

Chapter 2

Methods

2.1 Structural Study of Pleuromutilin Antibiotics

2.1.1 Crystallization and Data Collection

D50S crystals were obtained as previously described [1]. Individual crystals were soaked in harvesting solution at room temperature with 0.01 mM retapamulin (SB-275833) or SB-280080 for 12 h, or 0.1 mM SB-571519 for 8 h and then transferred into an anti-freeze solution [1] for 10 min in the presence of the pleuromutilin antibiotics at adequate concentrations. This was followed by flash cooling in liquid nitrogen. X-ray diffraction data were collected at 85 K. A few crystals were needed for yielding complete datasets of D50S complexes with SB-275833 and SB-280080. Importantly, a complete set with significant redundancy could be collected from a single crystal of the SB-571519 complex (Table 2.1).

2.1.2 Data Processing, Structure Solution and Refinement

Data were processed with HKL2000 [2] and CCP4 package suite [3]. The pleuromutilin derivatives binding sites were unambiguously located in a sigmaa-weighted difference electron density maps using the apo structure of D50S (PDB ID code 1NKW) as reference, after refinement by CNS [4]. Because no crystal structure of any isolated pleuromutilin derivative is available, initial models were generated from tiamulin coordinates (PDB ID code 1XBP) modified by using insight II (Accelrys, San Diego, CA) and CORINA (www.molecular-networks.com/software/category/gen3dcoord.html), followed by energy minimization with no X-ray terms. The maps were traced interactively using O [5] and COOT [6], followed by subsequent restraint CNS minimization for the entire complex. The same randomly selected 5% of the data were omitted for cross validation of all refinement

Table 2.1 Data statistics

Compound	SB-571519	SB-280080	Retapamulin (SB-275833)
Crystal information			
Space group	I222	I222	I222
a (Å)	170.4	170.5	170.1
b (Å)	405.8	412.7	405.9
c (Å)	703.8	696.7	695.2
Diffraction data statistics			
X-ray source	ID19, SBC/APS	ID19, SBC/APS	ID19, SBC/APS
Wavelength (Å)	1.033201	0.97933757	0.979290
Number of crystals	1	7	9
Crystal oscillation (°)	0.3–0.8	0.3–0.4	0.3–0.5
Resolution (Å)	30–3.50 (3.62–3.50)	30–3.56 (3.69–3.56)	30–3.66 (3.79–3.66)
Unique reflections	284,085	263,433	243,598
Observed reflections	1,400,909	1,541,408	1,973,738
Redundancy	4.9 (4.1)	5.9 (3.2)	8.1 (3.7)
Completeness (%)	92.7 (84.9)	90.7 (70.2)	93.1 (70.0)
$\langle I \rangle / \langle \sigma \rangle$	6.3 (1.5)	7.2 (1.5)	9.1 (1.4)
R-merge (%)	18.4 (79.7)	16.8 (70.2)	19.0 (79.1)
Refinement			
R-factor (%)	27.5	27.6	26.0
R-free (5%) (%)	33.4	33.8	33.4
rmsd bonds (Å)	0.008	0.008	0.008
rmsd angles (°)	1.4	1.4	1.4

procedures. The resulting coordinates have been deposited at the Protein Data Bank with PDB ID codes 2OGM, 2OGN, and 2OGO.

2.2 Comparative Structural Analysis to Reveal the Structural Basis for Cross-Resistance Between PTC Antibiotics

To perform general structural and statistical analysis, we generated a database holding information on the nucleotides involved in resistance or reduced susceptibility to PTC antibiotics (Table 2.2). This database includes information for each nucleotide, the antibiotics families affected by it, and the distance spans between this nucleotide to the most proximal atom of the bound antibiotic. Precisely, this distance represented the minimum value within a set of distances spans between all possible pairs of two atoms (excluding hydrogens); one in the relevant nucleotide and the other within the corresponding antibiotic molecule. Structural analysis was based on crystal structures of complexes of D50S with various antibiotics, including the phenicol chloramphenicol, the lincosamide clindamycin, the streptogramin_A dalfopristin, the pleuromutilin tiamulin, and the oxazolidinone linezolid. Crystal structures of D50S with other pleuromutilins, methymycin, and lankacidin were not included as currently no resistance data are available for them.

Biochemical data on antibiotic resistance in the archaeon *H. halobium*, and crystallographic data obtained for complexes of PTC antibiotics with the large ribosomal subunit from the archaeon *H. marismortui*, were also included. As these archaeons may resemble eukaryotes and since known pathogens are typically

Table 2.2 Resistance determinants in the PTC

Nucleotide	Appeared clinically [Y/N]	Antibiotic (family)	Bacterial strain	References
2032	N	Chloramphenicol (phenicol)	<i>E. coli</i>	[22]
			<i>B. hyodysenteriae</i>	[23]
		Clindamycin (lincosamide)	<i>E. coli</i>	[22]
		Linezolid (oxazolidinone)	<i>E. coli</i>	[24]
		Tiamulin (pleuromutilin)	<i>B. hyodysenteriae</i>	[23]
2055	N	Tiamulin (pleuromutilin)	<i>B. pilosicoli</i>	[23]
2057	N	Chloramphenicol (phenicol)	<i>E. coli</i>	[22, 25]
2058	Y	Chloramphenicol (phenicol)	<i>E. coli</i>	[22]
		Clindamycin (lincosamide)	<i>E. coli</i>	[22]
2059	N	Virginiamycin M1 and Pristinamycin II (streptogramins A)	<i>H. halobium</i>	[26]
2062	Y	Chloramphenicol (phenicol)	<i>H. halobium</i>	[27]
		Linezolid (oxazolidinone)	<i>H. halobium</i>	[28]
		Pristinamycin II (streptogramins A)	<i>S. pneumoniae</i>	[29]
		Pristinamycin I together with pristinamycin II or dalbapristin together with quinupristin (streptogramins A and B)	<i>S. pneumoniae</i>	[29]
2447	N	Linezolid (oxazolidinone)	<i>E. coli</i>	[24]
			<i>M. smegmatis</i>	[30]
		Tiamulin (pleuromutilin)	<i>B. hyodysenteriae</i>	[23]
			<i>E. coli</i>	[31]
		Chloramphenicol (phenicol)	<i>E. coli</i> and <i>B. stearothermophilus</i>	[32]
2451	N	Chloramphenicol (phenicol)	<i>E. coli</i> and <i>B. stearothermophilus</i>	[32]
2452	N	Chloramphenicol (phenicol)	<i>H. halobium</i>	[27]
		Linezolid (oxazolidinone)	<i>H. halobium</i>	[28]
2453	N	Linezolid (oxazolidinone)	<i>H. halobium</i>	[28]
2499	N	Linezolid (oxazolidinone)	<i>H. halobium</i>	[28]
		Tiamulin (pleuromutilin)	<i>B. hyodysenteriae</i>	[23]
2500	Y	Linezolid (oxazolidinone)	<i>S. aureus</i> (MRSA)	[33]
			<i>H. halobium</i>	[28]
			<i>E. coli</i>	[35]
		Tiamulin (pleuromutilin)	<i>E. coli</i>	[31]

(continued)

Table 2.2 (continued)

Nucleotide	Appeared clinically [Y/N]	Antibiotic (family)	Bacterial strain	References
2503	Y	Chloramphenicol and florfenicol (phenicols)	<i>E. coli</i>	[34]
			<i>S. aureus</i> and <i>E. coli</i>	[35, 36]
		Clindamycin (lincosamide)	<i>S. aureus</i>	[37]
			<i>S. aureus</i> and <i>E. coli</i>	[35, 36]
		Linezolid (oxazolidinones)	<i>S. aureus</i>	[37]
			<i>S. aureus</i> and <i>E. coli</i>	[35, 36]
		Tiamulin and Valnemulin (pleuromutilins)	<i>S. aureus</i> and <i>E. coli</i>	[35, 36]
2504	N	virginamycin M ₁ (streptogramins A) and dalfopristin/quinupristin (streptogramins A and B)	<i>S. aureus</i> and <i>E. coli</i>	[35, 36]
			<i>H. halobium</i>	[26]
		Linezolid (oxazolidinone)	<i>H. halobium</i>	[28]
2505	N	Tiamulin (pleuromutilin)	<i>Brachyspira pilosicoli</i>	[23]
			<i>B. hyodysenteriae</i>	[23]
2572	N	Linezolid (oxazolidinone)	<i>Enterococcus</i>	[38]
			<i>E. faecalis</i>	[39]
2576	Y	Tiamulin (pleuromutilin)	<i>B. hyodysenteriae</i>	[23]
2576	Y	Linezolid (oxazolidinone)	<i>Enterococcus</i>	[38]
			<i>Enterococcus</i>	[40]
			<i>S. aureus</i>	[41]
			<i>E. faecium</i>	[42]
			<i>S. aureus</i>	[43]
			<i>S. aureus</i>	[44]
			<i>E. faecium</i>	[45]
		Tiamulin (pleuromutilin)	<i>S. aureus</i>	[31]

Nucleotides are according to *E. coli* numbering system

eubacteria, distances and data analyses were performed separately for eubacteria and for archaea. Calculations and database analysis were performed by MATLAB (MathWorks, Inc., Natick, MA). In silico mutagenesis and structural analysis were performed by COOT [6] and PyMol (DeLano Scientific, Palo Alto, CA, USA). PyMol was also used for image rendering. PDB ID codes are shown when relevant.

2.3 Antisense Oligonucleotides for Targeting Functional Ribosomal Centers

2.3.1 Database Construction

The 23S rRNA of the large ribosomal subunit of eubacteria was selected as a model for initial study. First, we constructed a database that contained structural

and thermodynamical information (MathWorks, Inc., Natick, MA). The parameters that were incorporated into the database are as follows:

1. *Mutation sites*: Includes mutations in nucleotides that were previously related with one of the following categories: (i) antibiotic resistance or antibiotic enhancement, (ii) slow bacterial growth or reduce ribosomal activity and (iii) lethality. A total number of 132 mutations obeying at least one of these categories were selected out of the rRNA mutations database of Kathleen Triman (ribosome.fandm.edu).
2. *Stacking distance*: Including distances between the bases of each two sequent nucleotides (i.e. nucleotides i and $i + 1$), using N3 as a reference atom. The distances were calculated according to the coordinates of the large ribosomal subunit from *D. Radiodurans* (1NKW). A small N3 _{i} to N3 _{$i+1$} distance is a rough assessment for a stacking interaction between nucleobases, while a long distance indicates poor stacking.
3. *Base pairing*: Indicates Watson–Crick and Wobble pairs, as appeared in the 3D structure, detected by X3DNA [7].
4. *Surface accessibility*: Was calculated for each nucleotide separately; for the entire residue and for the backbone only (phosphate and ribose). Calculation done in the presence of all the large ribosomal subunit components (23S, 5S and r-proteins), using a probe radius of 1.4 Å (Crystallography & NMR System (CNS), [4]).
5. *Thermodynamics parameters*: Done by our collaborator, Joseph Sofaer, from the research group of Tamar Schlick, Faculty of Chemistry, NYU, using the computer program OligoWalk [8]. These parameters were calculated for all the possible 5–25 bases long antisense DNA oligonucleotides for the 23S rRNA of *E. coli*: a total sum of 60,690 theoretical antisense sequences. The following parameters were derived for each of them:
 - a. *Target structure energy*: The energy associated with braking the 2D structure of the target rRNA. The 2D structure of the 23S rRNA used to generate this parameter.
 - b. *Intramolecular energy*: Calculated for secondary structures that might be formed by the antisense itself.
 - c. *Intermolecular energy*: Considered antisense–antisense interactions.
 - d. *Duplex formation energy*: The energy gain of binding an antisense to the target sequence.
 - e. *Total binding energy*: Incorporating all of the thermodynamic parameters mentioned above to yield the overall binding energy.
 - f. *T_m of the duplex*: An estimate for the temperature by which 50% of the target sequences are bound to antisense oligonucleotides. This calculation neglected the structures of the target and of the antisense oligonucleotide, and assumed an excess of the antisense over the target. T_m calculated for antisense concentration of 1 μM.

2.3.2 *In Vitro* Transcription–Translation System for Ribosome Activity Assay

In order to characterize IC₅₀ values for the selected antisense oligonucleotides we constructed an *in vitro* transcription–translation system of *E. coli*, with firefly luciferase as a reporter gene. The procedure for *in vitro* transcription–translation system was used as previously described [9, 10], with some modifications.

2.3.2.1 *E. coli* Cell Extract Preparation

E. coli BL21(DE3) strain (Stratagene) fermented in LB medium (250 rpm at 37°C). Induction with 1 mM IPTG, for expression of T7 RNA polymerase, performed at 0.5 OD₆₀₀, followed by additional 4 h of incubation (250 rpm at 37°C) before harvesting (15 min under 4000 RCF at 4°C). Next, 20 g of bacterial paste were washed twice in 1 L and 500 ml buffer A (60 mM AcOK, 10 mM Tris–OAc pH 8.2, 14 mM Mg(OAc)₂, 155 µg/ml DTT, 0.5 µl/ml β-mercaptoethanol), followed by centrifugation (SPA-3000 rotor, 6000 rpm, 15 min, 4°C). The pellet was resuspended with 20 ml of buffer B (60 mM AcOK, 10 mM Tris–OAc pH 8.2, 14 mM Mg(OAc)₂, 155 µg/ml DTT). Cells were lysed using a French Press (American Instrument Company, Silver Spring, MA) at 8000 psi. The bacterial lysate was centrifuged to remove cell debris (SS-34 rotor, 11,600 rpm, 30 min, 4°C). The supernatant was re-centrifuged under the same conditions, and then dialyzed with SnakeSkin® 3.5 kDa molecular weight cut-off (MWCO) regenerated cellulose membrane (Pierce Biotechnology, Rockford, IL) against buffer B (750 ml × 4 times, 1–1.5 h each, 4°C). The solution was centrifuged (SS-34 rotor, 5,800 rpm, 10 min, 4°C) followed by incubation of 30 min at 37°C to reduce amounts of endogenous mRNA. Cell extract was divided into 500 µl aliquots, flash-frozen in liquid nitrogen, and stored at –80°C.

2.3.2.2 Transcription–Translation Reaction

Reaction mixtures included: 0.22 v/v *E. coli* cell extract, 1.8 ng/µl pIVEX2.6d plasmid carrying firefly luciferase under T7 promoter (donated by Roy Bar-Ziv, Weizmann Institute of Science), 900 µM of each amino acid (Sigma), 230 µg/ml Creatine Kinase from rabbit muscle (Roche), 150 µg/ml *E. coli* tRNA mixture (Sigma), 50 mM Hepes–KOH (pH 7.5), 9% PEG 8000, 180 mM KGlu, 1.6 mM DTT, 1.1 mM ATP, 0.8 mM GTP, 0.8 mM CTP and 0.8 mM UTP, 0.6 mM cAMP, 70 mM Creatine Phosphate, 30 µg/ml folinic acid, 25 mM NH₄OAc and 16 mM Mg(OAc)₂.

30 µl reaction mixtures were incubated for 60 min at 37°C in the presence of different concentrations of the various ODNs. Reactions stopped instantly with the addition of erythromycin solution to final concentration of 5 µM.

2.3.2.3 Luciferase Activity Assay for Translation Quantification

50 μ l Luciferase Assay System (Promega) were added to 10 μ l of the incubated reaction mixture in a black 96 well plate (Nunc, Roskilde, Denmark), and photoluminescence was instantly measured using SpectraFluor Plus plate reader (Tecan, Maennedorf, Switzerland). Dose response curves were fitted to the experimental data with MATLAB (MathWorks, Inc., Natick, MA) to produce IC₅₀ values.

2.3.3 Antisense Oligonucleotides Nomenclature

Antisense oligonucleotides were named according to their length and their first target nucleotide in *E. coli* numbering (e.g. antisense 463-10 is a 10 bases antisense that targets nucleotides 463–472 in *E. coli*).

2.4 Minimal Ribosomal Components with PTC Structure and Function

2.4.1 In Vitro RNA Transcription

DNA constructs were prepared using gene synthesis (purchased from GenScript, NJ) and were obtained in varies scales from few micrograms to few milligrams (Plasmid Mini, Midi, Maxi and Giga Kits, Qiagen). Plasmids were linearized with HgaI or BsaI (New England Biolabs, Madison, WI). RNA transcription and purification carried out from microgram to milligram scales, as described elsewhere [11], using a recombinant His-tagged T7 RNA polymerase (RNAP). T7 RNAP was overexpressed following standard protocols (*E. coli* BL21 strain carrying the gene in pQE8 expression vector was kindly donated by Dr. Irit Sagi and Dr. Barak Akabayov, Weizmann Institute). Mutations in RNA sequences were incorporated either by transcription from custom synthesized single strand DNA templates carrying the desired mutation (Sigma) or were incorporated to plasmids carrying the wild-type sequence using QuikChange Site-Directed Mutagenesis Kit (Stratagene), following manufacturer instructions.

2.4.2 Study of Dimerization Tendency

RNA samples were dissolved in double distilled water and annealed for 1 min at 90°C then flash cooled on ice. Incubation buffer was added to a final concentration of 89 mM tris–borate (TB) pH 7.5 and 15 mM MgCl. Unless otherwise indicated,

final RNA concentration in samples was 2 μ M. Samples were incubated at 37°C for 30 min and then placed on ice for additional 15 min. For non-denaturing gels 10 $\times$ glycerol loading dye (50% glycerol, 0.25% w/v bromophenol blue and 0.25% w/v xylene cyanol) was added and the samples were immediately loaded on ice-chilled 12% or 15% polyacrylamide (19:1 polyacrylamide:bisacrylamide) mini gel buffered with 89 mM TB pH 7.5 and 15 mM MgCl₂. For magnesium-free assays, MgCl₂ was replaced with 2 mM EDTA in both samples and gels. Non-denaturing gels were run overnight in icebox, under constant voltage of 40 V (Mini-PROTEAN[®], BioRad). For denaturing gels 2 $\times$ formamide loading dye (90% v/v deionized formamide, 0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol, 2 mM EDTA, 100 mM Tris-HCl, pH 7.3) was added to RNA samples following 10 min incubation at 65°C. Next, samples were briefly chilled on ice and loaded on denaturing 12% or 15% polyacrylamide gel with 7 M urea, buffered with 89 mM tris-borate pH 8.3 and 2.5 mM EDTA (TBE). Denaturing gels were run up to 2 h under constant voltage of 150 V (Mini-PROTEAN[®], BioRad). Gels were stained with 0.5 μ g/ml ethidium bromide (EtBr) and imaged using Bio-Rad Molecular Imager Gel Doc XR system, controlled by the Quantity One software (Bio-Rad).

2.4.3 Electrophoresis Mobility Shift Assay

The studied RNA was dephosphorylated using calf intestinal alkaline phosphatase (New England Biolabs) and 5'-end labeled with ³³P (gamma-³³P-ATP, Institute of Isotope Co., Ltd., Budapest, Hungary) or ³²P (gamma-³²P-ATP, PerkinElmer Inc.) using T4 polynucleotide kinase (New England Biolabs) as described elsewhere [11] or by KinaseMax[™] Kit (Ambion) according to manufacturer instructions. For homodimer Kd assessments non-labeled RNA of the same sequence in various concentrations was mixed with trace amount of ³³P or ³²P labeled RNA, and then was subjected to dimerization assay followed by non-denaturing polyacrylamide gel electrophoresis, as described above. For heterodimerization, labeled and unlabeled RNA of different sequences were used. Gels were transferred to Whatman 3 M paper and were dried under a vacuum at 80°C for 2 h using BioRad gel drying apparatus. Dried gels were exposed to a phosphorimager cassette (Molecular Dynamics) for periods ranging from overnight to one week, scanned (Phosphorimager Storm 820, Amersham Pharmacia Biotech) and analyzed (Quantity One software, Bio-Rad).

2.4.4 Size Exclusion Chromatography for the Separation Between Dimer and Monomer

SEC based separation between the dimer and the monomer was based on previous publications [12–14] with some modifications. Briefly, samples of the studied

RNA were annealed as described above for non-denaturing gels. 10–100 µg RNA samples were loaded on Superdex 200 10/300 GL column (Amersham Pharmacia Biosciences) equilibrated with 20 mM Tris–HCl pH 7.8, 40 mM KCl and 8 mM MgCl₂. Load volume was not exceeding 15 µl. Chromatography was performed at 0.5 ml/min at 4–7°C and fractions were collected manually into ice-chilled tubes.

2.4.5 Radiolabeling of Substrates for Peptidyl Transferase Activity Assay

CC-puromycin (CCPmn) or C-puromycin (CPmn) substrates (Dharmacon) were labeled with ³³P or ³²P as described above for 5' RNA labeling. 2× formamide loading dye (FD) added to labeled substrates prior to separation from unincorporated radiolabeled ATP by polyacrylamide gel electrophoresis (PAGE), using 12% acrylamide:bis-acrylamide ratio 29:1 gels buffered with 1× TBE and 7 M urea as denaturing agent. Gels run 1.5 h at 80 V (Mini-PROTEAN®, BioRad) in a tank containing 0.5× TBE. Under these conditions CCPmn labeled substrate co-migrated with the xylene cyanol dye. CCPmn and CPmn containing bands were sliced and eluted into Milli-Q water overnight at 4°C and quantified by scintillation counting.

2.4.6 Assay for Peptidyl Transferase Activity

Peptidyl transferase activity for various dimers performed as previously described [15] with some modifications. Briefly, RNA constructs were resuspended in Milli-Q water, annealed 1 min at 90°C and cooled down to room temperature before adding incubation buffer to final concentration of 120 mM KCl, 15 mM MgCl₂, 40 mM of either 2-(*N*-morpholino) ethanesulfonic acid (MES) pH 6.0, TRIS pH 6.8, HEPES pH 7.5 or HEPES pH 8.0. Substrates added to final concentration of 1 mM CCA-phenylalanine-caproic acid-biotin (Dharmacon) and > 2000 cpm ³³P or ³²P labeled CCPmn or CPmn. Samples contained 2 µM RNA. Control samples were incubated in the absence of RNA and the presence or absence of 1 µM 50S ribosomal subunit from *E. coli*. To check possible positive contribution of alcohol to peptidyl transferase activity, as was previously observed for similar reactions using larger substrates [16, 17], samples were incubated in the presence or absence of 30% methanol. 10 µl reactions were incubated for periods varying from 2 to 24 h at room temperature, 37 or 50°C. Reactions were quenched with the addition of 18.1 µl of 2× FD following with the addition of 8.1 µl of 5 mg/ml streptavidin (GenScript) aqueous in 150 mM NaCl and 10 mM potassium phosphate, pH 7.2. Samples were loaded on 7% acrylamide:bis-acrylamide 29:1 gels buffered with 1× TBE and 7 M urea as denaturing agent. Gels run 1 h at 80 V in a tank containing 0.5× TBE (Mini-PROTEAN®, BioRad), dried and scanned as described above.

2.4.7 RNA Two-Dimensional Structure Prediction

Two-dimensional structure prediction performed using mFold [18], version 3.2, using the default settings.

2.5 Numbering, Sequence Alignment, and Images

Nucleotides are numbered according to *E. coli* numbering system throughout, unless otherwise mentioned. Multiple sequence alignment was performed by ClustalW [19] and presented by JalView [20]. Figures of three-dimensional structures were generated by Pymol [21].

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