underlying promiscuous inhibitors from virtual and high-throughput screening. J. Med. Chem. *45*, 1712–1722.
17. Lee, Y.S., and Mrksich, M. (2002). Protein chips: from concept to practice. Trends Biotechnol. *20*, S14–S18.
18. Mrksich, M. (2002). What can surface chemistry do for cell biology. Curr. Opin. Chem. Biol. *6*, 794–797.
19. Lakowicz, J.R. (1999). Principles of Fluorescence Spectroscopy, Second Edition (New York: Plenum Press).
20. Klee, C.B., Ren, H., and Wang, X. (1998). Regulation of the calmodulin-stimulated protein phosphatase, calcineurin. J. Biol. Chem. *273*, 13367–13370.
21. Tang, W.J., Krupinski, J., and Gilman, A.G. (1991). Expression and characterization of calmodulin-activated (type I) adenylyl cyclase. J. Biol. Chem. *266*, 8595–8603.
22. Ladant, D., and Ullmann, A. (1999). Bordatella pertussis adenylate cyclase: a toxin with multiple talents. Trends Microbiol. *7*, 172–176.
23. Topcu, Z., and Borden, K.L. (2000). The yeast two-hybrid system and its pharmaceutical significance. Pharm. Res. *17*, 1049–1055.
24. Hidaka, H., and Tanaka, T. (1983). Transmembrane Ca2+ signaling and a new class of inhibitors. Methods Enzymol. *102*, 185–194.
25. Tokumitsu, H., Chijiwa, T., Hagiwara, M., Mizutani, A., Terasawa, M., and Hidaka, H. (1990). KN-62, 1-[N,O-bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine, a specific inhibitor of Ca2+/calmodulin-dependent protein kinase II. J. Biol. Chem. *265*, 4315–4320.
26. Shen, Y., Zhukovskaya, N.L., Zimmer, M.I., Soelaiman, S., Bergson, P., Wang, C.R., Gibbs, C.S., and Tang, W.J. (2004). Selective inhibition of anthrax edema factor by adefovir, a drug for chronic hepatitis B virus infection. Proc. Natl. Acad. Sci. USA *101*, 3242–3247.
27. Soelaiman, S., Wei, B.Q., Bergson, P., Lee, Y.S., Shen, Y., Mrksich, M., Shoichet, B.K., and Tang, W.J. (2003). Structure-based inhibitor discovery against adenylyl cyclase toxins from pathogenic bacteria that cause anthrax and whooping cough. J. Biol. Chem. *278*, 25990–25997.
28. Levin, L.R., and Reed, R.R. (1995). Identification of functional domains of adenylyl cyclase using in vivo chimeras. J. Biol. Chem. *270*, 7573–7579.
29. Shingare, M.S., and Ingle, D.B. (1977). Synthesis of some sulfonamide derivatives. J. Indian Chem. Soc. *54*, 705–708.