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Structure-based Inhibitor Discovery against Adenylyl Cyclase Toxins from Pathogenic Bacteria That Cause Anthrax and Whooping Cough*

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Edema factor (EF) and CyaA are adenylyl cyclase toxins secreted by pathogenic bacteria that cause anthrax and whooping cough, respectively. Using the structure of the catalytic site of EF, we screened a data base of commercially available, small molecular weight chemicals for those that could specifically inhibit adenylyl cyclase activity of EF. From 24 compounds tested, we have identified one quinazoline compound, ethyl 5-aminopyrazolo[1,5-*a*]quinazoline-3-carboxylate, that specifically inhibits adenylyl cyclase activity of EF and CyaA with ~20 μM K_i. This compound neither affects the activity of host resident adenylyl cyclases type I, II, and V nor exhibits promiscuous inhibition. The compound is a competitive inhibitor, consistent with the prediction that it binds to the adenine portion of the ATP binding site on EF. EF is activated by the host calcium sensor, calmodulin. Surface plasmon resonance spectroscopic analysis shows that this compound does not affect the binding of calmodulin to EF. This compound is dissimilar from a previously described, non-nucleoside inhibitor of host adenylyl cyclase. It may serve as a lead to design antitoxins to address the role of adenylyl cyclase toxins in bacterial pathogenesis and to fight against anthrax and whooping cough.

The 2001 anthrax attacks in the United States have spurred an intense effort to discover new drugs to combat this dangerous biowarfare agent (1). Anthrax is caused by the pathogenic bacterium *Bacillus anthracis*. The anthrax bacterium secretes three major exotoxins, protective antigen (PA),[1] lethal factor (LF), and edema factor (EF) (2). PA is a pH-dependent transporter that delivers LF and EF into host cells. To do so, 83-kDa PA (PA_{83}) first associates with the cell surface protein tumor endothelial marker 8 (TEM-8) (3). The N-terminal 20-kDa domain of PA_{83} is then cleaved by a surface furin-like protease to form a PA_{63} heptamer. Two PA_{63} domains within the PA_{63} heptamer form a surface to bind an EF or LF molecule so that up to 3 mol of EF/LF mixtures can be delivered by a PA_{63} heptamer (4). Upon endocytosis and acidification, PA forms a pore to deliver EF or LF into the cytosol of host cells (5). EF is a calmodulin (CaM)-activated adenylyl cyclase that can elevate intracellular cAMP to pathological levels (6). LF is a metalloprotease that can cleave and inactivate a family of mitogen-activated kinase kinases including mitogen-activated protein kinase/extracellular signal-regulated kinase kinase and p38 kinase (7, 8). All three toxins work in concert with a poly-D-glutamate capsule to make the anthrax bacterium deadly (9).

The molecular structures of all three anthrax toxins have been determined recently, providing an excellent starting point to develop specific inhibitors against the action of these toxins (6, 10, 11). Several peptide-based reagents including the extracellular domain of TEM-8, dominant-negative PA mutants, and oligomers of PA-binding peptides have been developed to block PA from interacting with TEM-8, forming a functional pore, and associating with EF/LF, respectively (3, 12, 13). In addition, sensitive assays to search for LF inhibitors and low nanomolar affinity inhibitors of LF have been developed recently (14, 15). However, to date no inhibitor against EF has been identified.

We have determined the molecular structure of EF with and without CaM (6). Based on the structure of EF, we have found that the catalytic site of this enzyme is different from host adenylyl cyclases. This contrast suggests that it should be feasible to identify small molecular weight compounds that can specifically inhibit the activity of EF without affecting host adenylyl cyclases. The deletion of the EF gene in *B. anthracis* not only impairs the germination of the anthrax bacterium in mouse peritoneal macrophages but also raises the LD_{50} value by 2 orders of magnitude in a rodent model (16, 17). These results suggest that the blockage of adenylyl cyclase activity of EF may significantly reduce the lethality of anthrax bacterium, thereby providing a wider window to treat patients with anthrax infection. The adenylyl cyclase domain of EF also shares homology with other adenylyl cyclase toxins, CyaA and ExoY (18, 19). CyaA is vital for the colonization of *Bordetella pertus-*

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[1] The abbreviations used are: PA, protective antigen; EF, edema factor; LF, lethal factor; CaM, calmodulin; EF3, catalytic domain of EF (amino acids 291–800); CyaA-N, catalytic domain of CyaA (amino acids 1–393); TEM, tumor endothelial marker; mAC, mammalian adenylyl cyclase.

sis in the respiratory tract; successful colonization results in whooping cough, a major health threat to infants (20). ExoY is a toxin delivered by the type III secretion system of *Pseudomonas aeruginosa*, a bacterium that accounts for 20% of hospital-acquired infections (19). In addition, a secreted fraction having adenylyl cyclase activity and a gene homologous to known adenylyl cyclase toxins were found in *Yersinia pestis*, a bacterium that causes bubonic and pneumonic plagues (21–23). Thus, molecules that can block the action of adenylyl cyclase toxins may have a broad usage to combat illness caused by several deadly human pathogens.

Here we describe the identification of specific inhibitors of EF and CyaA among commercially available chemicals. We first docked ~200,000 molecules from the Available Chemical Directory (MDL Information Systems Inc., San Leandro, CA) in multiple orientations and conformations into the ATP binding site of EF. Twenty-four high scoring molecules were selected for experimental studies to identify those that specifically inhibit EF and CyaA compared with corresponding host adenylyl cyclases and subsequently to block the intoxication of adrenocortical Y1 cells caused by edema toxin (a combination of EF and PA). This study identified a family of quinazoline compounds, the best of which specifically inhibited EF and CyaA with a K_i value of 20 μM without inhibiting mammalian type I, II, and V adenylyl cyclases.

EXPERIMENTAL PROCEDURES

Materials—Compounds 2-phenylaminoadenosine, (3*ar*,4*s*,7*r*,7*as*)-7-(carbobenzyloxyamino)-3*a*,4,7,7*a*-tetrahydro-2,2-dimethyl-1,3-benzodioxol-4-ol, and 2,7-diamino-3-cyano-4-phenyl-4*n*-benzopyrane were purchased from Sigma; *n*(2)-(4-nitrophenyl)[1,3]thiazolo[5,4-*d*]pyrimidine-2,7-diamine and 2-amino-4-(2-furyl)-5-oxo-5,6,7,8-tetrahydro-4*n*-chromene-3-carbonitrile were from Bionet (Camelford, UK); PU120 and PU574 were from Menai (Gwynedd, UK); 16/06-35 was from Buttpark (Bath, UK); 6,8-dibromoquinazolin-4-ol, 5-amino-8-(trifluoromethyl)pyrido[2,3-*e*][1,2,3]triazolo[1,5-*a*]pyrimidine-3-carbonitrile, 3-phenyl-8-(trifluoromethyl)pyrido[2,3-*e*][1,2,3]triazolo[1,5-*a*]pyrimidin-5-amine, (2-amino-4,5-dimethyl-3-thienyl)(4-chlorophenyl) methanone, ethyl 5-aminopyrazolo[1,5-*a*]quinazoline-3-carboxylate, (5-amino[1,2,3]triazolo[1,5-*a*]quinazolin-3-yl)(morpholino)methanone, *n*3-(4-pyridylmethyl)-5-amino [1,2,3]triazolo[1,5-*a*]quinazoline-3-carboxamide, 9-fluoro-5*n*-chromeno[4,3-*d*]pyrimidin-2-amine, 2-[(3-amino-4-oxo-4*n*-[1,2,4]triazino[3,4-*b*][1,3,4]thiadiazol-7-yl)thio]acetic acid, 7-methoxy-1,2-dihydrobenzo[*e*][1,2,4]triazolo[3,4-*c*][1,2,4]triazin-1-one, 8-(methylthio)-4,5-dihydrothieno[3′,4′:5,6]benzo[*c*]isoxazole-6-carboxamide, 6,8-difluoro-2,3-dihydro-1*n*-pyrazolo[4,3-*c*]quinolin-3-one, 4-amino-1-hydroxy-5,5-dimethyl-2-phenyl-3-imidazoline-3-oxide, 7-chloro-1,2-dihydrobenzo[*e*] [1,2,4]triazolo[3,4-*c*] [1,2,4]triazin-1-one, *n*3-ethyl-5-amino[1,2,3]triazolo[1,5-*a*]quinazoline-3-carboxamide, and (5-amino-7-chloro[1,2,3]triazolo[1,5-*a*]quinazolin-3-yl)(2-thienyl)methanone were from Maybridge (Cornwall, UK). Anthrax protective antigen was purchased from List Biological Laboratory (Campbell, CA), restriction enzymes were from New England Biolabs (Beverly, MA), and the QuikChange kit for site-directed mutagenesis was from Stratagene (La Jolla, CA). [α-^{32}P]ATP and the Big-Dye kit for automatic DNA sequencing were from PerkinElmer Life Sciences. Mouse adrenocortical Y1 cells were obtained from ATCC. Tissue culture reagents were obtained from Invitrogen and Cambrex Bio Science Walkersville, Inc. (Walkersville, MD).

Docking—The Northwestern University version (24–27) of DOCK (28, 29) was used to screen the Available Chemical Directory (version 2000.2, MDL) against the 3′-dATP binding site of the EF·CaM structure (Protein Data Bank code 1K90). To prepare the site for docking, 3′-dATP and all water molecules were removed. The observed ytterbium ion was treated as a magnesium ion, which is a tightly bound, non-displaceable group. Protonation of enzyme residues was done with Sybyl (Tripos, St. Louis, MO). To generate docking "spheres," which are used to orient ligand, we used both the positions of the 3′-dATP atoms and sphere positions identified by SPHGEN (28). Several selective spheres were labeled based on the chemical functionality of the nearby residues. The program DISTMAP was used to compute the excluded volume grid of EF (30), which is used as an initial steric filter in docking calculation. Electrostatic and van der Waals energy potential grids were calculated by DelPhi (31) and CHEMGRID (29), respectively.

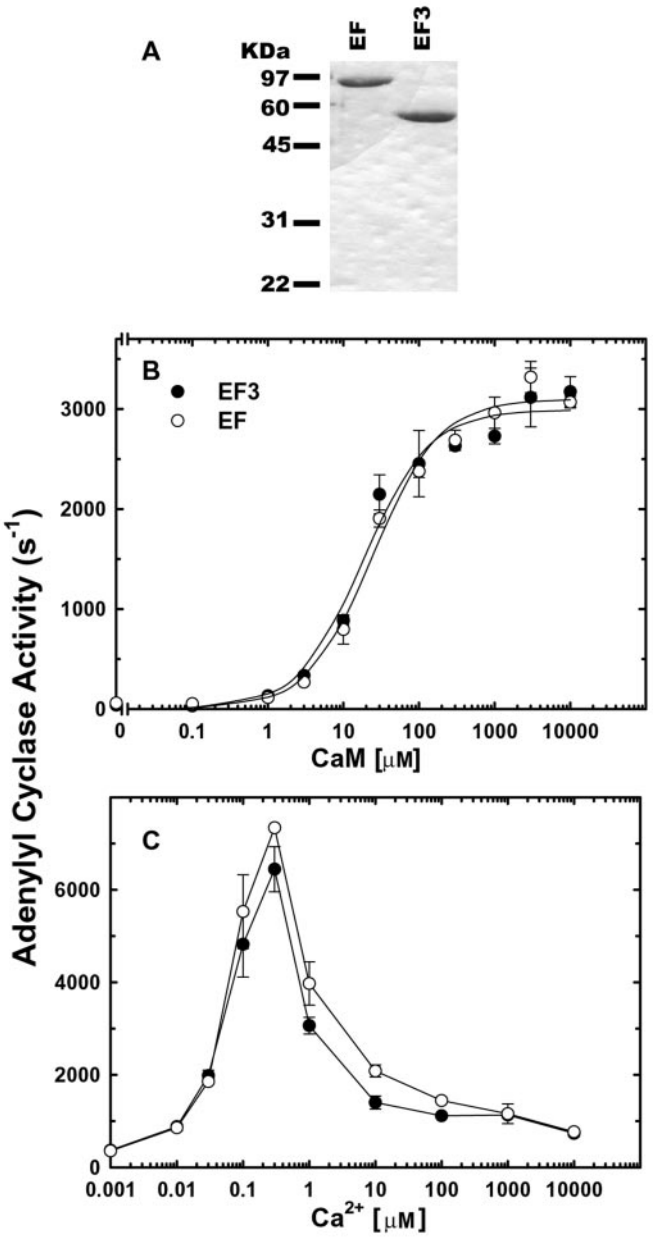

FIG. 1. **Effect of CaM and calcium ion on adenylyl cyclase activity of EF, a full-size EF, and EF3, a catalytic domain of EF.** *A*, purified EF and EF3. 1 μg of purified EF and EF3 were run on an SDS-polyacrylamide gel and stained by Coomassie Blue. *B*, adenylyl cyclase assays were performed with 0.78 nM EF (*open circles*) and 0.85 nM EF3 (*filled circles*) in the presence of 1.0 μM free Ca^{2+}. *C*, adenylyl cyclase activities were measured with 0.52 nM EF (*open circles*) and 0.56 nM EF3 (*filled circles*) in the presence of 10 μM CaM. Mean ± S.E. are representative of at least two experiments.

Ligand partial atomic charges and solvation energies were calculated with AMSOL (27, 32, 33). Conformations of ligand molecules were calculated with Omega (OpenEye, Santa Fe, NM) before docking, and up to 2000 conformers for each molecule were generated. An ensemble of conformations was stored and docked in a hierarchical manner that allows for rapid pruning of conformations that clash with the binding pocket (25). Alternative protonation states of ligand ionizable groups were sampled. The distance tolerance parameter (dislim) for orientation matching was set to 0.8 Å. The ligand and receptor bin sizes were each 0.3 Å, and ligand and receptor overlap were each 0.2 Å. Chemical matching (34) and "critical clusters" were used to guide the matching of ligand atoms to the spheres. Docking energies were corrected for the cost of desolvating the ligands (26, 27). Each ligand orientation that passed the steric filter was refined with up to 20 iterations of rigid body minimization (35). The 500 top scoring molecules of the 205,226 Available Chemical Directory molecules docked were displayed with Midas-

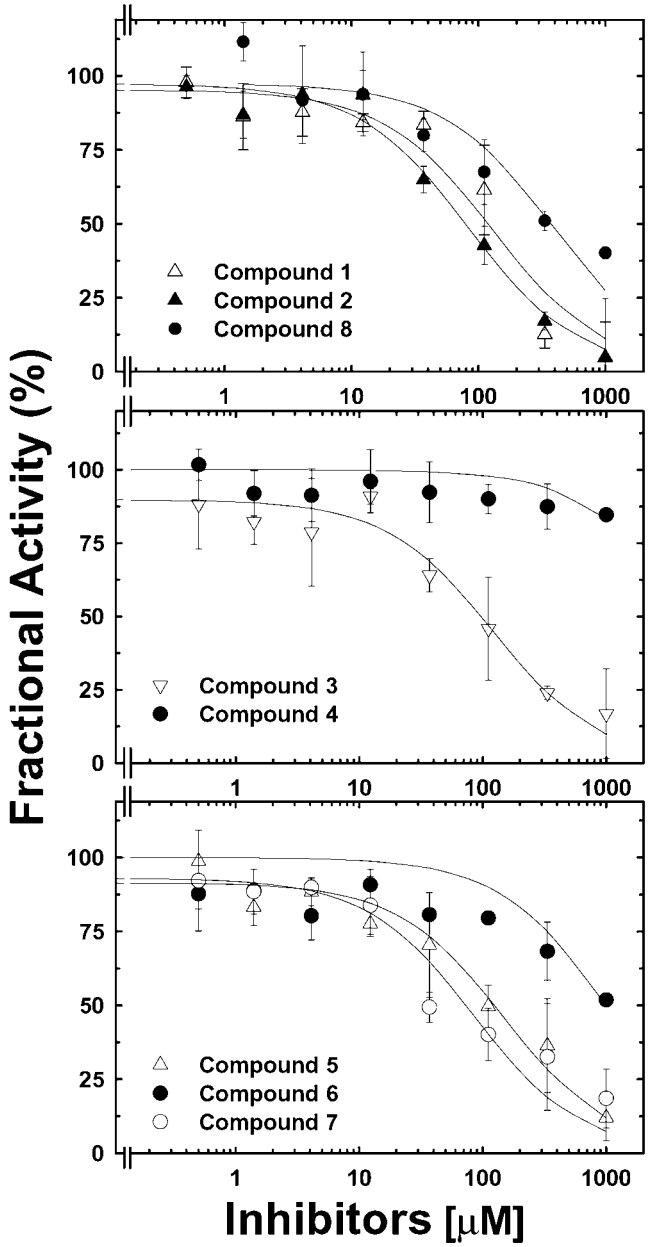

FIG. 2. **Effects of eight compounds on adenylyl cyclase activity of EF3.** The adenylyl cyclase assay was done in the presence of 16 pM EF3, 1 μM CaM, 1 μM free Ca^{2+}, and the indicated concentrations of compounds dissolved in Me_2SO. To avoid the effect of Me_2SO, only 1 μl of the compound solution (or Me_2SO as the solvent control) was added into the 100-μl reaction. Means ± S.E. are representatives of at least two experiments, and specific activities of EF3 without compounds in these experiments were in the range of 0.6–1.6 ms^{-1}.

TABLE I
Summary of eight compounds and their effect on EF

Compound	Structure	Docking rank[a]	IC_{50}[b]	Inhibition	Cell-Based assay[h]
1 (173463)		391	90[c]	Ambiguous[c]	N
2 (173464)		328	70[c]	Non-specific[d]	N
3 (119804)		412	60	Specific[e]	Y (125 μM)
4 (119805)		2033	>1000	N.A.[f]	N
5 (277890)		890	90	Specific	Y (125 μM)
6 (119808)		982	900	N.A.[f]	N
7 (120085)		403	25	Promiscuous[g]	N
8 (177532)		451	300	Promiscuous	N.D.[i]

[a] Out of 205,000 molecules docked.
[b] Apparent IC_{50} values were determined in the presence of 50 μM ATP.
[c] Showed sharp transition in the dose-response curve of EF inhibition and formation of aggregates based on dynamic light scattering
[d] Showed inhibition of β-lactamase activity.
[e] Neither showed promiscuity in inhibiting the activity of EF nor inhibition of β-lactamase activity.
[f] Not applicable.
[g] Showed sign of promiscuous inhibition to the activity of EF.
[h] Blocked the morphological change of adenocortical Y1 cells from spread to round-up morphology within 1–4 hr in response to the addition of PA and EF.
[i] Not done.

Plus (36), and 19 molecules were selected for experimental testing as inhibitors of EF. Following initial enzyme inhibition assays, the ISIS program (MDL) was used to select five high scoring analogs of compounds **1** and **2**, two initial docking "hits" that were found to inhibit EF, for testing.

Purification of EF, EF3, CyaA-N, and CaM—EF3 and CyaA-N, the catalytic domains of EF and CyaA, respectively, as well as calmodulin were purified as described previously (37, 38). To express edema factor that has a hexahistidine tag substituted for its leader peptide (amino acids 1–33) and can be delivered by anthrax-protective antigen into host cells, a plasmid, pProEx-H6-EF, was constructed as follows. The 3.2-kb *Eco*RI-*Xho*I fragment was excised from pSE42 (kindly provided by S. Leppla, National Institutes of Health) and inserted into pBluescript. A *Not*I site was then introduced at the sequence encoding amino acids 32–34 of EF by site-directed mutagenesis, and the mutation was confirmed by DNA sequencing. The 3.2-kb *Not*I-*Xho*I fragment encoding amino acids 35–800 of EF was subsequently moved into pProEx-H6. To make recombinant H6-EF, an N-terminal hexahistidine-tagged EF, pProEx-H6-EF was transformed into BL21(DE3) that harbored pUBS520, a plasmid that encoded tRNA for the AGA and AGG codons. The resulting cells were grown in a modified T7 medium with 50 μg/ml ampicillin and 25 μg/ml kanamycin at 30 °C to A_{600} = 0.4, induced by adding isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 200 μM, and harvested 19 h postinduction. The purification of EF was done by using a nickel-nitrilotriacetic acid column followed by Q-Sepharose column to yield ~20 mg from each liter of *Escherichia coli* culture. The protein concentrations of all recombinant proteins were determined by Bradford assay using bovine serum albumin as the standard.

Enzymatic Assays—Adenylyl cyclase activities were measured in the presence of 20 mM HEPES (pH 7.2), the indicated ATP concentration with a trace amount of [^{32}P]ATP, 1 mM EDTA, 10 mM $MgCl_2$, and 1 μM free calcium for a 10-min incubation at 30 °C. ATP and cAMP were separated by Dowex and alumina columns (39). Sf9 cell membrane containing type I, II, or V adenylyl cyclase was prepared as described previously (39). The compounds were dissolved in Me_2SO, and a minimal volume (1 μl) was added to the assay to avoid the inhibitory effect by Me_2SO. TEM-1 β-lactamase was expressed and purified to homogeneity as described previously (40). Kinetic measurements of TEM-1 were performed in 50 mM Tris buffer (pH 7.0) using 200 μM nitrocefin as a substrate in methacrylate cuvettes; reactions were monitored at 482

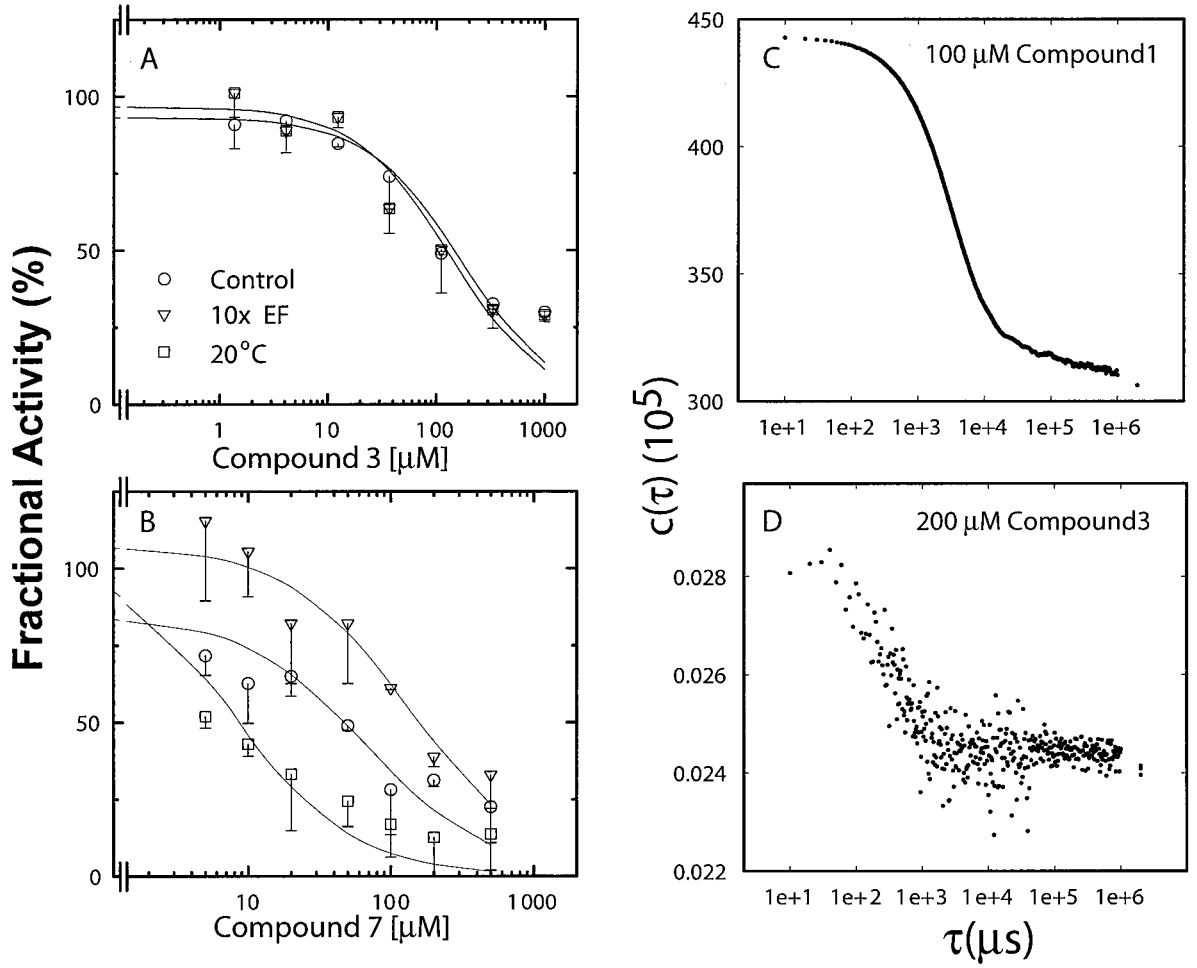

FIG. 3. **The promiscuity of compounds based on the *in vitro* adenylyl cyclase assays (*A* and *B*) and on dynamic light scattering (*C* and *D*).** Adenylyl cyclase assays were performed in the same way as in Fig. 2 except that 160 pM EF3 was used in the 10× EF condition and a 10-min incubation at 20 °C prior to the assay was done in the 20 °C condition. Means ± S.E. are representative of at least two experiments, and specific activities of EF3 without compounds in these experiments were in the range of 1.4–2.4 ms^{-1}. Autocorrelation functions from dynamic light scattering of 100 μM compound **1** (*C*) and 200 μM compound **3** (*D*) were performed in 50 mM KP_i.

nm. Reactions were initiated either by the addition of enzyme or, if inhibitor-enzyme preincubation was being tested, by the addition of substrate.

Dynamic Light Scattering—Compounds were dissolved to 20 mM in Me_2SO and diluted with filtered 50 mM pH 7.0 potassium phosphate buffer (KP_i). All compounds were analyzed with a 3-watt argon-ion laser at 514.4 nm with optical systems from Brookhaven Instrument Corp. The laser power and integration times were comparable for all experiments. Calculation of mean particle diameter was performed by the cumulate analysis tool of a 400-channel BI9000AT digital autocorrelator with the last four channels used for base-line measurement. The detector angle was 90°. Three to five independent measurements were performed for each concentration of each compound at 22 °C.

Cell Round-up Assay of Adrenocortical Y1 Cells—Y1 cells were maintained at 37 °C with 5% CO_2 in Dulbecco's modified Eagle's medium/F-12 supplemented with 2.5% fetal bovine serum and 12.5% horse serum. 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 (about 5×10^4 cells/well). For the round-up assay of Y1 cells, compounds were dissolved in Me_2SO at concentrations ranging from 50 mM to 390 μM in 2-fold dilutions, and 2 μl of each concentration were added to the appropriate wells. After a 1-h incubation, EF and PA were added to 3 and 25 ng/ml final concentrations, respectively. The morphology of Y1 cells was examined after 1 h, 4 h, and overnight incubation.

Surface Plasmon Resonance Spectroscopy—The ability of EF to bind cutinase-CaM was monitored by surface plasmon resonance spectroscopy as described previously (38, 41). In brief, EF (0.24 nM–2 μM) in the binding buffer (10 mM Tris-HCl, pH 7.0, 1.0 mM EGTA, 10 mM $MgCl_2$, 100 mM KCl, 0.96 mM $CaCl_2$) was mixed with compound **3** or **5**. This

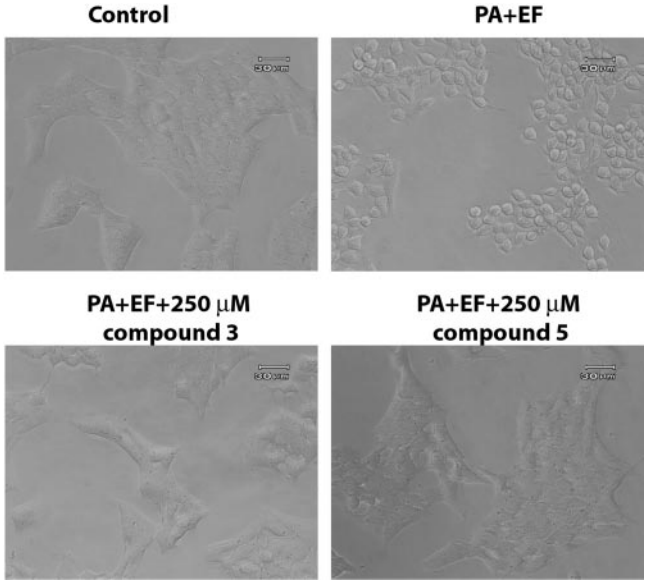

FIG. 4. **Effect of compounds 3 and 5 on the morphology of Y1 cells.** Pictures were taken 1 h after addition of EF and PA to final concentrations of 3 and 25 ng/ml, respectively. Y1 cells were incubated without EF and PA; with EF and PA; with EF, PA, and 250 μM compound **3**; or with EF, PA, and 250 μM compound **5** as indicated.

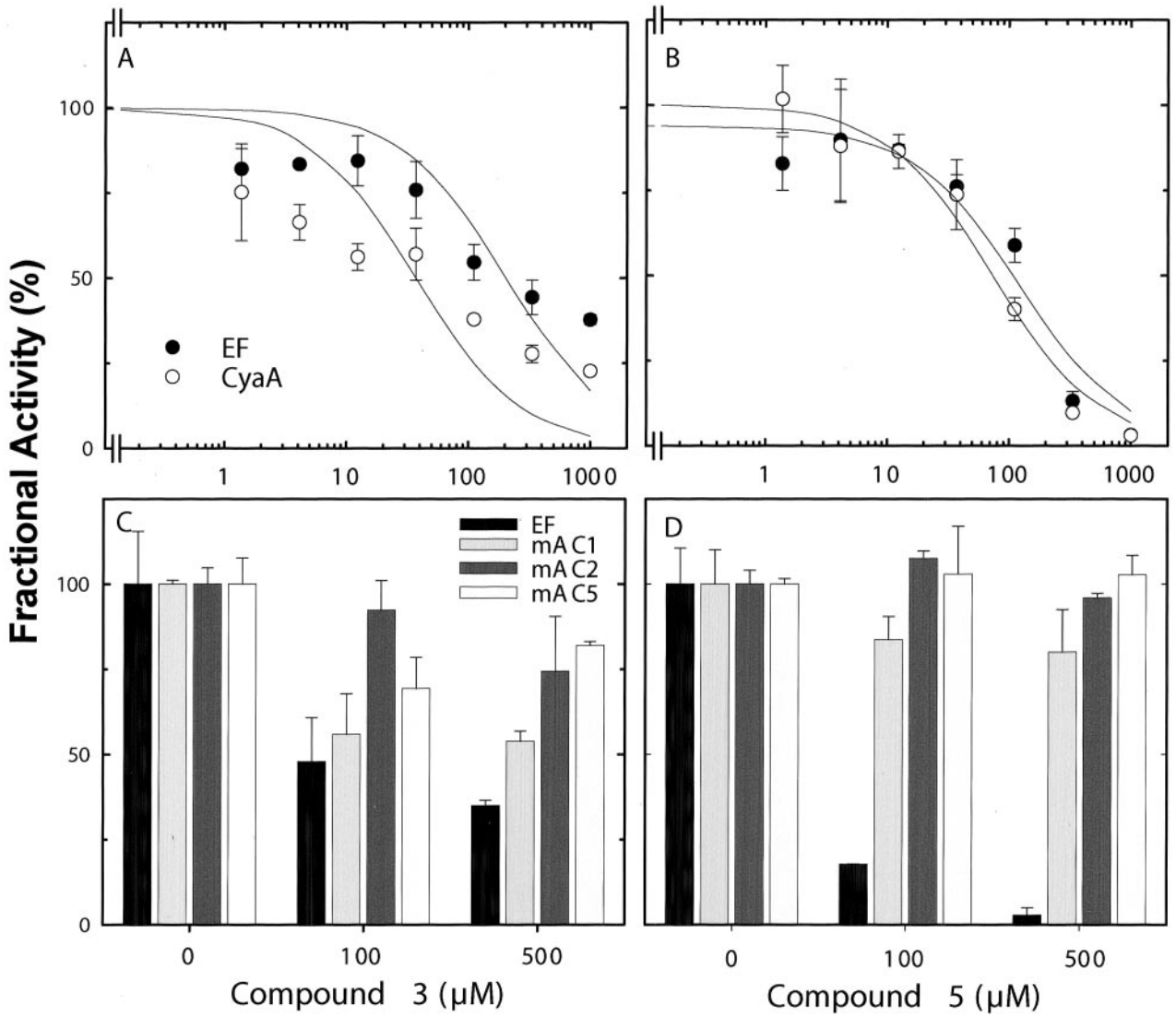

FIG. 5. **Effect of compounds 3 and 5 on bacterial (*A* and *B*) and mammalian (*C* and *D*) adenylyl cyclases.** The adenylyl cyclase assays with EF or CyaA were done in the presence of 16 and 22 pM enzyme, 1 μM CaM, 1 μM free Ca^{2+}, 100 μM ATP, and the indicated concentrations of compounds. The mammalian adenylyl cyclase assay was done using 20 μg of Sf9 membranes containing mAC1, mAC2, or mAC5 in the presence of 1 μM $G_s\alpha$, 50 μM forskolin, and 100 μM ATP. Mean ± S.E. are representative of at least two experiments.

mixture was then allowed to interact with cutinase-CaM immobilized on 2% phosphonate surface with a flow rate of 3 μl/min for 20 min, and the amount of bound EF was determined from the change of surface plasmon spectroscopic response.

RESULTS

Full-length EF (H6-EF) and the Catalytic Domain of EF (EF3) Have Similar Sensitivities to Calcium and CaM—Due to the problem in expressing the full-length EF, we have expressed and characterized the 60-kDa adenylyl cyclase domain of EF, named EF3 (37). By optimizing the expression, we now have effectively expressed and purified recombinant 90-kDa H6-EF, which contains both the catalytic domain and PA-binding domain of EF. After nickel-nitrilotriacetic acid and Q-Sepharose columns, ~20 mg of 90% pure H6-EF was obtained from each liter of *E. coli* culture (Fig. 1*A*), a 5-fold improvement over the previously reported expression and purification protocols (42). H6-EF can be stimulated by CaM with V_{max} and EC_{50} values identical to those of EF3 (Fig. 1*B*). We have recently shown that physiological calcium concentrations can promote the association between CaM and EF3 as well as directly inhibit the catalytic rate of EF3; such regulation is also found in H6-EF (Fig. 1*C*) (38). Thus, our data showed that the catalytic properties of EF are identical to those of EF3. For the subsequent studies, we used EF3 for the *in vitro* enzymatic assay to avoid the potential complication of the PA-binding domain of EF and used H6-EF for tissue culture cells where the PA-binding domain is required for EF to enter into cells.

Identification of Compounds That Can Inhibit the Catalytic Domain of EF—Our goal was to identify low molecular weight molecules that can specifically inhibit adenylyl cyclase toxins without affecting host adenylyl cyclases and block the cellular intoxication by edema toxin (PA and EF). To do so, we targeted our structure-based inhibitor discovery to the catalytic site of EF. The 3′-dATP binding site of the EF·CaM complex was screened against a data base of 205,226 small molecules. On average, each compound was sampled in 447 orientations and 294 conformations, and overall 2.7×10^{10} configurations were scored. Top scoring molecules were visually examined in the context of the binding site, and 19 compounds were initially chosen based on electrostatic or polar complementarity as well as favorable nonpolar interactions. These compounds were purchased and tested for their ability to inhibit adenylyl cyclase activity of EF3, a recombinant protein containing only the catalytic portion of EF. Two pyrido[2,3-*e*][1,2,3]triazolo[1,5-

a]pyrimidine-5-amines (compounds **1** and **2**) and one phenylmethanone (compound **8**) were found to have IC_{50} values lower than 300 μM (Fig. 2 and Table I). Five high scoring analogs (compounds **3–7**) of compounds **1** and **2**, which have IC_{50} values of about 100 μM, were picked from the Available Chemical Directory based on chemical similarity and were also tested for inhibition of EF (Fig. 2 and Table I). Three of these (compounds **3**, **5**, and **7**) have IC_{50} values lower than 100 μM.

Filtering the Active Compounds by Promiscuity Assays—Chemical compounds may form aggregates that promiscuously inhibit the activity of EF instead of specifically occupying its active site; such phenomena have been observed for many inhibitors from virtual and high throughput screening as well as for protein kinases (43–45). To eliminate the compounds with this unwanted effect, we first investigated the effect of preincubation of compounds with EF (Fig. 3, *A* and *B*; not shown for compound **8**). We found that compounds **7** and **8** had reduced IC_{50} values, an indication of promiscuous inhibition. In addition, the same set of compounds also had increased IC_{50} values when 10-fold more EF was used, which also suggested promiscuous inhibition. We then investigated the activities of compounds **1**, **2**, **3**, and **5** against a completely unrelated enzyme, β-lactamase. Compounds **1**, **3**, and **5** at 200–300 μM concentrations did not inhibit β-lactamase and showed no preincubation effect, while compound **2** at 70 μM almost completely inhibited β-lactamase activity. The inhibition of β-lactamase by compound **2** increased after preincubation and decreased when the enzyme concentration was raised by 10-fold (data not shown). We then used dynamic light scattering experiments to test whether compounds **1**, **3**, and **5** can form aggregates, which is a characteristic of some promiscuous inhibitors (43, 45). Compound **1** at a concentration comparable to its IC_{50} for EF showed high intensity scatter that decayed on the 1,000–100,000 μs time scale, suggesting that particles larger than 1 μm in diameter were present (Fig. 3*C*). Compounds **3** and **5**, at up to 200 μM, gave low intensity, poorly defined autocorrelation functions, consistent with the absence of particles (Fig. 3*D*, not shown for compound **5**). These phenomena are consistent with the notion that compounds **3** and **5** are classical, specific inhibitors of EF, while part of the inhibition of EF by compounds **1**, **2**, **7**, and **8** may be caused by the aggregation-based mechanism (43, 45).

Compounds 3 and 5 Block Round-up of Adrenocortical Y1 Cells Induced by EF and PA—The increase of intracellular cAMP can cause actin-cytoskeleton rearrangement and rounding up of mouse adrenocortical Y1 cells (46); such changes are commonly induced by bacterial toxins (47). To monitor whether compounds **1–7** can block the production of cAMP by EF, we took advantage of the rapid morphological change (within 1 h) seen in mouse adrenocortical Y1 cells in response to agents that increase cAMP. When H6-EF and PA were added to cells together, we observed the expected round-up of Y1 cells (Fig. 4). However, neither H6-EF nor PA alone also could induce round-up of Y1 cells (not shown). We then tested compounds **1–7** in Table I and found that only compounds **3** and **5** could block the cAMP-induced round-up of Y1 cells at concentrations of 125 μM and above (Fig. 4 and Table I). The other compounds had no effect on the round-up of Y1 cells even at a concentration of 1 mM (data not shown). These data together with the results above led us to focus our subsequent analysis on compounds **3** and **5**.

Specificity of Compounds 3 and 5—We then tested the specificity of compounds **3** and **5** on CyaA-N, the adenylyl cyclase domain of CyaA, which is an exotoxin secreted by *B. pertussis* (Fig. 5, *A* and *B*). We found that compounds **3** and **5** inhibited CaM-activated activity of CyaA-N with IC_{50} values of 40 and 80

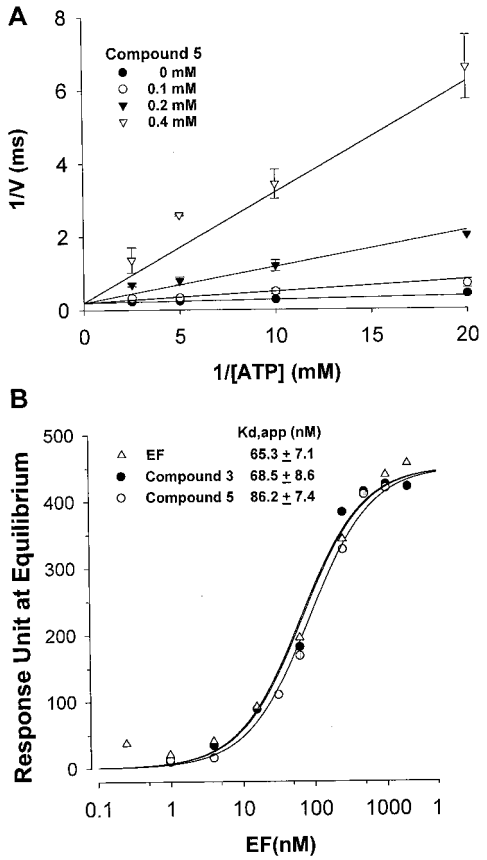

FIG. 6. **Characterization of compounds 3 and 5 on the mechanism of inhibition.** *A*, kinetic properties in the inhibition of EF by compound **5**. The adenylyl cyclase assay was done in the presence of 16 pM EF3, 1 μM CaM, 1 μM free Ca^{2+}, and the indicated concentrations of compounds. The V_{max}, K_m, and K_i values were estimated to be 5 ms^{-1}, 50 μM, and 20 μM, respectively. *B*, the effect of compounds **3** and **5** on the binding of EF to CaM. Apparent K_d was calculated using a simple one-to-one-binding model: $R_{eq} = R_{Max} \times ([EF]/([EF] + K_d^{app}))$ where, R_{eq}, R_{Max}, and K_d^{app} are responses at equilibrium, maximum response at excess EF, and apparent dissociation constant, respectively. The SPR spectroscopy was performed in the presence of Me_2SO alone (*open triangles*), 500 μM compound **3** (*filled circles*), and 500 μM compound **5** (*open circles*). Mean ± S.E. are representative of at least two experiments.

μM, respectively. There are nine isoforms of membrane-bound adenylyl cyclase found in mammals (48). We expressed three of them (type I, II, and V adenylyl cyclase) using Sf9 cells and tested whether compounds **3** and **5** could modulate the activity of these enzymes (Fig. 5, *C* and *D*). All three enzymes are activated by forskolin and recombinant $G_s\alpha$, the α subunit of the stimulatory G protein G_s. We found that, up to 500 μM, compound **3** reduced by 20–45% the activity of all three mammalian adenylyl cyclases, while compound **5** only marginally reduced the activities of those three enzymes.

Mechanism of Inhibition of EF by Compounds 3 and 5—We also examined the mechanism of how compounds **3** and **5** inhibit the catalytic activity of EF3 (Fig. 6). By varying substrate and inhibitor concentrations, the kinetics of inhibition by compounds **3** and **5** were found to fit well for a competitive inhibition mechanism, indicating that they compete directly with the binding of ATP (Fig. 6*A*, data not shown for compound **3**). The estimated K_i values were 50 and 20 μM for compounds **3** and **5**, respectively. Both EF and CyaA are activated by CaM. By loading a cutinase-CaM fusion protein to a self-assembled monolayer using active site-directed immobilization, we have used surface plasmon resonance spectroscopy to show that EF can specifically bind to the immobilized CaM in a calcium-de-

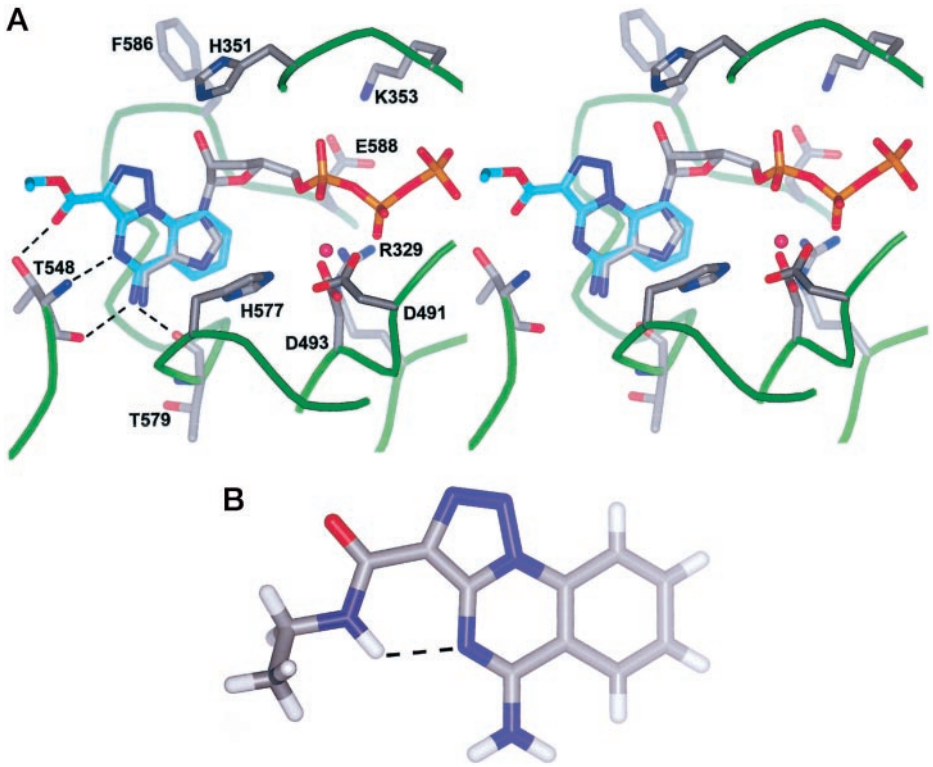

FIG. 7. **Data interpretation using the structural models.** *A*, docked structure of compound 5 compared with the observed structure of 3′-dATP bound to the EF·CaM complex. Pictures are in stereoview. Protein backbones are represented as *green tubes*. 3′-dATP and selective active residues are shown in *stick* representation. Carbon atoms of the proteins are colored in *gray*, and carbon of compound **5** is in *cyan*. Oxygen atoms are in *red*, nitrogen is in *blue*, phosphorus is in *orange*, and magnesium ion is in *magenta*. Four hydrogen bonds between EF and compound **3** are illustrated by *dashed lines*, and the bond distances are between 2.8 and 3.4 Å. *B*, the modeled conformation of compound **6**. The intramolecular hydrogen bond is illustrated by a *dashed line*, and the bond distance between the two nitrogen atoms is 2.9 Å.

pendent manner (38). Using this method, we then examined whether compounds **3** and **5** affected the interaction between EF and CaM (Fig. 6*B*). We found that the addition of compounds **3** and **5** did not change the affinity of EF to CaM at 10 μM free Ca^{2+}. This result indicated that these compounds did not affect the interaction between EF and CaM.

DISCUSSION

Compound **5** is a novel, specific inhibitor of adenylyl cyclase toxins from *B. anthracis* and *B. pertussis*. It blocks the morphological change in Y1 cells induced by edema toxin without the inhibition of mammalian type I, II, and V adenylyl cyclases. Despite its modest affinity (20 μM), its specificity and activity in cell culture make it a potentially good lead for an antitoxin against anthrax and whooping cough. Thus, it is appropriate to consider how the affinity of the inhibitor might be improved. In the absence of a crystal structure of an EF·inhibitor complex, we turned to the docking-predicted geometries to understand the binding of this compound. Based on our docking model, compound **5** overlaps primarily with the adenine group of the 3′-dATP structure, consistent with our data that the mechanism of inhibition is competitive (Fig. 7*A*). The quinazolino ring fragment fits snugly into the pocket where the adenine group binds where it would form the same three hydrogen bonds with the backbone atoms of residues Thr-579 and Thr-548 as the adenine group does (distances are between 3.0 and 3.4 Å). In addition, compound **5** appears to form a hydrogen bond with the Oγ of Thr-548 through its ester oxygen atom (distance is 2.8 Å). The ethoxyl group of compound **5** fits into a shallow groove on the enzyme surface.

Our model suggests that it will be possible to improve the affinity of this compound to EF without compromising the specificity. EF binds the ribose moiety of ATP in a manner that differs significantly from mAC. His-351 of EF is believed to interact with the 3′-OH of the ribose, while a catalytic metal is proposed to serve as the catalytic base. In addition, a hydrophobic pocket centered around Phe-586 of EF has also been shown to play a vital role in the binding of 3′-anthranyl group of the 2′-deoxy-3′-anthranyl-ATP of EF·CaM complex (38). This pocket is proximal to the putative binding site of compound **5** but is not currently used by this compound; derivatives might be able to do so, thus improving affinity. Finally the highly positively charged pocket formed by a catalytic metal and a group of basic amino acids (Arg-329, Lys-346, Lys-353, and Lys-372), which interacts with the phosphate groups of the nucleotide substrate, is not exploited by compound **5** (6).

Compound **5** represents the first non-nucleoside-based inhibitor of adenylyl cyclase toxins. It is dissimilar from nucleoside analogs and NKY80, a previously described non-nucleoside-based inhibitor of host adenylyl cyclases (49, 50). Several triazolo[1,5-*a*]quinazoline compounds, which are structurally similar to compound **5** (5-aminopyrazolo[1,5-*a*]quinazoline-3-carboxylate) have been synthesized and characterized recently (51). A subset of triazolo[1,5-*a*]quinazoline compounds is found to act as antagonists of the adenosine receptor and the benzodiazepine receptor. Together with our result, this suggests that azolo[1,5-*a*]quinazolines may be well suited to mimic adenine.

Our data show that substituting the ester of compound **5** with a secondary amide (compound **6**) decreased the affinity by 10-fold. The presence of an intramolecular hydrogen bond in compound **6** (Fig. 7*B*) may favor a conformation that poorly fits the catalytic site of EF. As a tertiary amide, compound **3** cannot form this intramolecular hydrogen bond and adopts a conformation better suited to the binding site. The lack of activity of compound **4**, which also has a secondary amide, is consistent with this view. Other possibilities such as the difference in solvation energy may also explain our observation. Further structure-activity studies are required to resolve this issue.

Our data also show that inhibitors against the catalytic site of EF from *B. anthracis* can be identified by structure-based inhibitor discovery. The hit rate in this computational approach, about 5%, is consistent with a recent docking screen for novel inhibitors of β-lactamase as is the potency of the inhibitors discovered (52). The hit rate is almost 10-fold lower than that in a large scale effort against a tyrosine phosphatase (53)

and is lower than that found by several other docking programs (for a recent review, see Ref. 54). However, we have gone to considerable effort to consider only non-promiscuous, biologically active molecules as "true" hits, which diminished their numbers. We note that promiscuous, aggregating small molecules appear to be relatively common in hit lists from both virtual and high throughput screening (44), and even widely used biological reagents such as kinase inhibitors rottlerin (against protein kinase C-δ) and K-252c (against cAMP-dependent protein kinase and protein kinase C) can act this way at micromolar concentrations (45). Therefore, care must be taken to exclude these promiscuous aggregators from hit lists in inhibitor discovery projects.

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