Discovery of a Structurally Unique Small Molecule that Inhibits Protein Synthesis

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INTRODUCTION

The advent of antibiotic use in the 1940’s dramatically improved infectious disease outcomes, preventing countless complications and deaths over the past seven decades. Nevertheless, the overuse of antibiotics in modern medicine, livestock, and agriculture has allowed for the expansion of drug-resistant strains, limiting the clinical efficacy of many drugs and instigating the augmenting global threat of antibiotic-resistant outbreaks [1].

Resistance to one antibiotic often severely limits the clinical utility of the entire class of structurally similar drugs. Examples include the expression of beta-lactamase in resistance to penicillins, expression of export proteins in resistance to tetracyclines, and mutation of ribosomal proteins in resistance to aminoglycosides like streptomycin [2]. Clinically, antibiotic repertoires are severely bound by the limited number of antibiotic structural classes as well as the limited number of molecular targets like prokaryotic ribosomes and bacterial cell wall enzymes [3]. Finding new structural or target-based classes of antibiotics that act by novel mechanisms is one strategy to combat the persistence of drug-resistance in the clinic.

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†Abbreviations: CFPS, Cell Free Protein Synthesis; poly(U), Polyuridylic acid; poly(Phe), Polyphenylalanine; DMSO, Dimethylsulfoxide; FAGE, Formaldehyde agarose gel electrophoresis; MOPS, 3-(N-morpholino)propanesulfonic acid; PEG20k, Poly(ethylene glycol) 20k; MPD, 2-methyl-2,4-pentanediol; n-BuLi, n-butyllithium; THF, Tetrahydrofuran; TBAF, Tetrabutylammonium fluoride; TLC, Thin layer chromatography; CAM, Ceric ammonium molybdate; NMR, Nuclear magnetic resonance; s, Singlet; d, Doublet; m, Multiplet; br, Broad; J, Coupling constant; Hz, Hertz; pT7, T7 promoter; prom, Promoter; term, Terminator; RBS, Ribosome binding sequence (AGGAGA); Stop, Tandem stop codons (TAATAA); ori, Origin; amp, Ampicillin; R, Resistance.

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In this study, we discuss a small molecule with translation-inhibiting properties. It was chosen for further study among other hits from high-throughput screening because of a structure that is unlike any known class of antibiotics. The compound, T6102, has a hydrophobic adamantane group and is reminiscent of the structures of current drugs used for neurological disease, diabetes, and viral infections, such as amantadine and rimantadine, as well as of investigational drugs such as the antitumor compound 2, 2-Bis (4-(4-amino-3-hydroxyphenoxy)phenyl) adamantine [4]. Adamantane structures are increasingly utilized in medicinal chemistry due to their versatility as rigid scaffolds, steric bulk for protecting intramolecular reactions, and lipophilic groups to increase partition coefficients [5]. Adamantane derivatives, due to their hydrophobicity, have also been used as substrituents in inhibitor design to bind to hydrophobic clefts in protein targets [6]. T6102, for example, was originally synthesized as a precursor for compounds used for hydrophobic tagging of target proteins to induce proteasomal degradation [7,8].

As far as antibiotics go, there have been reports of a few potential novel antimicrobials with adamantane moieties including antitycobic bacterial compounds (SQ109, N’-(Adamantan-2-ylidene)thiophene-2-carboxyazide, 17, 4-(adamantan-1-yl)quinoline, substituted 5-(1-adamantyl)-1,2,4-triazole-3-thiols) [9-12] and antimalarial compounds (adamantyl dihydroartemisinin) [13]. However, unlike T6102, most of these involve derivatization of current antimicrobial structures. The antitycobic bacterial compound SQ109, for example, was identified in a screen for compounds with activity against tuberculosis from a library of derivatives of ethambutol, an antibiotic that is currently one of the first line treatments against tuberculosis infections [5]. While T6102, like these investigational drugs, also contains an adamantane group, T6102 is unique in that the rest of its structure does not resemble any known class of antibiotics.

There is clearly a dire need for novel, structurally unique antibiotics that interact with a variety of potential targets by diverse mechanisms. Here we report the discovery and synthesis of a new compound, T6102, which
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Inhibits bacterial processes both in vitro and in vivo (in Bacillus subtilis) and may be a useful starting point for the development of structurally novel antibiotics.

RESULTS

In order to identify small molecule inhibitors of prokaryotic translation, we developed an economical, high-throughput, Escherichia coli lysate-based, cell-free protein synthesis screen for luciferase synthesis inhibition with a kanamycin positive control (Figure 1). 872 crude extracts of natural products from the Yale Rainforest Natural Product Collection [14] and 286 synthetic compounds from the Yale Compound Repository (Yale Center for Molecular Discovery, New Haven, CT) were screened. Among synthetic assay hits, including some that resembled currently utilized antibiotics (Figure 2), T6102 (Figure 3a) was the most structurally unique compared to known classes of antibiotics and was chosen for further analysis. We synthesized T6102 for additional testing of inhibition of the cell-free protein synthesis reaction, and this consistently revealed dose-dependent inhibition as assayed by luciferase activity, with an IC$_{50}$ of 453.7 +/- 39.3 uM (Figure 3b).

The cell-free protein synthesis system used for this assay was modified from Jewett et al, 2008 [15] and involves four major reactions: transcription, translation, energy regeneration, and aminoacyl-tRNA charging, in addition to luciferase activity. In order to investigate the mechanism of protein synthesis inhibition by T6102, inhibition of the cell free protein synthesis reaction was assayed by incorporation of radiolabeled amino acids with different templates. T6102 exhibited dose-dependent inhibition of protein synthesis from a circular luciferase DNA template (Figure 4a), further validating the initial hit and suggesting that T6102 inhibits protein synthesis rather than primarily inhibiting luciferase activity. When the same reaction was incubated with luciferase mRNA in vitro-transcribed separately by run-off transcription, T6102 again exhibited similar dose-dependent inhibition.
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Testing for synergy on bacterial growth inhibition when combined with other known classes of antibiotics may provide additional insights into the mechanism of action of T6102’s antibacterial activity.

DISCUSSION

While the search for new antibiotics has yielded several prokaryote-specific translation inhibitors that have been utilized in the clinic or have provided insight into the structure and function of the ribosome, the current repertoire of antibiotics is insufficient to combat the rise of drug-resistant bacteria. It is well known that bacterial resistance to one antibiotic often renders resistance to the entire class. Thus novel classes of antibiotics are needed to meet current and future therapeutic needs.

Here we describe the discovery and characterization of one potential candidate for a structurally novel class of antibiotics, T6102. Through in vitro assays, we demonstrate that T6012 specifically inhibits bacterial translation, rather than acting through inhibition of transcription, inhibition of energy regeneration, or nonspecific disruption of cellular function. Moreover, through in vivo growth inhibition assays, we show that T6102 inhibits the growth of B. subtilis in culture, making T6102 a promising antibiotic candidate.

One limitation in the study of T6102 was its relative insolubility and precipitation at concentrations higher than 1,500 uM. Along these lines, inhibition of human cell growth could not be reliably assessed due to variability in cell growth and precipitation at high concentrations (data not shown). Nevertheless, chemical analogs of T6102 that may have improved aqueous solubility and potency should be pursued and assessed for eukaryotic cell toxicity to assess potential for clinical antibiotic use.

Figure 3. T6102 exhibits dose-dependent inhibition of protein synthesis. (a) Chemical structure of T6102. (b) Percent inhibition of the Cell Free Protein Synthesis (CFPS) assay, from a circular DNA template and measured by luciferase activity, is plotted against the log (base 10) of the concentration of T6102 incubated in the protein synthesis reaction. Error bars represent the standard deviation for each condition.
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through a novel antimicrobial mechanism. T6102, unlike previously reported antimicrobial agents, appears structurally unlike any known class of translation inhibitors and may thus represent a novel class of antibiotics. In this study, T6102 was also shown to specifically inhibit bac-

terial translation in vitro as well as B. subtilis growth in vivo, making it a promising antibiotic candidate. In the growing context of emerging resistant infectious agents, novel classes of antibiotics, such as one suggested by T6102, are urgently needed to meet current and future therapeutic needs.

METHODS

Cell-Free Protein Synthesis Reaction. An Escherichia coli in-house lysate-based cell-free protein synthesis system similar to Seidelt et al, 2009 [17] was optimized for reporter protein, DNA or RNA template,
time, temperature, Mg\(^{2+}\) concentration, volume, T7 RNA polymerase concentration, and organic solvent tolerance. Cell-free protein synthesis reactions were set up according to [17] with the following exceptions: Reactions were performed in 25 uL final volumes with 15 mM magnesium glutamate (Sigma), 60 mM sodium pyruvate (Sigma), 8 mM sodium oxalate (Sigma), 170.6 ug/mL *E. coli* MRE-600 tRNA (uncharged) in water (Sigma), 15 mg/mL circular pIVEX-2.3d-luc (Figure 1b) DNA, 1 mg/mL T7 RNA polymerase (in house), and 2 percent dimethylsulfoxide (DMSO) (Sigma). Reactions were incubated for 1.5 hours at 30°C after which kanamycin (Sigma) was added to a final concentration of 1.2 uM to stop the reaction.

**Luciferase Assay.** 180 uL of room-temperature luciferase stabilization buffer [18] was added to each well of a black, opaque-bottom 96-well plate (Corning). 20 uL of each completed 25 uL cell-free protein synthesis reaction was added to individual wells. On a Synergy 4 Plate Reader (Biotek) at 25°C, background luminescence was measured, 15 uL of Steady-Glo Luciferase Assay reagent (Promega) at 25°C was added to each well, the plate was shaken for 10 seconds, and luminescence was measured. Final luminescence per sample was calculated by subtracting the sample’s background luminescence units from the sample’s measured luminescence units.

**Protein Synthesis with Radiolabeled Amino Acids.** 25 uL cell-free protein synthesis reactions were performed as described above with the following exceptions: the 2 mM L-amino acids alanine, arginine, aspartic acid, glutamic acid, histidine, isoleucine, leucine, lysine, phenylalanine, serine, threonine, tryptophan, and valine were replaced with their \(^{14}\)C-labeled counterparts (Perkin-Elmer NEC445E) at 0.1 mCi/mL. Completed reactions were assayed for protein incorporation of radioactivity by trichloroacetic acid precipitation as previously described [19].

**Poly-U Translation.** Poly-U translation was performed according to Boddeker et al, 2002 [20] with the following modification: reactions contained a final concentration of 2 percent DMSO.

**B. subtilis growth.** Aliquots of overnight culture of *B. subtilis* were diluted one hundred-fold into fresh LB medium. The diluted culture was incubated with 4 uL of DMSO or antibiotic in DMSO to a final volume of 200 uL for 12 hours at 37°C alongside non-inoculated LB medium. The optical density at 630 nm was measured on a Synergy 4 Plate Reader (Biotek).

**High Throughput Screening.** Cell free protein synthesis reactions were performed as described above with the following exceptions: 10 uL final volumes were used and the reactions were incubated at 28°C. For high-throughput screens, liquid handling was performed by hand or by Multidrop (Thermo) in Corning(C) white, non-binding surface 384 well plates (Sigma). Compounds in DMSO were added as 20 nL additions using a robotic pintool PlateMate2x2 (Matrix) to 20 nM final concentrations against 1.2 uM kanamycin positive controls in 10 uL cell-free protein synthesis reactions. Plates were sealed and incubated at 28°C for 1.5 hours, after which 35 uL of room-temperature Steady-Glo Luciferase Assay Reagent (Promega) diluted two hundred fold in 70 mM HEPES pH 7.7, 7 mM MgSO\(_4\), 3 mM dithiotreitol, and 1 percent Bovine Serum Albumin was added to the reactions followed by brief orbital shaking, a 5 minute incubation at room temperature, and

![Bacterial Growth](image-url)
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Trifuge at 4°C to pellet pyrophosphate and precipitated with 7.5 M LiCl, 50 mM EDTA pH 8.0 as described in the manufacturer’s protocol (Ambion) and visualized by formaldehyde agarose gel electrophoresis (FAGE) in 1.2 percent agarose gels containing 20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, 1X SYBR Gold Stain (Invitrogen) and 5 percent formaldehyde (v/v) pre-equilibrated in 20 mM 3-(N-morpholino)propanesulfonic acid (MOPS), 5 mM sodium acetate and 1 mM EDTA running buffer for 30 minutes at 4°C. Samples were mixed with 5 mM MOPS, 0.8 mM sodium acetate, 0.25 mM EDTA, 6 percent formaldehyde (v/v), 16 percent formamide (v/v), and xylene cyanol, incubated at 65°C for 10 minutes, and loaded and run on FAGE at 60 V and 4°C for 1.5 hours.

**In vitro transcription.** Run-off transcription reactions were performed using 100 ug/mL linear template DNA, 50 ug/mL T7 RNA Polymerase P266L mutant (in-house), and 5 mM of each rNTP pH 7.5 in 80 mM Tris-HCl pH 7.6, 24 mM MgCl₂, 2.5 mM spermidine, and 50 mM DTT in DEPC-treated water and incubated at 37°C for 2.0 hours.

**Transcription Inhibition Counterscreen.** A plasmid containing the *Vibrio cholera* VC1422 glycine riboswitch [21] for use as a test transcription product was linearized by restriction digest and *in vitro* transcribed by run-off transcription as described above but with the following modification: compounds dissolved in DMSO were added to a final concentration of 2 percent DMSO. Transcription products for the Transcription Inhibition Counterscreen were mixed with 12.5 mM EDTA pH 8.0, 50 percent formamide (v/v), xylene cyanol and bromophenol blue and incubated at 65°C for 10 minutes before visualization on 6 percent denaturing polyacrylamide gels containing 7 M urea in 1X TBE buffer (10 mM Tris, 90 mM Boric Acid, 1 mM EDTA) run at 30 W for 40 minutes. Gels were stained for qualitative visualization of RNA with Toluidine Blue, 0.01 percent Toluidine Blue (w/v) and 0.1 percent sodium tetaborate (w/v), and destained with water. RNA was quantified by Quant-iT luminescence measurement on Envision (PerkinElmer) plate reader. A luciferase inhibition counterscreen was performed exactly as above except 20 nL additions of compounds were performed after the 1.5 hour incubation. Greater than 98 percent inhibition of luciferase readout as compared to a 20 nL DMSO control was considered luciferase inhibition, and these hits were not pursued in downstream analysis. Signal analysis and significance of hits were analyzed with the assistance of Janie Merkel of the Yale Center for Molecular Discovery.

**Cloning of pIVEX2.3d-luc.** The firefly luciferase gene, *luc*, was polymerase chain amplified using Phusion High-Fidelity DNA Polymerase (NEB) according to the manufacturer’s protocol with pBESTluc plasmid template (Promega) and primers (5’- TATAACCATTGGAGACGCCAATAAACATAAGAAGG, 5’- TATATCTCGAGTTATTACAATTTGGACTTTCCGCCC, W.M. Keck Foundation). The product and vector pIVEX2.3d (5Prime) were digested with NcoI (NEB) and XhoI (NEB) and ligated after treatment of the vector with Calf Intestinal Phosphatase (NEB) using T7 DNA Ligase (NEB) all according to the manufacturers’ protocols. Plasmids were transformed into *E. coli* XL10Gold cells (Agilent Technologies, Inc.), and DNA was isolated and purified using QIAGEN plasmid isolation kits according to the manufacturers’ protocols. DNA was visualized and purified by agarose gel electrophoresis performed in 0.8 percent agarose gels containing 1X SYBR Gold Stain (Invitrogen). Gels were submerged in 1X TAE Buffer (40 mM Tris-acetate, 1 mM EDTA pH 8.3) and run against 1 kb or 100 bp DNA ladders (Invitrogen).

**Template mRNA Preparation and Analysis.** pIVEX-2.3d-luc was digested with XhoI (NEB) according to the manufacturer’s protocol for the generation of an mRNA template. Transcription was performed by run-off transcription as described above except for the addition of 1 U/mL SUPERase·In (Ambion) to reactions. After transcription reactions were performed, reactions were spun for 30 minutes at 13,000 rpm on a table-top centrifuge at 4°C to pellet pyrophosphate and precipitated with 7.5 M LiCl, 50 mM EDTA pH 8.0 as described in the manufacturer’s protocol (Ambion) and visualized by formaldehyde agarose gel electrophoresis (FAGE) in 1.2 percent agarose gels containing 20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, 1X SYBR Gold Stain (Invitrogen) and 5 percent formaldehyde (v/v) pre-equilibrated in 20 mM 3-(N-morpholino)propanesulfonic acid (MOPS), 5 mM sodium acetate and 1 mM EDTA running buffer for 30 minutes at 4°C. Samples were mixed with 5 mM MOPS, 0.8 mM sodium acetate, 0.25 mM EDTA, 6 percent formaldehyde (v/v), 16 percent formamide (v/v), and xylene cyanol, incubated at 65°C for 10 minutes, and loaded and run on FAGE at 60 V and 4°C for 1.5 hours.

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**Figure 6. Synthesis of T6102.** Compound labels and reaction scheme are described in Methods under Materials and Purification.
RiboGreen RNA Reagent and Kit (Invitrogen) assays according to the manufacturer’s protocol. Briefly, 1 U per ug of template DNA of RQ1 RNase-free DNase (Promega) was added to each reaction and incubated at 37°C for 20 minutes. 100 ug of 1:1000 dilutions of DNase-treated transcription reactions in RNase/DNase-free 1X TE Buffer (10 mM Tris-HCl, 1 mM EDTA pH 7.5) were prepared in black, opaque bottom 96 well plates. Background fluorescence was read using Excitation 485/20, Emission: 530/25 filter wheels. 100 ug of freshly made 2X RiboGreen solution was added to each well, the plate was shaken for 2 minutes and incubated for another 3 minutes at room temperature, and fluorescence was read on a Synergy 4 Plate Reader (Biotek). RNA was quantified against serial dilutions of an E. coli 16S and 23S ribosomal RNA standard (Invitrogen) and compared as a percentage of the uninhibited control.

Crystal Soaks. 70S ribosomes from T. thermophilus HB8 were prepared and crystallized as previously described [22]. Briefly, sitting drop vapor diffusion trays were set up with a 500 ul reservoir solution of 2.9 percent poly(ethylene glycol) 20k (PEG20k), 9 percent 2-methyl-2,4-pentanediol (MPD), 175 mM arginine and 100 mM Tris-HCl pH 7.6. 5 ug sitting drops contained ~2.5 ug of 12-13 mg/ml 70S T. thermophilus ribosomes (from D. Bulkley) in 5 mM HEPES pH 7.5, 10 mM ammonium chloride, 10 mM magnesium acetate, 50 mM KCl and ~2.5 ul of well solution before equilibration. Crystals were cryoprotected by increasing the MPD concentration from 25 percent to 30 percent to 35 percent in 2.9 percent PEG20k, 100 mM Tris-HCl pH 7.6, 10 mM magnesium acetate, 10 mM ammonium chloride, 50 mM KCl. Antibiotics were soaked into the cryo-stabilized crystals for several hours to overnight at concentrations around 100 ug/ml in 40 percent MPD. Data was collected at Brookhaven National Laboratory on beamline X29 and the Advanced Photon Source on beamlines 24-ID-E and 24-ID-C and processed using XDS [23]. Structures were solved by molecular replacement using the apo T. thermophilus 70S as a model (from Y. Polikanov) and refined on PHENIX [24].

Calculations. Percent inhibition was calculated by dividing each condition’s measurement by the average of the negative controls’ measurement (DMSO only), and then subtracting this number from 1. All experiments in Figures 3, 4, and 5 were performed in triplicate. The average of percent inhibition was taken of all of the replicates for each condition. IC₅₀ values were calculated using the IC₅₀ Toolkit (http://www.ic50.tk/).

Spectroscopic Analysis. ¹H and ¹³C spectra were recorded on Bruker Avance DPX-500 Nuclear Magnetic Resonance (NMR) spectrometers. ¹H NMR spectra are represented as follows: chemical shift, multiplicity (s = singlet, d = doublet, m = multiplet, br = broad), integration, and coupling constant (J) in Hertz (Hz). ¹H NMR chemical shifts were reported relative to CDCl₃ (7.26 ppm). ¹³C NMR were recorded relative to the central line of CDCl₃ (77.00 ppm).

Materials and Purification. The synthesis of T6102 is shown in Figure 6. 1-Adamantyl methyl ketone, ethynyltrimethylsilane, n-butyllithium (n-BuLi, 2.5 M solution in hexanes), cerium (III) chloride, and tetrabutylammonium fluoride (TBAF, 1.0 M solution in tetrahydrofuran) were purchased from Sigma-Aldrich. Tetrahydrofuran (THF) was distilled from sodium/benzophenone. Thin layer chromatography (TLC) was performed using glass plates precoated with silica gel (0.25 mm). TLC plates were stained by submersion into aqueous ceric ammonium molybdate (CAM) followed by brief heating on a hot plate. Flash column chromatography was performed using silica gel 60 (230-400 mesh, Merck) with the indicated solvents.

To a solution of ethynyltrimethylsilane 2 (206 mg, 2.1 mmol) in anhydrous THF (1.5 mL) at -78°C under N₂, was added dropwise n-butyllithium (2.5 M solution in hexanes, 0.8 mL, 2.0 mmol). The mixture was allowed to warm to room temperature for 1.0 hour. This mixture was added to a stirred suspension of cerium (III) chloride (493 mg, 2.0 mmol) in anhydrous THF (2.5 mL) at -78°C via cannula. After stirring at -78°C for 1.0 hour, a solution of 1-adamantyl methyl ketone 1 (178 mg, 1.0 mmol) in anhydrous THF (1.5 mL) was added to the mixture. The resulting mixture was stirred at -78°C for 0.5 hours, quenched with saturated aqueous NH₄Cl solution (10 mL), and extracted twice with diethyl ether. The extracts were washed with brine, dried over anhydrous Na₂SO₄, filtered, and concentrated. The crude product 3 was used without further purification for the next reaction.

TBAF (1.0 M solution in THF, 2 mL, 2.0 mmol) was added dropwise to a solution of the crude residue 3 in THF (4 mL) at 0°C. The reaction mixture was stirred at 0°C for 3.5 hours, quenched with saturated aqueous NH₄Cl solution, and extracted twice with diethyl ether. The extracts were washed with brine, dried over anhydrous Na₂SO₄, filtered, and concentrated. The residue was chromatographed (eluting with 100 percent hexanes initially, grading to 10 percent ethylacetate in hexanes) on silica gel to afford T6102 (137 mg, 67 percent) as a white solid. ¹H NMR (500 MHz, CDCl₃) d 2.43 (s, 1H), 3.03 (brs, 3H), 1.82 (s, 1H), 1.72 (d, J = 2.9 Hz, 6H), 1.72-1.68 (m, 3H), 1.65-1.62 (m, 3H), 1.42 (s, 3H). ¹³C NMR (125 MHz, CDCl₃) d 87.1, 73.8, 72.3, 39.0, 36.9, 36.1, 28.4, 23.4. TLC (20 percent EtOAc in Hexanes), Rₜ 0.68 (CAM).
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