

Eukaryotic Ribosomal Expansion Segments as Antimicrobial Targets

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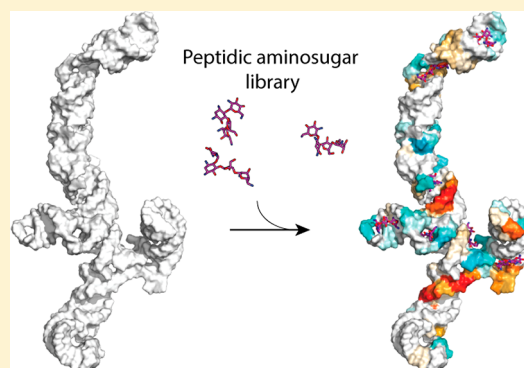
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S Supporting Information

ABSTRACT: Diversity in eukaryotic rRNA structure and function offers possibilities of therapeutic targets. Unlike ribosomes of prokaryotes, eukaryotic ribosomes contain species-specific rRNA expansion segments (ESs) with idiosyncratic structures and functions that are essential and specific to some organisms. Here we investigate expansion segment 7 (ES7), one of the largest and most variable expansions of the eukaryotic ribosome. We hypothesize that ES7 of the pathogenic fungi *Candida albicans* (ES7_{CA}) could be a prototypic drug target. We show that isolated ES7_{CA} folds reversibly to a native-like state. We developed a fluorescence displacement assay using an RNA binding fluorescent probe, F-neo. F-neo binds tightly to ES7_{CA} with a K_d of 2.5×10^{-9} M but binds weakly to ES7 of humans (ES7_{HS}) with a K_d estimated to be greater than 7 μ M. The fluorescence displacement assay was used to investigate the affinities of a library of peptidic aminosugar conjugates (PAs) for ES7_{CA}. For conjugates with highest affinities for ES7_{CA} (NeoRH, NeoFH, and NeoYH), the lowest dose needed to induce mortality in *C. albicans* (minimum inhibitory concentration, MIC) was determined. PAs with the lowest MIC values were tested for cytotoxicity in HEK293T cells. Molecules with high affinity for ES7_{CA} *in vitro* induce mortality in *C. albicans* but not in HEK293T cells. The results are consistent with the hypothesis that ESs represent useful targets for chemotherapeutics directed against eukaryotic pathogens.



Many antibiotics bind to regions of rRNAs (rRNAs) that are common to all organisms. These universal regions of rRNAs, called the common core,^{1,2} contain the A site, the P site, the exit tunnel, the peptidyl transfer center, and the decoding center.³ These functional centers are targeted by macrolides, aminoglycosides, tetracyclines, streptogramins, pleuromutilins, chloramphenicol, linezolid, and puromycin.⁴ Increasing resistance to these antibiotics^{5,6} motivates a search for new antibiotic targets.⁷

Eukaryotic pathogens, including various fungi, are gaining drug resistance in parallel with bacterial pathogens.⁸ Unlike those of prokaryotes, eukaryotic ribosomes contain species-specific rRNA expansions. To our knowledge, eukaryotic rRNA expansion segments (ESs)^{2,9–13} are substantially unexplored as drug targets. ESs emerge from the surface of the common core² and are characterized by idiosyncratic structures and functions that are essential and specific to various species. ESs are important for ribosome biogenesis,^{14,15} rRNA processing and stabilization,^{16,17} translation initiation, and recruitment of factors

such as chaperones, enzymes, aminoacyl tRNA synthetases, and signal recognition particles.^{18–21} Expansion segment 7 (ES7), the largest and most variable ES of the ribosome, is an extension of the ribosomal large subunit (LSU) rRNA.

Here we use ES7 of *Candida albicans* (ES7_{CA}) to investigate the general utility of ESs as targets for chemotherapeutic agents and to develop new approaches for eukaryotic drug targeting. *C. albicans*, along with *Cryptococcus neoformans*, *Coccidioides immitis*, and *Pneumocystis jiroveci* affect immunocompromised populations including AIDS and cancer patients, organ-transplant recipients, low-birth-weight infants, and individuals with inherited diseases.^{22,23} Candidiasis is the fourth leading cause of healthcare associated blood infections in the United States.²⁴

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C. albicans and related species show resistance to antimicrobial agents such as fluconazole.^{25,26}

ES7 rRNA of fungi, which is conserved in secondary structure, averages around 210 nucleotides (nts) in length (Table S.3, Figure S.1). ES7_{CA} is 209 nts with ~55% GC content (*C. neoformans*, 234 nts, ~50% GC; *C. immitis*, 210 nts, ~60% GC; *P. jiroveci*, 223 nts, ~50% GC). By contrast, ES7 of *Homo sapiens* (ES7_{HS}) is different from ES7_{CA} in size (876 nts), composition (~80% GC content), sequence, and structure.

We demonstrate that isolated ES7_{CA} like ES7 from *S. cerevisiae* (ES7_{SC}),¹⁹ and ES7_{HS} (Lizzette M. Gómez Ramos and Loren Dean Williams, unpublished) folds reversibly to a near-native state. We investigate the interactions of isolated ES7_{CA} with aminosugars, which are known to bind to bacterial ribosomes. An assay to screen library of peptidyl aminosugars (PAs) for binding to ES7 was developed. Relative affinities of PAs (Figure 1) for

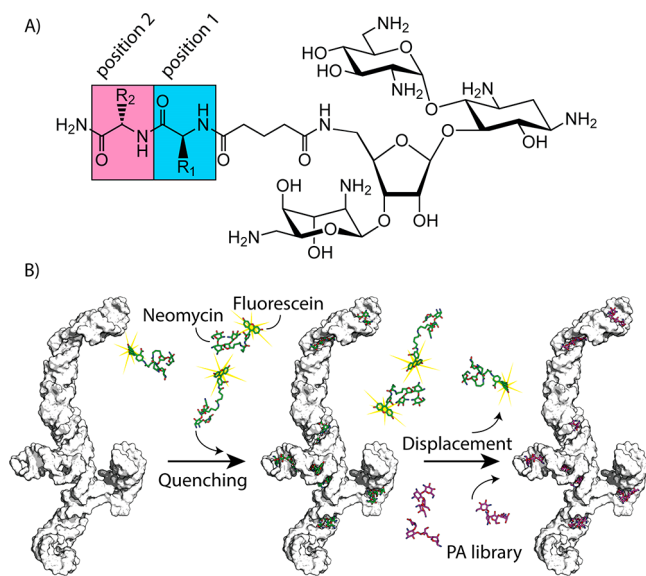


Figure 1. Affinities of a library of PAs (peptidyl amino sugars) for ES7_{CA} were investigated by a fluorescence displacement assay. (A) Structures of PAs. Position 1 is blue. Position 2 is pink. R₁ and R₂ are amino acid side chains. (B) Schematic diagram of the assay showing displacement of fluorescein-neomycin (F-neo, green) by a competitive ligand (PAs, purple) from a three-dimensional model of ES7_{CA}. The yellow star indicates fluorescence.

ES7_{CA} were assessed with fluorescence displacement assays and differential thermal melting studies. Members of the PA library with high affinity for ES7_{CA} were investigated to determine the minimum inhibitory concentration (MIC) needed to induce mortality of *C. albicans*. PAs with the lowest MICs were tested for cytotoxic effects in HEK293T cells. PAs that bind with high affinity and specificity to ES7_{CA} *in vitro*, in comparison to ES7_{HS}, induce mortality in *C. albicans* but not in HEK293T cells. Our results suggest selectivity and activity of selected PAs for specific rRNAs and species, and indicate that ESs might represent useful targets for the development of new chemotherapeutics directed against eukaryotic pathogens.

MATERIALS AND METHODS

Synthesis of ES7_{CA} and ES7_{HS} rRNAs. The DNA encoding ES7_{CA}, appended to the T7 promoter, flanked by restriction sites for *Eco*RI (5' end) and *Hind*III (3' end), was constructed by recursive PCR.²⁷ The DNA was ligated into restricted,

dephosphorylated pUC19, and transformed into competent DH5α cells. Colonies were selected on X-gal Blue-White screening (Thermo Scientific). Oligomer and primer sequences used in the recursive PCR are shown in Tables S.1 and S.2. Oligomers, primers, and DNA sequencing were obtained from Eurofins-MWG Operon. The gene encoding ES7_{HS}, with the T7 promoter and restriction sites for *Eco*RI (5' end) and *Hind*III (3' end), in pUC57-Kan was purchased from Genewiz.

The SHAPE structural cassette²⁸ was incorporated onto the 3' end of ES7_{CA} with the Q5 Site-directed mutagenesis kit (New England BioLabs) to allow SHAPE mapping of the entire ES7_{CA}. Primer sequences are shown in Table S.4. ES7_{CA} and ES7_{HS} RNAs were transcribed using the HiScribe T7 high yield RNA synthesis kit (New England BioLabs) and purified as described previously.¹⁹

SHAPE Reactions. Selective 2'-hydroxyl acylation analyzed by primer extension (SHAPE) was adapted from published protocols.^{28,29} SHAPE monitors local nucleotide flexibility through reactivity of 2' oxygens to an electrophile. Experiments on ES7_{CA} were performed as described previously.¹⁹ Data were collected in 200 mM Na⁺ and no Mg²⁺ to promote and analyze secondary structure.^{30–32} Tertiary interactions were characterized by changes in SHAPE upon addition of Mg²⁺ ions without other changes in the experimental procedure. Na⁺ only and Na⁺/Mg²⁺ SHAPE data for ES7_{CA} were normalized, and Na⁺ reactivities were subtracted from Mg²⁺ reactivities at each nucleotide position as previously described.³³

The effect of neomycin (Neo) was investigated by monitoring changes in SHAPE upon addition of Neo. Since the hydroxyl groups of the Neo ribose are expected to be modified by the SHAPE reagent, Neo concentration was elevated in the SHAPE reactions (10 mM). Control and Neo SHAPE data for ES7_{CA} were normalized, and control SHAPE reactivities were subtracted from Neo reactivities at each nucleotide position.

Thermal Folding/Unfolding. Thermal melting of ES7_{CA} RNA was monitored by absorbance at 260 nm in a thermostated Varian Cary-1E UV spectrophotometer. The melting buffer consisted of 180 mM NaCl, 20 mM Tris-HEPES (pH 8.0), and various concentrations of Neo. Melting, data reduction, and fitting were performed as described.^{19,34}

Interactions of ES7_{CA} with PAs. A library of 215 PAs (Figure 1), which use Neo as the scaffold,³⁵ was screened for binding to ES7_{CA} using a fluorescence displacement assay.³⁶ Fluorescein-neomycin (F-neo) was used as the reporter molecule and was synthesized as described.

K_d's of Neomycin and ES7. Binding of F-neo to ES7_{CA} was assayed in triplicate in binding buffer [50 mM NaCl, 0.4 mM EDTA, 10 mM HEPES (pH 7.0)]. F-neo concentration was fixed at 10 nM in each well of a 96-well plate, which was titrated with 2× serial dilutions of ES7_{CA} from 1.58 × 10^{−8} M to 7.63 × 10^{−12} M. Binding of F-neo to ES7_{HS} RNA was assayed at fixed F-neo (100 nM) titrated with 2× serial dilutions of ES7_{HS} from 2.5 × 10^{−5} M to 1.22 × 10^{−8} M. F-neo was excited at 485 nm and monitored at 525 nm using a Genios Pro plate reader (Tecan). Fluorescence intensity decreased with increasing RNA because the interaction with RNA quenches F-neo emission.

The stoichiometry of binding of F-neo to ES7 was estimated from plots of fluorescence of F-neo versus molar ratio of ES7_{CA} to F-neo. Separately from the stoichiometric calculation, K_d's were estimated from mass balance describing the relationship between bound F-neo, [F-neo]_b, and the total concentration of F-neo, [F-neo]_{total}, and the total concentration of RNA, [RNA]_{total}:

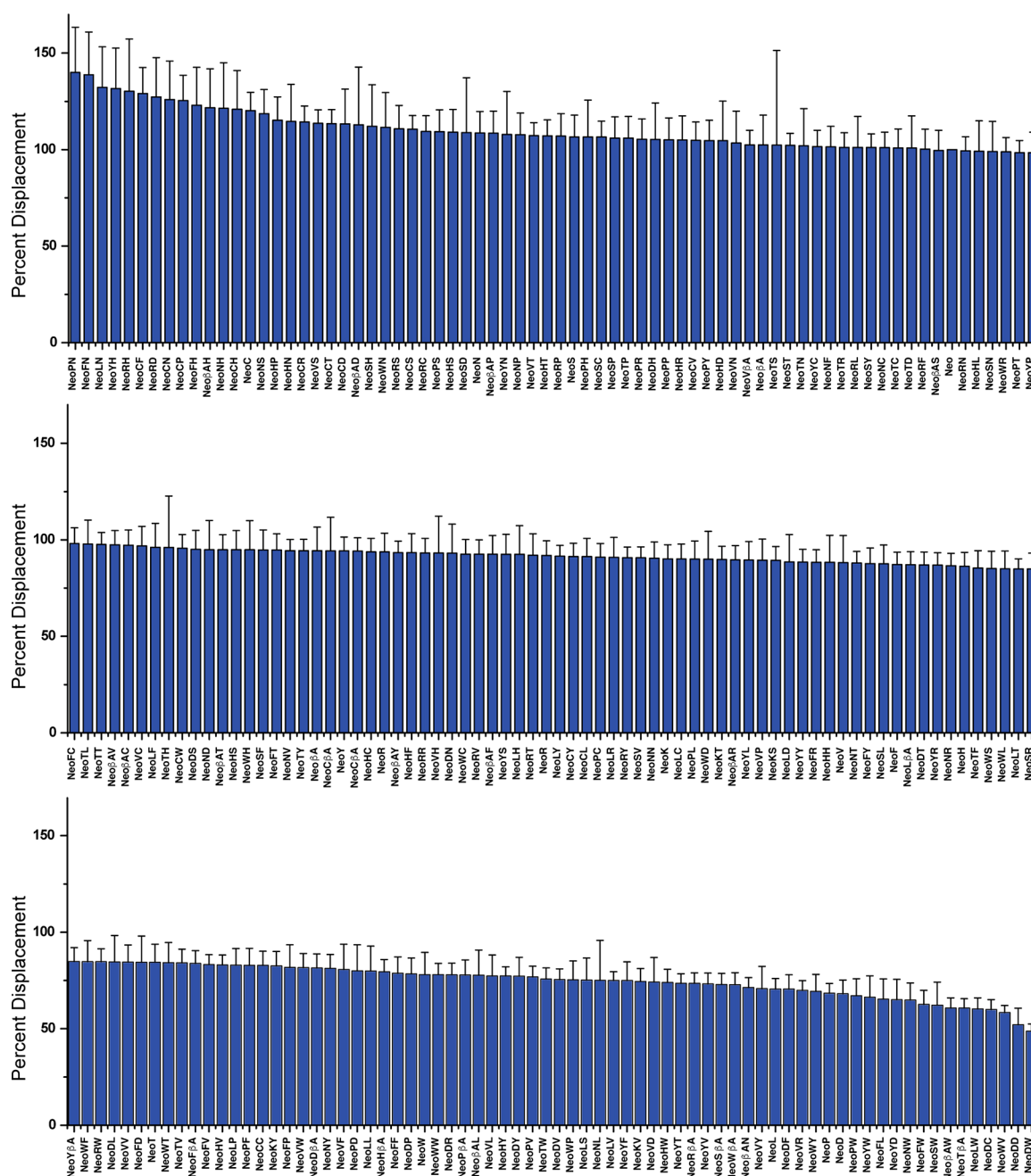


Figure 3. Ranking of 215 PAs by their tendency to displace F-neo from ES7_{CA} normalized by the displacement of F-neo by Neo. Each experiment was performed in duplicate. Standard deviations are shown with error bars.

binding buffer using the fluorescence displacement assay.³⁶ PAs (1 μ M) were added to the F-neo/ES7 mixture (100 nM F-neo and 11.6 nM ES7 RNA, final concentrations) previously aliquoted into 96-well plates. All plates contained two sets of controls, the F-neo/ES7 complex and the F-neo/ES7 complex with Neo. The displacement of F-neo by PAs was measured by the increase in fluorescence (ΔF) after addition of PAs and was compared to fluorescence of the F-neo/ES7 complex. The extent of displacement of F-neo by PAs was calculated from changes in fluorescence upon addition of PAs to the F-neo/ES7 complex (ΔF_{PA}) divided by the change in fluorescence obtained by the addition of Neo to the F-neo-ES7 complex (ΔF_{Neo}).

$$\% \text{displacement} = (\Delta F_{PA} / \Delta F_{Neo}) \times 100$$

IC₅₀ Experiments. IC₅₀ experiments were performed using a fluorescence displacement assay. The F-neo/ES7 complex was maintained at constant concentration (100 nM F-neo and 11.6 nM ES7_{CA} RNA) and was titrated with PAs in a 96-well plate. Curve fitting was performed with Igor Pro. The IC₅₀ is the inflection point of the sigmoidal fit.

Minimum Inhibitory Concentrations. Stocks of PAs were stored at 1 μ M in deionized water at -80°C . *C. albicans* SC5314 was cultured in yeast extract-peptone-dextrose (YPD) agar plates at 30°C for 24 h. Colonies were picked and diluted in YPD media to OD₆₀₀ $\approx$ 0.1. Eighty microliters of cultivated media were aliquoted in 96-well clear flat bottom plates. Twenty microliters of diluted PA was added to each well (final concentrations of 200, 100, 50, 25, 12.5, 6.25, 3.13, 1.57, 0.78,

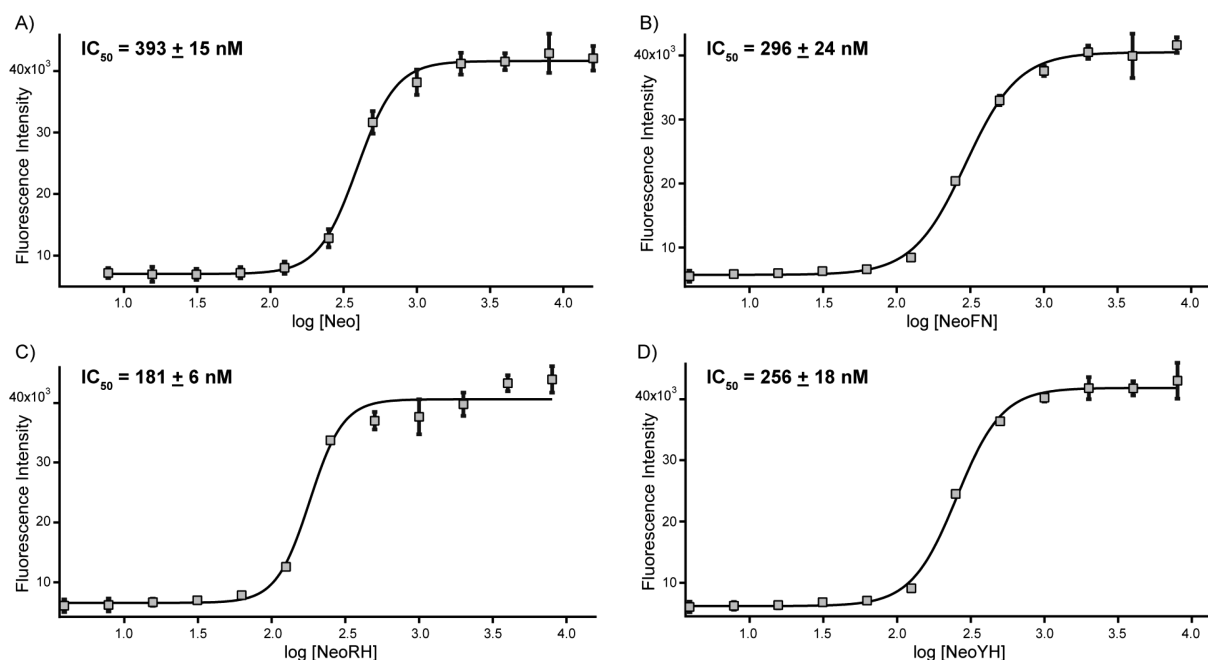


Figure 4. IC₅₀ measurements of PAs. Titration of ES7_{CA} was performed with (A) Neo, (B) NeoFN, (C) NeoRH, and (D) NeoYH. F-neo displacement was monitored. IC₅₀ values are estimated to be Neo = 393 ± 15 nM, NeoFN = 296 ± 24 nM, NeoRH = 181 ± 6 nM, and NeoYH = 256 ± 18 nM.

0.39, 0.20, and 0.10 μM) to a final volume of 100 μL per well. Controls include 20 μL of water and 80 μL of YPD media. Standard growth was measured in 20 μL of water and 80 μL of YPD. Plates were shaken continuously at 30 °C for 24 h in a Synergy H4 Hybrid Multimode plate reader (Biotek). Absorbance at 600 nm was measured every 10 min. The effect of PA on growth was assayed by taking the absorbance at 600 nm with PA (A_{PA}) minus baseline (media alone, A_{media}), normalized by the absorbance of the standard culture (A_{ST}) minus baseline:

$$\%growth = (A_{PA} - A_{media}) / (A_{ST} - A_{media}) \times 100$$

MIC values were determined after incubation of cells with PAs for 24 h.

Cytotoxicity. HEK293T cells were grown in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum. Cells were incubated at 37 °C in humidified 5% CO₂. Three thousand cells were seeded in 96-well plates, with total volumes of 100 μL per well. Cell adhesion to the plates was allowed in an initial incubation period of 24 h. PAs were added to each well (100 μM final concentrations) followed by incubation for 72 h. The CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) was used to determine cell viability. Absorbance was measured at 490 nm.

Modeling of ES7_{CA}. The initial secondary model of ES7_{CA} was obtained by homology with the known secondary and three-dimensional structure of ES7_{SC}.^{19,37} The ES7_{CA} secondary model predicted by mFold³⁸ is consistent with SHAPE data. The resulting user defined secondary structure (Chart 1) was used to construct three-dimensional models using RNAComposer.³⁹ Three dimensional models were ranked based on energy,⁴⁰ confirmed in secondary structure with RNApdbee,⁴¹ and used in docking studies.

RESULTS

Interactions with PAs with ES7_{CA} were evaluated with SHAPE, fluorescence displacement assays, and thermal melting. Members of the PA library with greatest estimated affinities for ES7_{CA} were

investigated to determine MIC values in *C. albicans*. PAs with the lowest estimated MICs were tested for cytotoxicity in HEK293T cells. The secondary structure of ES7_{CA} appears to be conserved when is isolated from the rest of the rRNA, as indicated by SHAPE, computational melting, and modeling.

Affinities of F-neo for ES7_{CA} and ES7_{HS}. F-neo binds with high affinity and specificity to ES7_{CA} relative to ES7_{HS}. The extent of binding was estimated from changes in fluorescence of F-neo during titration with RNA. The dissociation constant (K_d) for binding to ES7_{CA} was estimated from nonlinear fitting of data to mass balance equations. The K_d of F-neo for ES7_{CA} is estimated to be 2.5×10^{-9} M ± 9×10^{-10} M (Figure 2A). The K_d of F-neo for ES7_{HS} could not be accurately determined because the affinity is lower than the practical limits of the assay >7 μM (Figure 2B). The affinity F-neo for ES7_{CA} is estimated to be 2000 times greater than for ES7_{HS}. By contrast, K_d 's for association of Neo with A site RNA, its cognate binding site, were previously estimated to be 0.053 μM for *Escherichia coli* and 0.26 μM for human.⁴² This difference in A site binding (4-fold) is less than the difference in K_d of F-neo with ES7_{CA} versus the K_d of F-neo with ES7_{HS}.

Stoichiometric coefficients were estimated by fitting the binding data of ES7_{CA} to F-neo⁴³ (Figure 2C). Fluorescence of F-neo versus the molar ratio of ES7_{CA} to F-neo is linear in the limiting regions of the binding curve. The limiting lines intersect at a molar ratio of ~0.077, suggesting a stoichiometric coefficient of F-neo of approximately 13 in the binding reaction. We assume that each binding event has the same affinity, and thus K_d implies $K_{d,apparent}$. We also assume that the stoichiometries of binding to ES7_{CA} are conserved between F-neo and Neo. The binding stoichiometry for the interaction of F-neo to ES7_{HS} could not be estimated due to the low affinity of F-neo for ES7_{HS}.

The Library. We investigated the affinities of a 215-member library of PAs (Figure 1) for ES7_{CA} by several methods.³⁵ The library consists peptides linked by glutarate to Neo at the 5' position of the ribose ring. Some members of the PA library are mono-peptidic, with amino acids at position one only and some are di-peptidic, with amino acids at positions one and two.

High Throughput Screening of the PA Library for Affinity for ES7_{CA}. The extent of binding of PAs to ES7_{CA} was estimated through HTS using a fluorescence displacement assay (Figure 2).^{35,36} In this assay, ES7_{CA} is first incubated with F-neo, which is then competitively displaced by a PA. The assay allows ranking of members of the PA library and is normalized so that F-neo displacement by Neo equals 100%. PAs with displacements greater than 100% bind to ES7_{CA} with greater affinity than Neo. PAs with displacements less than 100% bind with less affinity than Neo. Around 60 compounds from the PA library showed greater affinity than Neo for ES7_{CA} in the HTS (Figure 3). A few of these are single amino acid PAs; the others are double amino acid PAs. The low affinity of F-neo for ES7_{HS} precluded estimation of the affinities of the PA library for ES7_{HS}.

IC₅₀ Measurements of Affinity for ES7_{CA}. To confirm results obtained from the HTS, we performed IC₅₀ measurements on several conjugates that showed F-neo displacements above the median. In the IC₅₀ assay, complexes of F-neo and ES7_{CA} were titrated with PAs and displacement of F-neo was monitored at various ratios of PA to ES7_{CA}. Binding affinities are 2.2-fold greater than Neo for NeoRH, 1.5-fold greater for NeoYH, and 1.3-fold greater for NeoFN. Thus, the IC₅₀ measurements are generally consistent with the HTS results.

Minimum Inhibitory Concentrations. Inhibition of microbial growth by PAs was initially assessed through a single-dose assay. The 10 strongest binders identified in HTS (above) were tested at 200 μ M for inhibition of growth of *C. albicans*. All the PAs except NeoRD and NeoPN inhibited *C. albicans* growth under these conditions.

MICs, which are more accurate measures of inhibition of growth than single dose assays, are consistent with the results obtained in the single-dose assay (Table 1). MICs for *C. albicans*

Table 1. Minimal Inhibitory Concentrations (MIC)^a

name	PA no. ^b	MIC (μ M)
Neo		>5000
NeoPN	1151	>200
NeoFN	1150	200
NeoLN	1149	200
NeoYH	1182	100
NeoRH	1173	100
NeoCF	1212	200
NeoRD	1159	>200
NeoCN	1156	200
NeoCP	1226	200
NeoFH	1178	100
fluconazole		>200

^aMIC is the concentration of PA that inhibits *C. albicans* growth by 95% at 30 °C after 24 h. Averages of triplicate experiments are reported. ^bPreviously published PA numbering system.³⁵

were evaluated for PAs that showed activity in single dose assays. MIC is the lowest concentration of PA that inhibits *C. albicans* growth by 95% after 24 h. NeoFH, NeoRH, and NeoYH exhibited MICs of 100 μ M, which are the lowest MICs obtained. NeoFN, NeoLN, NeoCN, NeoCF, and NeoCP exhibited MICs of 200 μ M. NeoPN and NeoRD exhibited MICs greater than 200 μ M. After 24 h at 200 μ M, NeoPN and NeoRD fail to inhibit growth. Neo has a MIC of greater than 5 mM. While the MICs of the PAs evaluated here lie above the useful therapeutic range, some of them show ~50 fold improvement over Neo and provide a starting point to further modify and optimize the PAs.

Fluconazole is a standard treatment against fungal infections. Some PAs exhibit MIC values that are superior to those of fluconazole. MICs for fluconazole determined here in a variety of experimental conditions are consistent with previous work.⁴⁴

Cytotoxicity Assays. Useful antimicrobial agents must have target selectivity and lack of toxicity toward the host. Cytotoxicity studies suggest that PAs are not toxic to human cells (Figure 5). HEK293T cells were incubated for 72 h with Neo, NeoRH, NeoFH, and NeoYH at their MIC values of 100 μ M. The results indicate a survival rate of around 70% or higher.

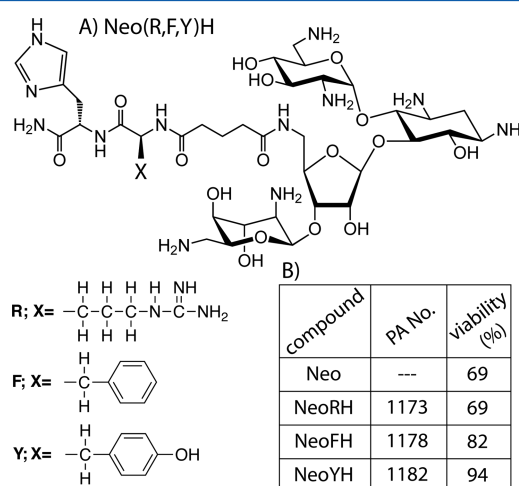


Figure 5. Cytotoxicity of PAs in human cells. PAs were incubated with HEK293T cells for 72 h at 100 μ M. (A) Chemical structures of NeoRH, NeoFH, and NeoYH. (B) Cell viability is reported in comparison to that of PA-free cells.

General Trends in Structure and Function. The HTS results suggest that 10 of the 20 best ES7_{CA} binders in the PA library contain aromatic amino acids. The results also suggest that single amino acid conjugates that exhibit higher affinity than Neo for ES7_{CA} (NeoS, NeoC, and NeoN) contain amino acids that are polar and uncharged, including serine, cysteine, and asparagine (Figure 3). An exception to this is Neo β A, which contains a nonpolar amino acid. Single amino acid conjugates, NeoD and NeoP, show the lowest affinity for ES7_{CA}, displacing F-neo at 68% under our experimental conditions (Figure 3). The low affinity of anionic NeoD is expected since RNA is also negatively charged.

Double amino acid conjugates with positively charged and polar amino acids, including PAs containing asparagine, histidine, or cysteine at either the first or second position, exhibit higher affinities for ES7_{CA}. NeoPN displays the highest affinity for ES7_{CA} with 140% displacement, which is very similar to that of NeoFN, NeoLN, and NeoYH. The conjugation of two positive charges to the PAs does not seem to enhance the binding to ES7_{CA} (Figure 3). Negative charge is unfavorable. Three of the 10 worst binders contain an aspartic acid in the first position. Conjugation to a tryptophan also appears detrimental for binding to ES7_{CA}; 7 of the 10 worst binders belong to this category. The worst binding double amino acid conjugates contain negatively charged or bulky amino acids.

IC₅₀ results are generally consistent with the HTS results. IC₅₀ results obtained with double amino acid conjugates confirm that binding to ES7_{CA} is strengthened when the Neo scaffold is conjugated to positively charged and polar amino acids. IC₅₀ results also suggest that conjugation to amino acids with smaller

aromatic systems and greater hydrogen bonding capabilities can improve the affinity toward ES7_{CA}. Of all the conjugates tested, NeoRH displays the greatest affinity. This PA contains the smallest aromatic system and the most hydrogen donors of the three PAs tested for IC₅₀.

MIC experiments suggest that the second amino acid position is important for PA activity *in vivo*. Compounds conjugated to a histidine in the second amino acid position exhibit the lowest MICs. Overall, the amino acids conjugated at this position follow a well-defined pattern; most of them are polar, with hydrogen bonding capabilities. NeoRD is an exception and exhibited higher values of MIC. Histidine is the smallest aromatic amino acid and possesses two sites for hydrogen bonding. It seems likely that a combination of geometry and hydrogen bonding capabilities at the second amino acid position offer interactions with the PA-ES7_{CA}. No specific contribution could be deduced for the first amino acid position, as no definite trend is observed.

Secondary Structure of ES7_{CA}. Isolated ES7_{CA} folds to a native-like secondary structure in the presence of Na⁺, with four primary helices (ES7a, ES7b, and ES7c and Helix 25, Figure 6). The secondary structure of isolated ES7_{CA} was determined by SHAPE footprinting,^{45,46} computational folding,³⁸ and secondary structure homology modeling. As shown in Figure 6A, nucleotides in helical regions are the least reactive in the SHAPE assay.

ΔMg²⁺ Heat Maps of ES7_{CA}. The conformation of isolated ES7_{CA} changes upon addition of Mg²⁺. SHAPE reactivity obtained in the presence of Na⁺ alone (Figure 6A) was subtracted from reactivity in the presence of both Na⁺ and Mg²⁺ (Figure 6B) to give a ΔMg²⁺ heat map (Figure 6C). The ΔMg²⁺ heat map identifies nucleotides that exhibit Mg²⁺-dependence of SHAPE reactivity, which can be either positive or negative. Helical regions are invariant to Mg²⁺ (white on the ΔMg²⁺ heat map) as expected,^{30,32} indicating that secondary structure forms in the presence of Na⁺ alone and is maintained upon Mg²⁺ addition. Mg²⁺-specific changes in SHAPE reactivity are focused in bulges, loops, and nonhelical regions. It seems possible that some of these nucleotides are involved in long-range interactions in the native state, which are favored by the addition of Mg²⁺.^{30,32} This work provides the first structural data on any rRNA of *C. albicans*.

Comparison of SHAPE data obtained for ES7_{CA} (Figure 6) and ES7_{SC}¹⁹ show that the ES7s of these fungi share similar secondary and tertiary structures. Our SHAPE data obtained previously for isolated ES7_{SC} matches the predicted models of the ES7_{SC} secondary structure¹⁹ and the SHAPE reactivity of the ES7_{SC} of the assembled *S. cerevisiae* ribosome.⁴⁸ ES7 for both organisms consists of four helices and a primary junction where Helices ES7a, ES7c, and 25 are joined. This junction is the region of greatest changes in SHAPE upon addition of Mg²⁺. Many of the bulges and helical mismatches show changes in SHAPE upon addition of Mg²⁺. The loops and mismatches are conserved between ES7_{CA} and ES7_{SC}, as are changes in SHAPE reactivity of these regions upon Mg²⁺ addition.

ΔNeo Heat Maps of ES7_{CA}. SHAPE data, obtained under saturating Neo, allow characterization of ligand-induced conformational changes of RNA but do not allow determination of differential affinities of the multiple Neo binding sites. Neo binds to ES7_{CA} with high stoichiometry, as indicated by binding assays (see above) and is therefore assumed to occupy multiple binding sites during the SHAPE experiments. SHAPE reactivity obtained in the presence of saturating Neo was subtracted from the SHAPE reactivity obtained in the absence of Neo to give a

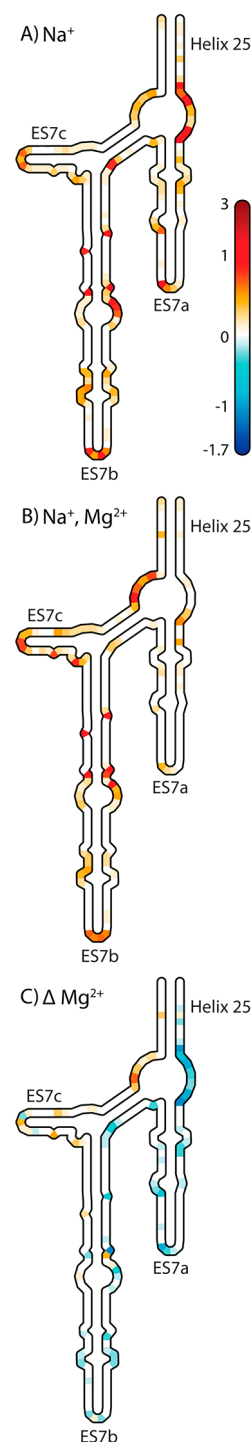


Figure 6. (A) SHAPE heat maps in the presence of Na⁺ alone are consistent with the proposed secondary structural model of ES7_{CA}. Helical regions are unreactive (white), while loops and bulges are reactive (red). (B) Addition of Mg²⁺ does not disrupt the secondary structure. (C) A delta map (ΔMg²⁺), showing changes in reactivity upon addition of Mg²⁺ indicate Mg²⁺-induced changes in the structure of ES7_{CA}. Nucleotides with positive values (red) indicate higher reactivity upon addition of Mg²⁺. Nucleotides with negative values (blue) indicate decreased reactivity. All reactions contained 200 mM NaOAc, 50 mM NaHEPES (pH 8.0). Figures were generated with the program RiboVision.⁴⁷

ΔNeo heat map (Figure 7). We have obtained ΔNeo heat maps in the presence and absence of Mg²⁺.

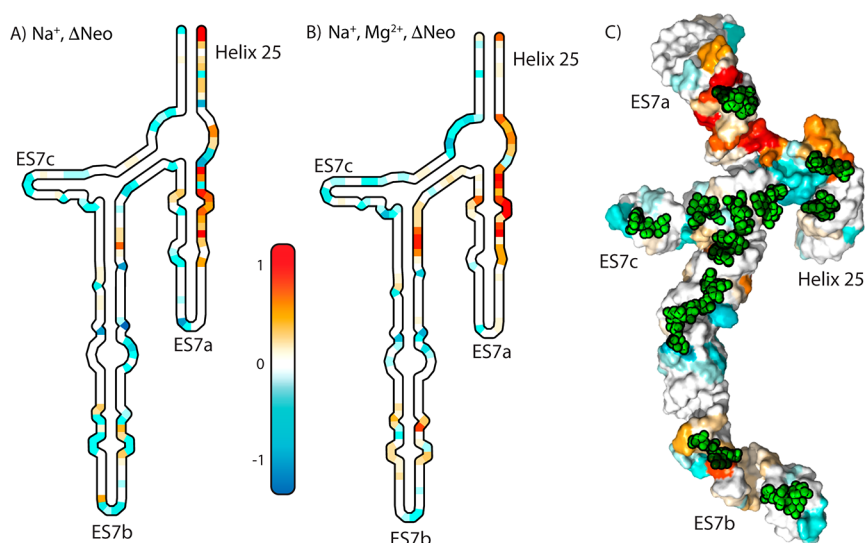


Figure 7. Δ Heat maps showing changes in SHAPE reactivity of ES7_{CA}. (A) Changes in reactivity upon addition of Neo in Na⁺ only. (B) Changes upon addition of Neo in Na⁺/Mg²⁺. (C) Docking of Neo (green) on a three-dimensional model of ES7_{CA}. Δ Neo data from panel B is mapped onto a three-dimensional model of ES7_{CA}. Red indicates increase in reactivity. Blue indicates decrease in reactivity. Experimental conditions and methods of data analysis are the same as in Figure 6. Three dimensional figures were generated with the program PyMOL.⁵⁴

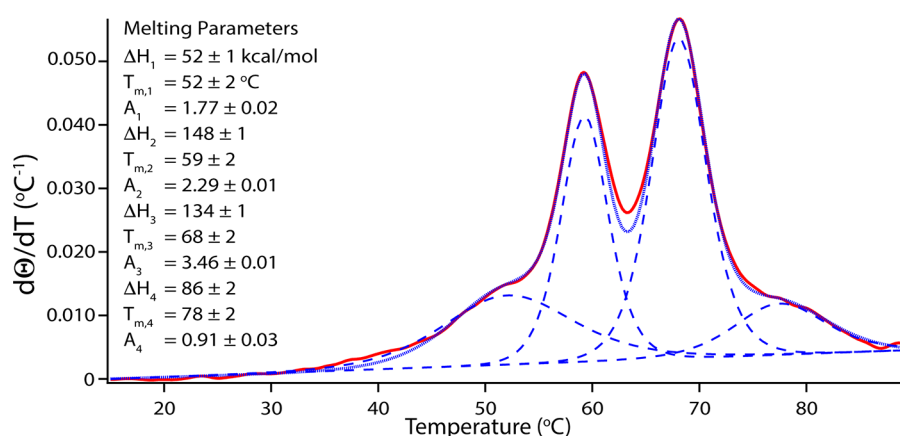


Figure 8. Melting profile of ES7_{CA} rRNA, showing the derivative of the fraction unfolded with respect to temperature, monitored at 260 nm. The melting profile was fit to a nonsequential, independent transition model, with four transitions with the program Igor Pro. The enthalpy (ΔH_n), melting temperature ($T_{m,n}$), and the relative hyperchromicity (A_n) were estimated for each transition, n . The observed profile is red, the fit is solid blue, and the deconvolution of the fit is dashed blue.

Δ Neo heat maps in both the presence and absence of Mg²⁺ (Figure 7) show that Neo dependent-changes in reactivity are broadly distributed throughout ES7_{CA}. This distribution is consistent Neo-induced changes in conformation and base pairing. Nucleotides located in the main junction, helical loops, single-stranded, and mismatch regions exhibit the most intense signals in both Δ Neo heat maps. The locations of Neo-dependent changes in SHAPE reactivity are consistent with base pairing and conformational changes in regions of noncanonical secondary structure.^{49–53} The induction of large scale conformational fluctuations are not inconsistent with our data. Similarities in the Δ Neo heat maps obtained in the absence and presence of Mg²⁺ shows that binding is not substantially altered by formation of tertiary structure. Mg²⁺ does not appear to compete with Neo, and it is not required for Neo binding to ES7 (Figure 7A,B).

Thermal Folding/Unfolding of ES7_{CA}. Isolated ES7_{CA} is well-behaved in solution. As temperature changes, ES7_{CA} folds and unfolds reversibly in a multistate process. The number of melting transitions of ES7_{CA} were estimated, along with the T_m 's

and thermodynamic parameters, from nonlinear fitting of observed melting profiles to predictions of a multistate model with independent transitions (Figure 8).^{34,55} Thermodynamic parameters were estimated previously for ES7_{SC}¹⁹ and ES7_{HS} (Lizzette M. Gomez Ramos and Loren Dean Williams, unpublished). Melting profiles are plots of the derivative of the fraction unfolded ($d\theta/dT$) versus temperature.

The unfolding profile of ES7_{CA} is similar to that of ES7_{SC}.¹⁹ The best fits for both these fungal ESs were obtained with four transition models. For ES7_{CA} in 180 mM NaCl, the melting temperatures of the four unfolding transitions ($T_{m,n}$) are $T_{m,1} = 52$ °C, $T_{m,2} = 59$ °C, $T_{m,3} = 68$ °C, and $T_{m,4} = 78$ °C. Under the same conditions, $T_{m,1}$ is the same for ES7_{CA} and ES7_{SC}. However, $T_{m,2}$, $T_{m,3}$, and $T_{m,4}$ for ES7_{CA} are around ~ 5 °C less than the corresponding T_m 's of ES7_{SC}. The results suggest that ES7_{CA} is less stable than ES7_{SC}. As observed for ES7_{SC}, the T_m 's of ES7_{CA} are constant with varying RNA concentrations, indicating that the transitions are unimolecular.

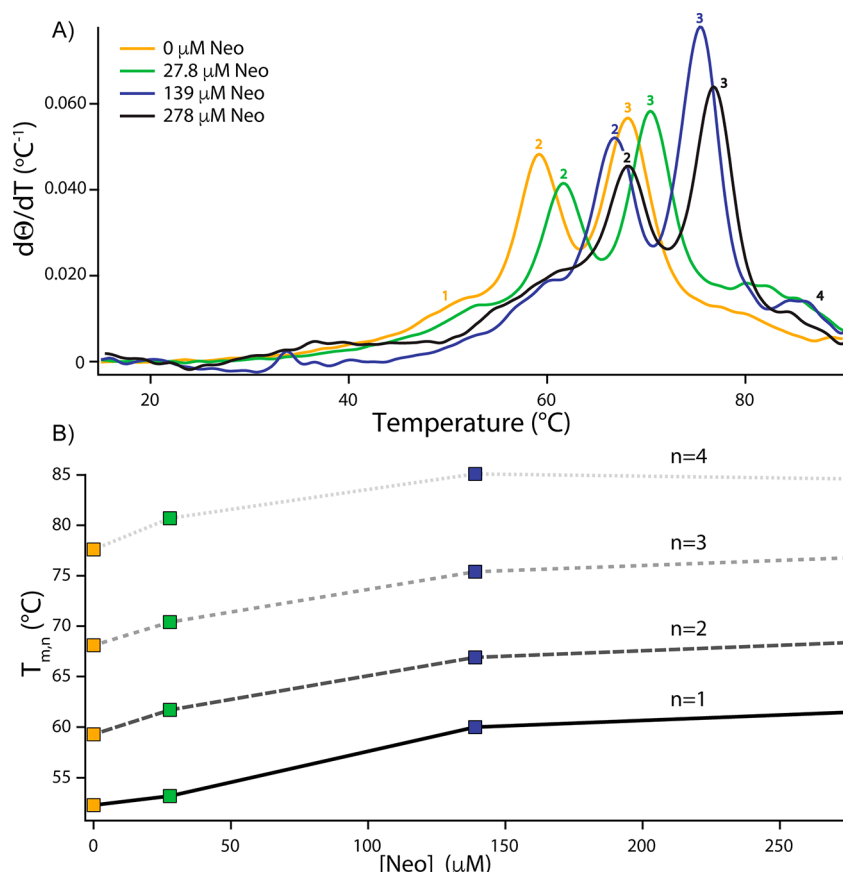


Figure 9. Melting profiles of ES7_{CA} at various concentrations of Neo. (A) The derivative of the fraction unfolded with respect to temperature at 260 nm suggests four melting transitions ($n = 1, 2, 3, 4$). Increased [Neo] causes each $T_{m,n}$ to increase. (B) The four $T_{m,n}$ of ES7_{CA} plotted versus [Neo]. The melting transition number n is indicated for each peak in both panels.

Neo stabilizes ES7_{CA}. Melting profiles for ES7_{CA} were obtained at five molar ratios of Neo to ES7_{CA} (0 μM Neo, 2.78 μM, 27.8 μM, 139 μM, and 278 μM, at constant 278 nM ES7_{CA}). The overall stability of ES7_{CA} increases with increasing concentration of Neo as indicated by monotonic changes in all four $T_{m,n}$'s (Figure 9). The results are consistent with a model (above) in which Neo binds to ES7_{CA} at broadly distributed sites, of roughly equivalent affinities. It appears that Neo binds to both native and partially unfolded intermediates. Additional information on fitting procedures and parameter estimations are provided in [Supporting Information](#).

DISCUSSION

Translation is the most conserved^{1,56–58} and highly networked⁵⁹ cellular system. Perturbations to a variety of functional sites of the ribosome can have dramatic consequences, often leading to toxicity.⁴ Eukaryotic ESs are attractive drug targets because they are idiosyncratic, are located on the ribosomal surface, and are putative binding sites for a variety of proteins and cofactors. Although bacterial ribosomes are important drug targets,⁶⁰ thus far fungal ribosomes have not been explored as drug targets. Fungal ribosomes are considered poor targets in part because of acute similarities in sequence and structure between fungal and human ribosomes in the A site, P site, exit tunnel, and peptidyl transfer center regions. Instead, chemotherapeutics target fungal target cell walls, membranes, and nucleotide biosynthetic pathways.⁶¹ The diversity of RNA functions, which are only recently being understood, suggests that small molecule binding to RNAs may provide new avenues to useful therapeutics.⁶²

Targets have emerged including ribozymes, riboswitches,⁶³ microRNAs,⁶⁴ and mRNAs.⁶⁵

Human and fungal ribosomes are highly divergent in their ESs, and therefore fungal ESs are inviting drug targets. Our goal is to determine if ES rRNAs can be developed as useful targets for chemotherapeutics directed at eukaryotic pathogens. Fungal translation can be targeted with specificity as evidenced by the activity of Sordarins, which inhibit ribosomal translation in fungi by interacting with elongation factor 2 (EF-2).⁶⁶

Aminoglycosides such as Neo are useful lead compounds for a discovery process for targeting fungal ribosomes. Aminoglycosides bind to rRNA and are active against Gram-positive and Gram-negative bacteria.⁶⁷ In bacteria, Neo associates with the A site decoding region of 16S rRNA, inducing changes in mRNA-tRNA conformation leading to amino acid misincorporation in the nascent protein.^{68,69} Aminoglycoside-induced mistranslation is thought to promote protein misfolding and cell death.^{70,71}

Here we show that Neo binds tightly and selectively to ES7_{CA} *in vitro*. The K_d of F-Neo for ES7_{CA} is in the low nanomolar range, similar to the K_d of Neo for the bacterial A site.⁷² Neo appears to be specific for *C. albicans* ES7 over human ES7. F-Neo binds to ES7_{CA} with 2000× greater K_d than to ES7_{HS}. ES7_{CA} and ES7_{HS} share low similarity in sequence, size, and structure. ES7_{HS} is four-times larger than ES7_{CA} with significantly greater GC content.

HTS of a 215 member PA library (Figure 1) reveals 63 compounds that appear to bind with greater affinity than Neo to ES7_{CA}. This library was previously screened for antibacterial activity in Gram-negative and Gram-positive bacteria and for

binding to *E. coli* A site rRNA.³⁵ PAs in which polar and positively charged amino acids are conjugated at the second position (Figure 3) exhibit the greatest affinity for ES7_{CA}. NeoFH and NeoYH have lower IC₅₀ values than Neo and NeoFN. MIC values were determined for the 10 best ES7_{CA} binders from the HTS using *C. albicans*. NeoFH, NeoRH, and NeoYH exhibited the lowest MIC values (100 μ M). Our results suggest that the geometry and hydrogen bonding capabilities of the second amino acid position are important for binding and antifungal activities. PAs with histidine at the second position exhibit the lowest IC₅₀ values and MIC values.

Thermal melting, SHAPE, binding assays, and modeling suggest that ES7_{CA} has multiple binding sites for Neo. Saturation plots suggest that there are ~13 binding sites for Neo in ES7. Neo is known to interact with various rRNA structures including hairpins,^{49,50} internal bulges,⁵⁰ single-stranded RNA,⁵¹ telomeric DNA,⁷³ DNA triple helices,^{53,74–76} RNA triple helices,^{52,53} and DNA–RNA hybrid helices.⁵² SHAPE experiments suggest that Neo causes conformational changes to internal loops, hairpins, single-strand regions, and mismatches of ES7_{CA}.

The results here are consistent with a model in which ES7_{CA} is a target for certain PAs. This model is supported with tight binding to ES7_{CA} *in vitro* and with the MIC results. Binding of PA-compounds to ES7_{CA} *in vivo* might disrupt core ribosomal function or could disturb interactions with nonribosomal proteins that are essential for cell viability.¹⁹ In sum, ESs appear to be reasonable targets for future drug target studies and that ESs may emerge as useful new targets for antimicrobials.

It seems unlikely that Neo or PAs bind tightly to the A site of *C. albicans* rRNA, which is very similar in sequence and structure to the human A site (Figure S.4). Arya demonstrated that Neo binds with low affinity to the human A site.³⁵ Our cytotoxicity studies here suggest that PAs are nontoxic to human cells.

Conclusions. Isolated ES7_{CA} is stable, folds autonomously, and adopts a near-native state. PAs bind with high affinity and selectivity to ES7_{CA}. Moreover, PAs with high affinity for ES7_{CA} induce mortality on *C. albicans* but not in HEK293T cells. Additional experiments are required to confirm that the mechanism of mortality in *C. albicans* is related to binding of the lead compounds to ES7.

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.7b00703.

Design of ES7_{CA} rRNA (Table S.1–S.4), the docking of F-neo and Neo onto the three-dimensional structure of ES7_{CA}, the sequence alignment of ES7 rRNAs (Figure S.1), the goodness of fit for the estimation of dissociation constants (Figure S.2), the thermal melting of ES7_{CA} rRNA at 260 nm (Figure S.3), the estimation of melting parameters for ES7_{CA} melting (Table S.5), and the secondary structures of the A site of *E. coli*, *C. albicans* and *H. sapiens* (Figure S.4) (PDF)

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Notes

The authors declare the following competing financial interest(s): D.P.A. has ownership interest in NUBAD, LCC.

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