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Virtual screening to identify lead inhibitors for bacterial NAD synthetase (NADs)

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ABSTRACT

Virtual screening was employed to identify new drug-like inhibitors of NAD synthetase (NADs) as antibacterial agents. Four databases of commercially available compounds were docked against three subsites of the NADs active site using FlexX in conjunction with CScore. Over 200 commercial compounds were purchased and evaluated in enzyme inhibition and antibacterial assays. 18 compounds inhibited NADs at or below 100 μM (7.6% hit rate), and two were selected for future SAR studies.

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With the increasing threat of pathogens, such as *Bacillus anthracis*, being used as bioweapons,[1] and the rise in the incidence of multi-drug resistant bacteria,[2] the need for new antibiotics that act at novel targets has never been greater. Previous studies within this group[3–5] have revealed that inhibition of one such target, the amidotransferase enzyme nicotinamide adenine dinucleotide (NAD) synthetase (NADs), could hinder both spore outgrowth and vegetative growth, which would provide antibacterial action at two different steps in the bacterial life cycle.[6–10]

The first class of NADs inhibitors designed by this group consisted of tethered dimers that contain two hydrophobic groups linked by a polymethylene tether, and a positively charged nitrogen on one end.[3–5] These inhibitors were antibacterial, and there was a correlation between the potencies of enzyme inhibition and antibacterial effects. However, the permanent positive charge and detergent-like properties of this class of compounds were unattractive for further drug development.[11,12] More drug-like lead inhibitors were, therefore, sought.

Virtual screening of compound databases using the detailed structure of the drug target can serve to greatly enhance success in the lead discovery process.[13–17] Here we use the *in silico* screening program FlexX 1.20.1 (BiosolveIT GmbH®) for the virtual screening of commercially available compounds within the catalytic site of NADs to identify new classes of lead inhibitors. In this study, four commercial compound databases were filtered according to Lipinski's rule of 5 using Tripos' program Unity: Maybridge (58,650 after filtering), ChemBridge (404,132), Tripos' LeadQuest (72,660), and ComGenex (82,737). Because these docking studies predate our solution of the crystal structure of *B. anthracis* NADs (PDB code 2PZB),[18] the highest available resolution crystal structure of *B. subtilis* NADs,[19] reported by our group, was utilized for docking (PDB code 1KQP[19]). The crystal structures of *B. anthracis* and *B. subtilis* NADs reveal that the binding sites are nearly identical, with all active site residues being conserved.[18]

NADs is a large homodimer of approximately 60 kDa that contains two identical binding sites. The crystal structure of the protein from *B. subtilis* reveals two identical long, linear binding sites containing the adenylated reaction intermediates lying partly within the dimer interface on the NaAD end, and in a buried cavity within one monomer on the ATP end. Due to the enormity of the NADs homodimer catalytic site, and considering our limited computational resources at that time, three smaller binding subsites were constructed to be used in the virtual screening study. To accomplish this, a sphere with radius 25 Å around one of the bound intermediates was extracted from the whole protein structure to produce a partial protein structure which consisted of the three shells of amino acid residues immediately surrounding the binding cavity and which fully contained one complete binding site. All crystallographic waters and metals were removed, hydrogens were added, and the protonation states of active site residues were adjusted to their dominant ionic forms assuming a local physiological pH. The "active site," as needed for use by FlexX, was further defined by creating a smaller sphere of radius 17 Å which consisted

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of the first two shells of amino acids surrounding the bound substrate, resulting in a rather large active site: 31 Å in length, and a width ranging from 7 Å on the NaAD end to 16 Å on the ATP end.

As explained earlier, the complete catalytic site was then divided into three overlapping subsites: the NaAD binding subsite, the ATP subsite, and a center subsite which bridges the two end sites. The resulting NaAD binding subsite is the most confined and is approximately 16 Å long and 7 Å wide, appearing as a "canyon" near the homodimer interface; the center subsite is shaped like a tunnel, and is 14 Å long and 9 Å wide; the ATP subsite is buried within a single monomer and is the largest of the three at 21 Å long and 16 Å in width. The bound ligand was excluded from all docking runs.

Each of the four commercial databases was docked into each of the three subsites employing FlexX 1.20.1, which has been shown to be suitable for exploring many kinds of binding sites,[14,20] and routinely produces hit rates comparable to other highly regarded programs.[21–23] FlexX was accessed using the SYBYL 6.9 suite of programs (Tripos, Inc.®), and default parameters were used for each docking run. For our purposes, automatic base fragment selection was employed. Within each of the three subsites, the core subpocket was defined as all residues which interact directly with the bound substrate. Formal charges were assigned, and 5 poses for each ligand were saved. Docking began on a 64 bit dual processor SGI Octane computer running Unix, and was completed in parallel using a 64 bit PQS 4-processor Opteron Quantum Cube running Linux. After all databases were screened against all sites and ranked according to FlexX score, the best poses from each run were combined and re-ranked using a consensus scoring[24] program, CScore.[25] A total of 22,240 compounds were ranked with CScore, and all compounds with a CScore of 5 were reviewed according to several criteria: realistic orientation within the binding pocket, a predicted binding conformation that is energetically reasonable, structures that are chemically simple and can be easily modified synthetically, and compounds representative of chemically diverse structural classes that are considered medicinally interesting. Additionally, selected compounds with both a CScore of 4 and a good FlexX score were reviewed if they were structurally unique. Representatives from the most interesting structural classes were purchased and screened in our NADs enzyme inhibition and *B. anthracis* antibacterial assays.

The high-throughput assay utilized by us for previous synthetic NAD synthetase inhibitors[11] monitored production of NAD via enzymatic conversion to NADH, and the latter was detected by both fluorescence and UV absorption. However, this assay was unsuitable for many commercial compounds because they interfered with the fluorescence and/or absorbance at the wavelengths observed. Further, some compounds gave false positives due to direct reaction with NADH. Therefore, an alternate HPLC assay was designed and is presented here for the first time.

In this new assay the reaction product NAD was directly monitored. Sample plates were prepared using a BioMek® FX liquid handling system and the reaction volume was 200 μL. The reaction mixture contained 60 mM HEPPS, pH 8.5, 0.5 mM NH_4Cl, 20 mM KCl, 10 mM $MgCl_2$, 0.1 mM NaAD, 0.2 mM ATP, 6 μg/ml purified *B.* anthracis NADS, 2.5% (v/v) DMSO, 0.3% BOG and inhibitors at various concentrations. Compounds were assayed beginning at 600 μM and at doubling dilutions down to 0.6 μM. The reaction was initiated by adding 0.2 mM ATP, and quenched after 10 min by adding 50 μL of 6 M guanidine-HCl. The plates were sealed by aluminum tape, and centrifuged at 2500 rpm for 10 min in order to pellet any precipitation that may have been caused by the inhibitors. Plates were stored at 4 °C prior to the HPLC analysis.

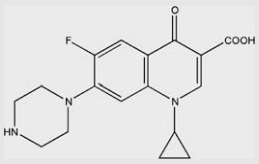
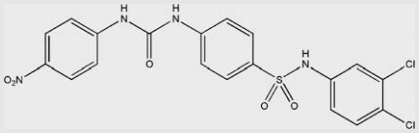
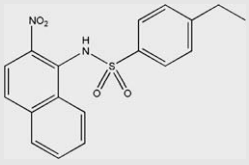
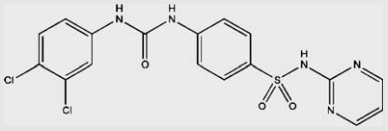
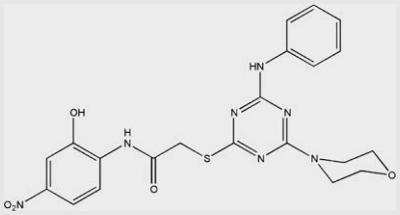
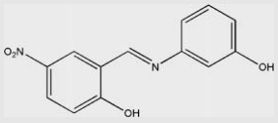
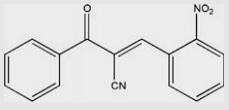
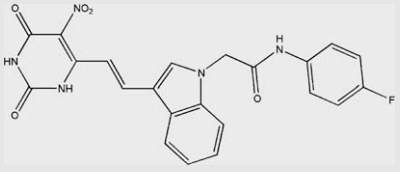
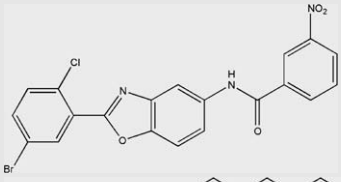
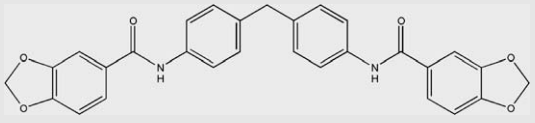
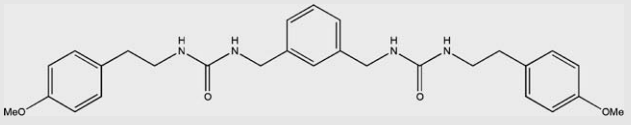
The HPLC procedure utilized a Gilson® 215 liquid handler, two Gilson® 306 pumps, and a Gilson® 170 diode array detector. A Phenomenex® Luna 5 μm, C5, 100 Å, 100 × 4.60 mm column was used for separations. The mobile phase was **A**: 20 mM NaH_2PO_4 pH 6.90 and **B**: acetonitrile. The gradient was 100% **A** from 0–3 min, to 5% **A**/95% **B** from 3–4 min for each 20 μL injection. The flow rate was 1.0 mL/min and DAD detection was 190–400 nm. Peak height estimation for NAD was based on baseline integration. The percentage inhibition at each inhibitor concentration was calculated by the difference in peak height of NAD compared to reactions without inhibitor. The IC_{50} was determined from the plot of NAD peak height vs. inhibitor concentration, and is defined as the concentration of inhibitor required to produce NAD peak height at 50% of the uninhibited reaction. Peak areas were used to calculate the IC_{50} for selected active compounds, and similar results were obtained. Each compound was tested in duplicate, and the IC_{50} is reported as the average IC_{50} obtained from duplicate runs. False positives due to promiscuous inhibition were excluded by including detergents in the inhibition assay.

All purchased commercial compounds were also screened against *Bacillus anthracis Sterne* in an antibacterial assay as previously reported[8,11] with the following modifications. *B. anthracis Sterne* spores were subcultured from stock cultures into Luria-Bertani (LB) broth and incubated for 2–3 h at 37 °C in ambient air until the OD_{600} measurement reached 0.5 to 0.6, when the bacteria were in mid-log phase. The cultures were diluted 1:1 into LB Broth with

Table 1
Commercial compounds identified by FlexX studies to be NAD synthetase inhibitors at or below 300 μM, the subsites in which they were predicted to bind, and their biological activities

ID	MW	NADs subsite	IC_{50} (μM)	MIC_{100} (μM)
5379	278.27	NaAD	51	120
5588	466.84	ATP	78.5	>215
5589	378.34	center	136.6	>264
5591	364.32	center	160	>274
5597	446.48	ATP	86.1	>224
5599	356.40	center	168.1	3.8
5604	450.54	ATP	141	>222
5605	368.37	ATP	145.9	>259
5606	422.37	center	141.1	>237
5609	490.61	ATP	70	>204
5615	449.40	ATP	55.4	>223
5616	404.21	center	207.5	>247
5617	438.29	center	77.5	15
5660	258.23	NaAD	22.5	>387
5679	303.71	NaAD	262	>329
5684	440.26	NaAD	99.5	>227
5691	430.25	NaAD	106	>232
5707	424.43	ATP	253	>240
5710	327.39	NaAD	128.5	>240
5724	443.44	NaAD	290.6	>240
5731	506.92	center	270.7	>240
5737	354.39	NaAD	235.3	>240
5749	527.76	NaAD	219.8	>240
5763	472.89	NaAD	232.1	>240
5764	505.96	NaAD	97.2	>240
5768	455.50	center	170.5	>240
5775	432.33	NaAD	290	>240
5785	426.39	center	108.6	>240
5792	346.35	NaAD	76	>240
5793	465.52	NaAD	78.8	>240
5798	472.68	NaAD	61.8	>240
5799	479.45	NaAD	174.8	>240
5802	411.42	NaAD	225.2	>240
5806	413.44	NaAD	67.8	>240
5807	401.40	NaAD	123.9	>240
5815	404.47	NaAD	185.6	>240
5818	494.51	NaAD	65.7	>240
5821	411.80	NaAD	103.6	>240
5822	424.46	NaAD	107.1	>240
5824	481.32	NaAD	10	1.9
5830	441.49	NaAD	198.2	>240
5831	451.89	NaAD	243.3	>240
5833	483.51	NaAD	78.3	15

Table 2
Examples of NADs inhibitors from the most common structural classes identified through in silico screening

Cmpd. ID	Structure	NADs IC_{50} (μM)	B.a. MIC (μM)
Cipro		–	0.5
5824		10	1.9
5599		168.1	3.8
5617		77.5	15
5833		78.3	15
5660		22.5	>387
5379		51	120
5615		55.4	>223
5798		61.8	>240
5818		65.7	>240
5609		70	>204

an absorbance at 600 nm measuring 0.25–0.3, then were added to plates containing 240 μM samples of the compounds to be tested. Compounds were tested at a final DMSO concentration of 1%. The plates were incubated at 37 °C, and absorbance at 600 nm was read at 0 h and every hour for 5 h. Any compounds which inhibited growth of the vegetative cell (as compared to the control containing only DMSO) were screened in a full MIC determination starting at 240 μM and creating doubling dilutions down to 1.88 μM in quadruplicate wells. A plot of cell density vs. time yields inhibition of growth results, and the MIC is defined as the lowest concentration of compound required to completely inhibit growth (100% inhibition). MIC_{100} is reported as the average of the four data points acquired for each compound. Controls for each assay measured sterility, *B. anthracis Sterne* viability, and included a commercial antibiotic positive control (ciprofloxacin hydrochloride from MP Biomedicals).

Among the NADs subsites, the best FlexX scores were obtained from docking in the larger ATP subsite, presumably due to the many residues capable of charge-charge interactions. A total of 211 commercial compounds were purchased based on the CScore rankings: 135 from the NaAD, 31 from the center and 45 from the ATP subsites; 43 (20%) of those compounds were found to have IC_{50}'s less than or equal to 300 μM against NADs (Table 1). It should be noted that ranking compounds solely by their FlexX scores produced fewer hits than when compounds were ranked using consensus scoring. At 100 μM or below, 16 compounds (7.6% hit rate) were active against NADs (a cutoff routinely used to define virtual screening hit rates)[15,17,26], while 4 were active at or below 50 μM. The hit rate at 100 μM is similar to those obtained by other virtual screening studies against different enzymatic targets.[17,26,27] Of these active compounds, 27 inhibitors resulted from their predicted binding in the NaAD subsite, while 9 and 7 were predicted to bind in the center and ATP sites, respectively. The hit rates (100 μM) based on the number of compounds purchased from the NaAD, center, and ATP subsites were 8.1%, 6.5%, and 8.9%, respectively. Only a few compounds scored well in more than one subsite, and none of those screened were enzyme inhibitors.

The most significant result of this study was the identification of drug-like compounds that have good activities against both NADs and *B. anthracis*: **5617**, **5824**, and **5833**. However, unlike our earlier tethered dimer inhibitors, there is a poor correlation between enzyme inhibition and antibacterial effects. Several enzymatically inactive commercial compounds were found to behave as antibacterial agents, while only 4 compounds that inhibited NADs were also effective against the vegetative cell, with MIC's at or below 15 μM. As mentioned earlier, this is in contrast to our results for earlier libraries of tethered dimer NADs inhibitors, which exhibited a linear correlation between enzyme inhibition and antibacterial activity.[10] Possible explanations for active enzyme inhibitors that do not show a good MIC include: (1) low permeability into the bacterial cell; (2) loss via efflux pumps;[28] (3) metabolism by the bacterial cell into inactive forms. It can also be inferred that those compounds which confer antibacterial activity against the vegetative cell but do not inhibit NADs must be acting on a different target(s). Our preliminary studies support the identity of a second target that explains the antibacterials with no enzyme activity, and these results will be reported separately upon completion.

Among the enzyme inhibitors identified, several different structural classes have emerged (Table 2), and those that also inhibit bacterial growth are considered most interesting for further optimization. **5379** is an acrylonitrile—potentially a good Michael acceptor, and thus not an ideal drug candidate. Other structural classes that produced NADs inhibitors include sulfonamides (**5599**, **5617** and **5824**), ureas (**5609**, **5617**, and **5824**), complex amides (**5615**, **5798**, **5818** and **5833**), and Schiff bases (**5660**). Except for **5833**, all of the antibacterial inhibitors (**5599**, **5617** and **5824**) contain a sulfonamide, a urea, or a combination of both. While all four of these antibacterial inhibitors meet the requirements for moderate molecular weight in a drug-like structure, with the possibility for further analog generation, we selected **5617** and **5824** as compounds that best meet these requirements. **5833** appears less suitable for facile synthetic modifications, and the *o*-nitronaphthylamine moiety of **5599** contains two lower ranking functionalities relative to drug potential (e.g., the nitro and naphthalene groups). Compounds **5617** and **5824** reveal some similarities; both contain three aryl rings linked by a urea and a sulfonamide, and both contain a 3,4-dichlorophenyl ring. This class of urea-sulfonamides was chosen for future SAR analysis via parallel library synthesis.

During submission of this report, a related online prepublication[29] appeared describing modest inhibitors of NADs from mycobacteria—the only other reported inhibitors of NADs—although these compounds did not block mycobacterial growth.

In conclusion, virtual screening has provided the first reported drug-like small molecule inhibitors of NAD synthetase with antibacterial activity.

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Supplementary data

Supplementary data, including (a) structures of compounds from Table 1 not shown in Table 2, (b) graphical representations of the binding sites used and poses of selected docked ligands, and (c) a sample HPLC-chromatogram used in the enzyme assay. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.02.034.

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