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Small molecule inhibitors of anthrax edema factor

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Abstract

Anthrax is a highly lethal disease caused by the Gram-(+) bacteria *Bacillus anthracis*. Edema toxin (ET) is a major contributor to the pathogenesis of disease in humans exposed to *B. anthracis*. ET is a bipartite toxin composed of two proteins secreted by the vegetative bacteria, edema factor (EF) and protective antigen (PA). Our work towards identifying a small molecule inhibitor of anthrax edema factor is the subject of this letter. First we demonstrate that the small molecule probe 5'-Fluorosulfonylbenzoyl 5'-adenosine (FSBA) reacts irreversibly with EF and blocks enzymatic activity. We then show that the adenosine portion of FSBA can be replaced to provide more drug-like molecules which are up to 1000-fold more potent against EF relative to FSBA, display low cross reactivity when tested against a panel of kinases, and are nanomolar inhibitors of EF in a cell-based assay of cAMP production.

Graphical Abstract

Keywords

Anthrax; edema factor; covalent inhibitor

Anthrax results from infection by *Bacillus anthracis* and is highly lethal unless quickly diagnosed and treated. *B. anthracis* makes two bipartite toxins generated by the combination of three proteins secreted by the bacteria. Edema Toxin (ET) composed of edema factor (EF), an adenylate cyclase,¹ plus protective antigen (PA), and Lethal Toxin (LT), made of

lethal factor (LF), a zinc-dependent metalloproteinase,² plus PA, are considered major contributors to the pathogenesis and death of humans exposed to *B. anthracis*.³

At present, antibiotics and antibody therapies are available to prevent the onset of disease following exposure; however, once a patient is symptomatic these agents rapidly lose their effectiveness. This is due to the release of ET and LT immediately following germination,⁴ and the rapid uptake of these toxins by cell surface receptors which are ubiquitous throughout the host. Once inside cells, EF and LF are no longer accessible to antibodies and initially act to promote the infection by disarming the body's immune system.⁵ Both EF and LF target cell signal pathways resulting in abnormal cell function and cell death.⁶ Recent studies using mice modified for expression of anthrax toxin receptors have demonstrated that lethality from exposure to ET and LT results from each toxin targeting specific tissues of the host.⁷ In the case of ET, hepatocytes appear to be the major target, whereas LT primarily induces lethality through its effects on cardiomyocytes and vascular smooth muscle cells. Cardiovascular system damage has been linked to the intracellular activity of LF in the rabbit model⁸ while EF is the cause of massive edema seen in some human patients infected by *B. anthracis*.⁹ Recent *ex vivo* studies using a rat aortic ring model implicate ET as a contributor to shock in the host due to arterial relaxation.¹⁰ Due to their unique ability to evade normal clearance mechanisms of the host,¹¹ both toxins can contribute to mortality and morbidity of human patients long after the bacteria have been cleared from the blood stream by antibiotics.^{9, 12}

Competitive¹³ and allosteric¹⁴ inhibitors of EF have been reported. However, these compounds display low affinity, or are derivatives of ATP¹⁵ which exhibit poor drug-like properties making them unlikely candidates for therapeutic intervention to treat anthrax in humans. To address this unmet need, we present below our initial studies towards identifying potent small molecule inhibitors of anthrax EF.

The lack of an FDA approved small molecule drug to treat EF intoxication results in part from the challenging nature of the problem to be solved. In addition to the need for specificity, a second consideration is the very high turnover rate ($k_{cat} \sim 1500 \text{ s}^{-1}$) exhibited by this enzyme.¹⁶ A third issue is the very high intracellular concentrations¹⁷ ($\sim 5 \text{ mM}$) of its substrate, ATP, which presents a significant challenge for any competitive inhibitor.

One solution to these problems is to employ a covalent edema factor inhibitor (EFI) to irreversibly silence the enzyme. Covalent inhibitors appear well suited for the treatment of acute diseases and may be used at lower doses due to their higher efficiency and lower need for optimized PK parameters.^{18–20} This approach also addresses the problem associated with active toxin remaining after antibiotic treatment since any circulating and intracellular EF would be eliminated after reacting with a covalent EFI. Typical covalent inhibitors targeting kinases²¹ are composed of a recognition element for the ATP site combined with an electrophilic group that is reactive towards a cysteine residue. Although no cysteine residues are located near the ATP binding site of EF, an x-ray crystal structure of 3'-deoxy-ATP bound to EF²² does show three lysine residues in contact with the triphosphate group attached to the adenosine core structure (Figure 1). This observation suggests that all three

terminal amino groups have the potential to react as nucleophiles with an electrophilic group if correctly positioned in the ATP binding site of EF.

Although not as common as cysteines, lysine residues are capable of acting as nucleophiles and deactivating enzymes by covalent bond formation to small molecules substrates.^{23–27}

After surveying the known small molecule inhibitors which react with lysine residues, we selected the affinity label 5'-*p*-fluorosulfonylbenzoyl adenosine (FSBA) as a model compound for proof of concept studies (Figure 2). Being a derivative of adenosine, this compound appeared ideally suited to test the hypothesis that EF could be inactivated by a small molecule covalently modifying the ATP binding site. Examination of the protein crystal structure of 3'-deoxy-ATP bound to EF²² indicated that intermolecular hydrogen bonds between the adenine ring and Thr548 provided a *recognition element* for binding to the ATP site while the ribose portion of adenosine displayed only minimal interaction and appeared to act simply as a *linking group* to the triphosphate tail. This data combined with molecular modeling (Figure S7) provided the initial support that FSBA could indeed bind in a similar manner to EF with the fluorosulphonyl group capable of acting as the *electrophilic group* towards any of the three lysine terminal amino groups.

To test this hypothesis, we conducted concentration-time dependent studies²⁸ with FSBA. As shown in Figure 3A, FSBA was indeed capable of inactivating EF over time in a dose-dependent manner. Further, a plot of [FSBA] versus k_{obs} afforded a curved line (Figure 3B) displaying saturation kinetics which supports a two-step mechanism of EF inactivation where the initial binding of FSBA by the ATP site is followed by covalent bond formation in a second step.²⁹

To demonstrate that inactivation of EF by FSBA was irreversible, the enzyme was incubated with two concentrations of FSBA for 90 minutes followed by filtration of each sample through a size exclusion column to separate the protein from the unbound FSBA. An aliquot of each filtrate was checked for enzyme activity and compared to a control sample containing EF alone. EF exposed to FSBA resulted in decreased enzymatic activity in a dose dependent manner consistent with covalent (irreversible) binding to the enzyme (Figure 3C).

After demonstrating that EF could be inactivated in a time-dependent manner, we began our search for novel inhibitors of EF by using FSBA as a starting point for compound synthesis. An immediate goal was to identify possible replacements for the adenosine moiety (recognition element + linking group) of FSBA while retaining the phenylsulfonyl fluoride as the electrophile given the demonstrated ability of this functional group to inactivate EF. Our strategy to divide the molecule into three regions based on these functions (Figure 4) allowed us to design a modular synthesis of new EFIs and expedite preparation of a set of diverse analogs (Fig S 1–6).

Kinetic inactivation data³⁰ for analogs based on a bicyclic recognition element is shown in Table 1, and for monocyclic analogs, in Table 2. It is important to note that when comparing the relative potency of covalent inhibitors, the recommended parameter to consider is the ratio k_{inact}/K_I , which unlike IC_{50} values, is a second order rate constant independent of pre-incubation time and substrate concentration.²⁹ The data in Tables 1 and 2 are ordered by

increasing inhibitor potency relative to FSBA and include physicochemical, and cytotoxicity data for each of the EFIs. Given the potential for more than one lysine residue to react with the electrophilic group (Figure 1 and Figure S7) and therefore contribute to k_{inact} , any discussion of inhibitor SAR with respect to changes in the linking group or electrophilic group without knowing the specific ligand-nucleophilic lysine pair involved is highly speculative. Therefore, the discussion will focus on changes to the recognition element since these modifications are less likely to influence the final position of the electrophilic group relative to the remaining regions of the EFI.

Beginning with the adenine series (A and B), aliphatic based linking groups provided EFIs **2** and **3** with reduced activity (k_{inact}/K_i) relative to FSBA (**1**). Use of cyclic systems appeared to provide slightly better potency specifically with the 4-piperidine based analogs **5** and **6**. Modifying the recognition element by moving the N-atom at position 7 of the adenine ring to give the pyrazolylpyrimidine series (C and D) resulted in a clear improvement in EFI potency with **10** and **11** being 32-fold and 64-fold more potent relative to **5**. A further increase in potency was realized with a change to the pyrrolopyrimidine series (E and F) where **13** is 786-fold more potent compared to the adenine analog **5** and >1000-fold more potent than FSBA. Based upon this data, a clear trend towards increased potency is seen with increased hydrophobicity of the five-membered ring present in the recognition element. Addition of a methyl group at C2 of the recognition element slightly improved potency in the adenine series (*cf.* **5** vs **8**), however, the opposite trend was observed in analogs from the remaining series (*cf.* **10** vs **11**; and **12** vs **13**). Relative to **1**, the PC properties of these analogs are similar in logP, but show improved values for the calculated PSA and membrane permeability based on the observed PAMPA values³¹. Also, the lack of cytotoxicity supported further testing of these compounds in a cell-based assay of EF activity.

Overall, the monocyclic analogs (Table 2) were less active than those based upon a bicyclic recognition element (Table 1). The most potent is the pyrimidine analog **25** which is similar in inhibitory activity to **7** and **8** in the bicyclic series (Table 1). Analogs in the 2-amino pyridine series (G) were more active relative to those based upon the 6-methyl-2-aminopyridine series (H), where many in the latter series were found to be inactive up to 150 μM . Also any further increase in the size of the R-group at this position provided only inactive analogs (data not shown). This finding, combined with a similar trend seen in the bicyclic series suggests that alkyl group substitution at this position may destabilize the H-bonding of the recognition element to the amide linkage of the Thr548 in the ATP binding site of EF (Figure 4). Similar to the bicyclic series, a clear improvement in physicochemical (PC) properties relative to **1** was seen in this series of EFIs with no observed cytotoxicity. Perhaps the most important finding from this initial medicinal chemistry effort was the demonstration that replacement of adenosine with a less complex substructure could provide EFIs with superior potency and better drug like properties compared to **1**.

While the lack of observed cytotoxicity reduced our concerns, we decided it would be prudent to explore the topic of safety further by screening the two most potent analogs **12** and **13** against a panel of human kinases using staurosporine as a positive control (Table 3). Overall, low levels of inhibition were seen with both EFIs, with **13** displaying only moderate inhibition against 3 of the 19 kinases examined. The one exception is the strong inhibition of

CHK1 by **12**. Although these results are from single point studies, the data for these early stage non-optimized EFIs provides support for the potential to achieve good selectivity for EF using covalent inhibitors.

Our next steps were to test a subset of the EFIs for chemical and biological stability, and efficacy in a cell-based assay of EF inhibition.³² We first investigated the chemical stability of **13** over a broad range of pH values and found this EFI to be stable at low and physiological pH. However, less than 1% was present after 24 hours at pH 10 (Figure S8) with the hydrolysis product of the fluorosulphonyl group identified by LCMS to be the major component of the product mixture. Given the good stability seen with **13** at physiological pH, we examined the stability of this EFI in plasma as a prelude to testing in any *in vivo* models of EF intoxication. In this case, less than 5% of **13** remained after incubation for 3 hours in mouse plasma, indicating metabolic instability was an issue with this compound. To determine if the observed instability was core structure related or due to the fluorosulphonyl group³³, representative analogs from both the mono and bicyclic series were tested for their stability in mouse plasma. Unfortunately, for each of the analogs tested the observed half-lives were found to be less than 1.2 hours (Table S1) implicating the fluorosulphonyl group as the source of instability. It should be noted that this finding now called into question the total lack of cytotoxicity observed in our routine screening results mentioned above (Tables 1 and 2) since this assay was run in the presence of 10% fetal bovine serum (FBS). However, retesting these compounds in the absence of FBS also showed no observable cell toxicity (data not shown).

We then determined if inhibition of EF activity was possible in a cell-based model of EF activity by conducting a wash-out study with the most potent EFIs from Tables 1 and 2. As shown in Figure 5, a clear decrease in cAMP production occurs with increasing concentrations of inhibitor, supporting the ability of these EFIs to cross cell membranes and block the activity of EF within cells. Compound **13** showing the highest potency (k_{inact}/K_I , Table 1) also displays the best inhibition in the cell-based assay ($EC_{50} = 12.0 \text{ nM}$). Somewhat more interestingly, EFI **25** (Table 2) was only 3-fold less potent compared with **13** despite being kinetically much less efficient ($k_{\text{inact}}/K_I > 200$ -fold) at inhibiting EF. This finding indicates that factors other than simple kinetic potency contribute to the observed cell based inhibition activity, and also illustrates the efficiency that can be achieved with covalent inhibitors.

In summary, this work has demonstrated that anthrax edema factor is a viable target for inhibition by small molecule covalent inhibitors, and this inhibition can be achieved even after the toxin has entered cells. However, the low metabolic stability of the fluorosulfonyl group exhibited by this class of EFIs presents a significant challenge to their use as a drug to treat anthrax in humans. Future work will focus on identifying electrophilic groups with improved stability, and will continue to address the need for increased selectivity towards EF when targeting a binding site which uses ATP as the natural ligand.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
The EF-3' deoxy-ATP complex showing the three lysines present in the EF ATP binding site.

Figure 2.
Structures of ATP and FSBA

Figure 3.

A, Plot of Ln(%residual EF activity) vs. time; the slope of each line provides k_{obs} . Time dependent inactivation of EF by FSBA at various concentrations: ■: 100 μM ; ►: 75 μM ; ▼: 50 μM ; ◌: 25 μM ; ●: 16 μM ; ◻: 12.5 μM ; **B**, Plot of [FSBA] versus k_{obs} where $k_{\text{obs}} = (k_{\text{inact}} \times [\text{FSBA}]) / (K_I + [\text{FSBA}])$ and provides for the determination of $k_{\text{inact}} = 0.00148 \text{ min}^{-1}$ (SD = 0.00069) and a $K_I = 18.35 \text{ } \mu\text{M}$ (SD = 2.74); **C**, Dose dependent inactivation ($n = 3$) of EF by FSBA. Incubation of EF +FSBA for 90 minutes was followed by filtration (size exclusion) and assay for pyrophosphate production.

Figure 4.
Outline of medicinal chemistry strategy to identify novel EFIs using FSBA as a hit compound.

Figure 5.
Ability of select EFI to block intracellular cAMP production by EF in RAW264.7 cells

Kinetic and potency data along with physicochemical and cytotoxicity data for EFIs with bicyclic recognition elements

Table 1

EFI	Structure	Key ^a	k _{inact} (min ⁻¹)	K _i (μM)	K _{inact} /K _i (M ⁻¹ s ⁻¹)	K _{inact} /K _i (FSBA)	clogP ^b	PSA ^b	PAMPA (logP) ^c	CC ₅₀ (μM) ^d
1 (FSBA)	Fig.1		0.015	18.4	13.42	1.00	2.25	188.1	-7.20	>100
2	A3a		0.012	37.7	5.3	0.4	1.92	141.2	-5.18	>100
3	A1a		0.011	25.9	6.9	0.5	3.17	138.4	-5.47	>100
4	A5a		0.039	96.5	6.7	0.5	2.70	132.5	nd ^e	>100
5	A4a		0.010	9.4	18.0	1.3	2.25	132.5	-8.00	>100
6	B4b		0.028	14.7	31.2	2.3	2.16	115.4	-4.05	>100
7	D3a		0.121	34.8	58.0	4.3	2.11	141.2	-5.74	>100
8	B4a		0.062	16.5	62.8	4.7	1.64	132.5	-5.59	>100
9	D4b		0.052	4.6	190.0	14.2	2.92	115.4	-5.15	>100
10	D4a		0.072	2.1	581.2	43.3	2.41	132.5	-5.79	>100
11	C4a		0.260	3.7	1163.6	86.7	2.22	132.5	-6.10	>100
12	F4a		0.129	0.7	3143.9	234.3	3.11	119.6	-4.99	>100
13	E4a		0.282	0.3	14156.9	1054.9	2.80	119.6	-5.26	>100

^aKey: Capital letter (recognition element), number (linking group), lower case letter (electrophilic group).

^bdetermined using ADC (v12.01);

^cPAMPA (Parallel Artificial Membrane Permeability Assay) average value for n = 3;

^dMTT assay;

^end : not determined.

Kinetic and potency data along with physicochemical and cytotoxicity data for EFIs with monocyclic recognition elements

Table 2

EFI	Structure	Key ^a	k_{inact} (min ⁻¹)	K_I (μM)	K_{inact}/K_I (M ⁻¹ s ⁻¹)	K_{inact}/K_I (FSBA)	clogP^b	PSA ^b	PAMPA (logP _o) ^c	CC ₅₀ (μM) ^d
1 (FSBA)	Fig.1		0.015	18.4	13.42	1.00	2.25	188.1	-7.20	>100
14	G2a		0.028	127.4	3.6	0.3	2.29	119.8	-8.00	>100
15	G6a		0.008	38.9	3.5	0.3	2.44	111.0	nd ^f	nd
16	G-b		0.198	954.7	3.5	0.3	2.68	90.7	nd	86
17	G4a		0.049	139.8	5.8	0.4	3.08	110.9	-5.11	>100
18	G7a		0.003	10.9	4.9	0.4	1.79	111.0	nd	nd
19	G3b		0.063	222.4	4.7	0.4	2.86	102.7	-8.00	>100
20	G3a		0.043	88.1	8.1	0.6	3.10	119.8	-6.44	>100
21	H4b		0.020	42.2	7.9	0.6	3.38	93.9	-3.71	>100
22	G2b		0.029	49.6	9.6	0.7	2.52	102.7	-4.53	>100
23	I2a		0.158	286.3	9.2	0.7	2.01	132.7	-5.18	>100
24	G4a		0.069	59.1	19.3	1.4	2.94	93.9	-3.58	>100
25	J4a		0.112	27.6	67.8	5.1	2.11	132.7	-4.62	>100

^aKey: Capital letter (recognition element), number (linking group), lower case letter (electrophilic warhead).

^bdetermined using ADC (v12.01);

^cPAMPA (Parallel Artificial Membrane Permeability Assay) average for n = 3;

^dMTT assay;

^einactive = kinetic data not reproducible due to weak activity;

^fnd : not determined.

Table 3Kinase activity profiles for **12** and **13**; staurosporine (staur)

Kinase	staur	12	13
ATK1	61	24	20
Aurora A	84	47	39
CAMK4	88	27	7
CDK2	58	10	0
CHK1	86	97	18
CK1a1	51	0	0
CK1g1	38	16	23
CK2a1	64	16	9
DAPK1	86	13	4
GSK3B	8	48	9
IKKb	64	0	0
IRAK	85	0	10
LCK	89	20	4
MAPKAPK2	100	0	0
MINK	98	17	5
p38a	11	2	6
PAK1	84	0	0
ROCK1	85	19	6
TAK1	81	52	9

Single point kinase inhibition data for compounds tested at 10 μ M.