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Identification of Low-Molecular-Weight Compounds Inhibiting Growth of Corynebacteria: Potential Lead Compounds for Antibiotics

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The bacterial genus Corynebacteria contains several pathogenic species that cause diseases such as diphtheria in humans and "cheesy gland" in goats and sheep. Thus, identifying new therapeutic targets to treat Corynebacteria infections is both medically and economically important. CG2496, a functionally uncharacterized protein from *Corynebacterium glutamicum*, was evaluated using an NMR ligand-affinity screen. A total of 11 compounds from a library of 460 biologically active compounds were shown to selectively bind CG2496 in a highly conserved region of the protein. The best binder was identified to be methiothepin ($K_D = 54 \pm 19$ μM), an FDA-approved serotonin receptor antagonist. Methiothepin was also shown to inhibit the growth of *C. glutamicum*, but not bacteria that lack CG2496 homologs. Our results suggest that CG2496 is a novel therapeutic target and methiothepin is a potential lead compound or structural scaffold for developing new antibiotics specifically targeting Corynebacteria.

The genus *Corynebacterium* consists of nearly 70 species and is closely related to the genera *Mycobacterium*, *Nocardia*, and *Rhodococcus*. The characteristic traits of Corynebacteria include cells that are shaped like straight rods with clubbed ends, as well as an extra cell wall layer consisting of mycolic acids covalently bound to the peptidoglycan layer which adds an additional layer of protection against antibiotics. Corynebacteria are very well studied and outstandingly important for the large scale biotechnological production of amino acids and nucleotides.[1] Moreover, Corynebacteria produce several pathogens that affect humans and livestock. Toxins produced by *C. diphtheriae* and *C. ulcerans* cause diphtheria, a highly contagious respiratory infection in humans,[2] or diphtheria-like symptoms,[2,3] respectively. *C. pseudotuberculosis* causes "cheesy gland" disease in goats and sheep resulting in significant economic losses.[2,4] Moreover, while rare, many other Corynebacteria species have also been shown to cause infections. Therefore, elucidating the functional roles of uncharacterized proteins from Corynebacteria is of high biomedical and economic importance.

Protein CG2496 from *Corynebacterium glutamicum* (UniProtKB ID: Q6M3G5, Q8NNC9; Gene ID: CG2496, Cgl2275) is predicted to be an integral membrane protein comprised of 684 amino acids, where the N-terminal and C-terminal polypeptide segments relative to a single transmembrane helix are extracellular and cytoplasmic, respectively. In the NCBI RefSeq and Uni*i*ProtKB[5] databases, CG2496 is annotated as a chromosome segregation ATPase, but there is currently no experimental evidence for this particular annotation. Homologous proteins are found in genomes of 43 other species of Corynebacteria. A significant portion of the N-terminal domain of CG2496 (residues 63–171) belongs to the TPM domain (named after proteins **T**LP18.3, **P**sb32 and **M**OLO-1) family (Pfam[6] accession: PF04536), which currently contains 3085 protein sequences from 1821 species, including bacteria, plants, protozoa and lower metazoa, such as nematodes and lancelets. Two TPM domain-containing proteins, TLP18.3 from *Arabidopsis thaliana* and Psb32(Sll1390) from *Synechocystis sp.*, were shown to be involved in the photosystem II (PSII) repair cycle.[7] Phosphatase activity was reported for TLP18.3;[7b] however, the measured enzymatic activity levels were very low. In *Caenorhabditis elegans*, the TPM domain protein MOLO-1 acts as a modulator of the levamisole-sensitive acetylcholine receptor (L-AChR), but the function of TPM domain proteins in other organisms is still unknown. The Northeast Structural Genomics Consortium (NESG; http://www.nesg.org) recently determined the solution NMR structure[8] of the TPM domain of CG2496 comprising residues 41–180 (PDB ID: 2KPT; NESG target ID: CgR26 A) which revealed a distinct architecture and provided the first structural representative for PF04536.

Here we describe the identification of low-molecular-weight compounds binding to the extracellular N-terminal domain of protein CG2496 from *C. glutamicum* using an NMR-based screening approach (FAST-NMR).[9] We expect that the newly identified compounds will also support future functional characterization of protein CG2496. Furthermore, assuming that protein CG2496 plays an important role for proliferation of *C. glutamicum*, we investigated to which extent one of the compounds, that is, methiothepin, inhibits cell growth.

1D ^{1}H NMR screening of the FAST-NMR library of 460 low-molecular-weight compounds resulted in 13 initial hits which

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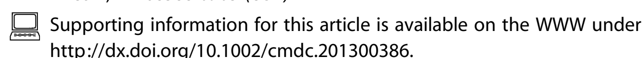

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Table 1. List of compounds from the FAST-NMR screen that bind CG2496(41–180)

Compound name	1D Hits[a]	2D Hits[b]	Perturbed residues[c]
N-Succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide	×	×	Y53, N56, V168
4-Methylpyrazole	×	×	Y53, N56, V168
Bay 11-708	×	×	Y53, N56, V168
Histamine	×	×	Y53, N56, V168
Methiothepin	×	×	Y44, L46, Y53, N56, T58, G62, V79, V90, D96, T104, N111, G112, G114, V116
Adenine	×	×	Y53, N56
1-Methylimidazole	×	×	Y53, N56, V168
Ethacridine	×	×	L46, Y53, N56, T58, F149, L162, V168
Adenosine-5′-triphosphate[d]		×	N56, T58
Serotonin[d]		×	Y53, N56, T58
Adenosine-5′-monophosphate[d]		×	Y53, N56, V168

[a] Indicates compounds that exhibited line broadening in 1D ^{1}H NMR screen in the presence of CG2496(41–180). [b] Indicates compounds that caused peak perturbations in the 2D [^{1}H,^{15}N]-HSQC of CG2496(41–180). [c] Identity of the residues (one-letter code) that were significantly perturbed (>1 standard deviation from mean perturbation) in the 2D [^{1}H,^{15}N]-HSQC of CG2496(41–180). [d] These compounds were evaluated in the 1D ^{1}H NMR screen but exhibited no line broadening. Evaluated in 2D [^{1}H,^{15}N]-HSQC screen to test potential CG2496(41–180) functions.

showed broadening of ^{1}H NMR peaks in the presence of CG2496(41–180) (Table 1). Specific interaction with CG2496(41–180) was revealed by assessing chemical shift perturbations in 2D [^{15}N,^{1}H]-HSQC spectra recorded for CG2496(41–180) in the presence of each of the 13 compounds. Additionally, five more compounds from the library, which did not show line-broadening in the 1D 1H NMR screen, were evaluated using the 2D [^{15}N,^{1}H]-HSQC screen. These compounds were selected to test hypotheses of CG2496(41–180) function. Of the 18 total compounds screened by 2D [^{15}N,^{1}H]-HSQC, 10 induced minor perturbations of a small number (<7) of 2D [^{15}N,^{1}H]-HSQC peaks. Only one compound, methiothepin, induced a significant number (≈25) of chemical shift perturbations in the 2D [^{15}N,^{1}H]-HSQC spectrum of CG2496(41–180) (Figure 1). An NMR titration of CG2496(41-180) with methiothepin determined a dissociation constant (K_D) of 54±19 μM. Intriguingly, residues exhibiting significant chemical shift perturbations upon the binding of methiothepin are strongly conserved within the TPM domain family and are part of a mostly neutral and hydrophobic surface cleft (Figure 2), which was previously predicted as the putative active site.[8] Every compound that induced a chemical shift perturbation in the 2D [^{15}N,^{1}H]-HSQC showed a perturbation for N56, while 10 of 11 of these compounds showed perturbations for Y53. These two residues are also found in the predicted active site. Notably, methiothepin is a serotonin receptor antagonist commonly used as a United States Food and Drug Administration (FDA) approved antipsychotic drug.[10]

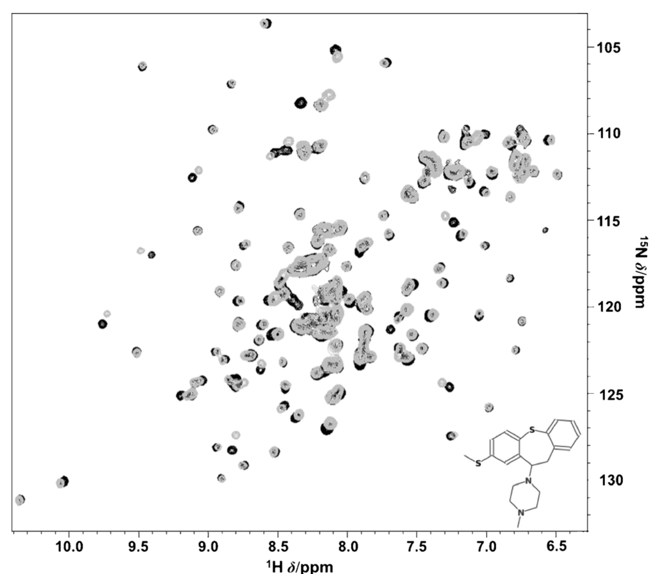

Figure 1. An overlay of 2D [^{15}N,^{1}H]-HSQC spectra of free CG2496(41–180) (black) and CG2496(41–180) bound with methiothepin (gray). The chemical structure of methiothepin is displayed in the lower right.

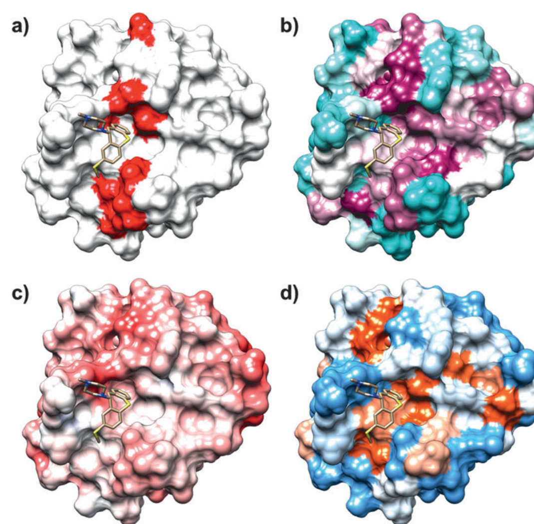

Figure 2. a) The CG2496(41–180)–methiothepin complex generated by AutoDock where residues with significant chemical shift perturbations are colored red. b) ConSurf[16] residue conservation surface representation of the CG2496(41–180)–methiothepin complex where highly conserved residues are magenta and poorly conserved residues are cyan. c) UCSF Chimera[17] hydrophobicity surface representation of the CG2496(41–180)–methiothepin complex where the hydrophilic surface is blue and hydrophobic surface is orange. d) Delphi[18] electrostatics surface representation of the CG2496(41–180)–methiothepin complex where the positively charged surface is blue and negatively charged surface is red.

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Despite the available three-dimensional structure and similarity with TPM domains of known function, the function of the CG2496(41–180) domain remains unknown. Sequence similarity searches with BlastP[11] identify only uncharacterized proteins from Corynebacteria, while searches for structurally similar proteins with PDBeFold[12] and DALI[13] identify several phosphatases and the C-terminal domain of an alanyl-tRNA synthetase. However, our binding results did not indicate any interaction with typical phosphatase substrates, such as *O*-phospho-L-serine. The STRING[14] database indicates relationships with primarily hypothetical proteins; however, a gene encoding a dGTP hydrolase and a gene encoding a histone N-acetyltransferase are loosely associated with CG2496(41–180). Furthermore, a comparison of the CG2496(41–180)–methiothepin binding site to a database of protein–ligand binding sites using CPASS[15] did not result in any hits above a 30% similarity threshold, which is the minimum score used to consider two proteins to have structurally and functionally similar binding sites.[15b]

Next, assuming that protein CG2496 plays an important role for *C. glutamicum*, we investigated if methiothepin inhibits cell growth. A disk diffusion assay (Figure 3) shows that methiothepin does indeed inhibit the growth of *C. glutamicum*. Additionally, a comparative minimum inhibitory concentration (MIC) test for methiothepin was performed for *C. glutamicum* and *Staphylococcus aureus*, which does not possess a homolog of CG2496. *C. glutamicum* can only grow in media with up to 20 mM methiothepin. Conversely, *S. aureus* is able to grow at higher methiothepin concentrations of at least 40 mM. The low solubility of methiothepin in complex cell culture media prevented the use of higher concentrations and the determination of a reliable MIC value. However, the growth inhibition of *C. glutamicum* by methiothepin and the corresponding lack of activity against *S. aureus* suggest CG2496 is the in vivo target of methiothepin. Correspondingly, methiothepin would be expected to be active against other Corynebacteria containing a homolog of CG2496.

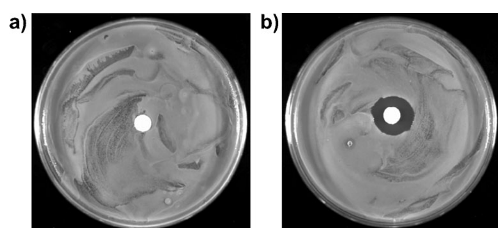

Figure 3. Disk diffusion assay. *C. glutamicum* was plated and grown in the presence of a) a disk soaked with water and b) a disk soaked with an aqueous solution containing 400 μM methiothepin.

A tiered ligand-affinity screen using the FAST-NMR approach revealed that methiothepin, an FDA-approved drug, binds to CG2496(41–180) and also inhibits the growth of *C. glutamicum*. The presence of CG2496 homologs in *Corynebacterium spp.* pathogens (e.g., the genomes of *C. ulcerans*, *C. diphtheriae*, and *C. pseudotuberculosis* encode homologs of CG2496 with 46%, 38%, and 43% sequence identity, respectively) suggests that methiothepin may bind to these proteins as well and may also act as an antibiotic for these species. These results identify the functionally uncharacterized CG2496 protein and its homologs as novel targets for drug discovery, and methiothepin as a potential lead compound to develop a new line of antibiotics against Corynebacteria.

Experimental Section

Details of the FAST-NMR ligand affinity screens, the CG2496(41–180)-methiothepin NMR titration experiment, the generation of the of CG2496(41–180)–methiothepin complex structure, and the disk diffusion assay are provided in the Supporting Information.

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