Catalytic Properties of Mutant 23S Ribosomes Resistant to Oxazolidinones.

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Oxazolidinones binding site and mechanism of resistance.

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SUMMARY

Kinetic analysis of ribosomal peptidyltransferase activity in a methanolic puromycin reaction with wild type and drug resistant 23S RNA mutants was used to probe the structural basis of catalysis and mechanism of resistance to antibiotics. 23S RNA mutants G2032A and G2447A are resistant to oxazolidinones both \textit{in vitro} and \textit{in vivo}, with the latter displaying a 5-fold increase in the value of $K_m$ for initiator tRNA and a 100-fold decrease in $V_{\text{max}}$ in puromycin reaction. Comparison of the $K_i$ values for oxazolidinones, chloramphenicol, and sparsomycin revealed partial cross-resistance between oxazolidinones and chloramphenicol; no cross-resistance was observed with sparsomycin – a known inhibitor of the peptidyltransferase A-site. Inhibition of the mutants using a truncated CCA-Phe-X-Biotin fragment as a P-site substrate is similar to that observed with the intact initiator tRNA, indicating that the inhibition is substrate independent and that the peptidyltransferase itself is the oxazolidinone target. Mapping of all known mutations that confer resistance to these drugs onto the spatial structure of the 50S ribosomal subunit allows for docking of an oxazolidinone into a proposed binding pocket. The model suggests that oxazolidinones bind between the P- and A-loops, partially overlapping with the peptidyltransferase P-site. Thus, kinetic, mutagenesis and structural data suggest that oxazolidinones interfere with initiator fMet-tRNA binding to the P-site of the ribosomal peptidyltransferase center.
INTRODUCTION

Oxazolidinones, the only novel class of antibiotics identified in the last two decades, are the focus of intensive discovery efforts [1-9]. Linezolid, an oxazolidinone, is approved for treatment of infections caused by Gram-positive bacteria that are resistant to other antibiotics. Emerging resistance to all known drugs, including the “last resort” vancomycin family, poses a serious threat to the public health worldwide. Understanding the mechanism of action of oxazolidinones at the molecular level, therefore, has a great importance for the development of the next generation of these novel antibiotics and, ultimately, for the outcome of the ongoing battle against drug resistant pathogens.

Oxazolidinones impose their action at the initiation stage of translation [4, 5], apparently via inhibition of pre-initiation complex formation [9]. Our recent finding that oxazolidinones interfere with binding of initiator tRNA to the ribosomal P-site [1], thus inhibiting formation of the first peptide bond, prompted a search for similarities between oxazolidinones and known inhibitors of peptidyltransferase. To address this and other mechanistic questions, we have studied catalytic properties of oxazolidinone-resistant ribosomes, and compared the mechanism of oxazolidinone inhibition to the action of known peptidyltransferase inhibitors, such as chloramphenicol and sparsomycin. To further define the oxazolidinone binding site, we have mapped resistant mutations onto the three-dimensional structure of the ribosomal 50S subunit to reveal an inhibitor binding pocket and docked an oxazolidinone inhibitor therein to show its fitness.

MATERIALS AND METHODS
Reagents and Materials. Puromycin, chloramphenicol, and tRNA$_{\text{Met}}$ were purchased from Sigma-Aldrich. SAM$^{90}$Biotin Capture plates were purchased from Promega. Microscint-20 and Microscint-40 were purchased from Packard Bioscience Co. [$^3$H]-Puromycin (50 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA). KaleidaGraph (version 3.5) was purchased from Synergy Software (Reading, PA). Sybyl Molecular Modeling Software, version 6.6, (1999) was purchased from Tripos, Inc. (St. Louis, MO). The fragment P-site substrate CCA-Phe-X-Biotin was synthesized as described in [1] at DuPont Pharmaceuticals Company. Sparsomycin was obtained from Drug Synthesis & Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute.

Preparation of oxazolidinone resistant 23S RNA mutant ribosomes. Oxazolidinone resistant mutant strains carrying a single mutated copy of ribosomal 23S RNA were selected and characterized by Thompson et al. [2, 33], using plasmids with site-directed mutations [10] and a recently described single plasmid-encoded rRNA operon system in Escherichia coli [11]. Cells from 4 liters of exponentially growing wild type or mutant E. coli culture (A$_{590}$ = 0.6) were pelleted by centrifugation in a Sorvall SLA3000 rotor at 9110 rpm for 15 minutes at 4°C. Cell pellets were resuspended in equal volume of buffer A (20 mM HEPES-KOH (pH 7.6 @ 0°C), 6 mM MgCl$_2$, 30 mM NH$_4$Cl, 4 mM β-ME, 0.1 mM PMSF, and 0.1 mM BH) and centrifuged at 9110 rpm in SLA3000 rotor for 10 min. The pellets were transferred into a mortar and ground with pre-chilled alumina, followed by the addition of an equal volume of Buffer A supplemented with β-ME, PMSF, and BH. After incubation with RNase-free DNase for 30 min. at 0°C, alumina and cell debris were removed by centrifugation at 15,000 for 15 min and 30,000 × g for
30 min, respectively. Supernatant was then layered over equal volume of 1.1 M sucrose cushion in Buffer B (Buffer A + 0.5 M NH₄Cl) and centrifuged at 100,000 × g for 15 hrs. After rinsing with Buffer A, resulting ribosome pellet was resuspended in 4 ml of Buffer A. The tight-coupled 70S ribosomes were divided into portions, frozen in liquid nitrogen and stored at –80° C.

**Synthesis of initiator fMet-tRNA<sub>Met</sub>.** *In vitro* enzymatic synthesis of [³⁵S]fMet-tRNA was performed as described in [1] using individual tRNA<sub>Met</sub> and S-100 extract as a source of formyltransferase and methionyl-tRNA synthetase. An average preparation had a specific radioactivity of 0.02-0.03 µCi/pmol.

**Puromycin peptidyltransferase reaction.** The *in vitro* peptidyltransferase assay was carried out in a 45 µl mixture containing following components: 36.7 mM Tris-HCl (pH 7.4 at 23° C), 10 mM MgCl₂, 266.7 mM KCl, 0.01 µM of *E. coli* 70S ribosomes, 0.01-0.4 µM f-[³⁵S]Met-tRNA<sub>Met</sub> (20 Ci/mmol), 10-200 µM puromycin, 33.3% MeOH, and 0.0083% DMSO. Reactions were incubated at room temperature for various time periods. 10 µl of 5 N KOH was added to stop the reaction and the quenched reactions were incubated for 20 min at 37°C, followed by the addition of 600 µl of 1 M NaHPO₄ (pH 7.0) and 250 ml ethyl acetate. The product [³⁵S]formyl-methionyl-puromycin was extracted into the ethyl acetate phase by shaking for 15 min and centrifugation for 5 min. 80 µl of the ethyl acetate phase was mixed with 125 µl Microscint-40 to the organic phase and counted in a Packard Topcount-NTX™ (Packard Instrument Co.).

**Fragment peptidyltransferase reaction.** Reactions were performed at room temperature and contained the following components in a 60 µl volume: 36.7 mM Tris-HCl (pH 7.4 at 23° C), 10 mM MgCl₂, 266.7 mM KCl, 0.15 µM of *E. coli* MRE600 70S
ribosomes, various concentrations of CCA-Phe-X-Biotin, 50 µM [³H]puromycin (0.5 Ci/ mmol), 33.3% MeOH, and 0.0083% DMSO. The reaction mixtures were stopped by addition of 0.5 volume of 7 M Guanidine-HCl with 20 mM EDTA(pH 8.0) at different incubation time points. The quenched reactions were then transferred to a SAM⁹⁶Biotin Capture plate pre-wetted with 2 M NaCl. After incubation for 10 min at room temperature, free [³H]puromycin was removed by filtration and subsequent washes with 2 M NaCl. The bound Biotin-phe-[3H]puromycin was measured using a Packard Topcount-NTX™ by adding 15 µl Microscint-20.

Mutant $K_m$ and $V_{max}$ determination. Assuming that the ribosomal peptidyltransferase has as an ordered mechanism of action [12], in which P-site substrate, A, and A-site substrate, B, associate with the ribosome, E, in an obligate order: the A-site substrate can only bind to the preformed P-substrate $*$ ribosome complex,

$$E + A \xrightarrow{K_A} EA + B \xrightarrow{K_B} EAB \xrightarrow{k_P} E + P$$

the corresponding velocity equation would be given by equation 1.

$$\frac{V_o}{V_{max}} = \frac{[A][B]}{K_A K_B} \left[ 1 + \frac{[A]}{K_A} + \frac{[A][B]}{K_A K_B} \right]$$

(1)

Determination of apparent $V_{max}$ and $K_m$ parameters for fMet-tRNA$_{i}^{\text{Met}}$ was performed under the pseudo first-order reaction conditions, where fixed saturating concentration of puromycin (200 µM) with 70S ribosomes at 0.005 µM has been used, and fMet-tRNA$_{i}^{\text{Met}}$ concentration was varied from 0.04 to 0.8 µM, depending on the mutant studied. To obtain corresponding puromycin $K_m$ values ($K_B$) a fixed saturating concentration of 0.8 µM fMet-tRNA$_{i}^{\text{Met}}$ was used for the wild type and all three mutants, while the puromycin
concentration was varied from 5 to 400 µM. Time courses at each set of conditions were performed and initial rates were determined by linear regression analysis of steady-state kinetics.

**IC50 and $K_i$ determination.** To determine $K_i$ values in the puromycin and fragment reactions initial rates of the reaction in the presence of different concentrations of inhibitors were evaluated by quantifying product at a single time point [1]. Incubation times for wild type and different mutants have been selected based on linearity of time courses for different peptidyltransferases. The puromycin reaction was performed using 50 µM of puromycin, 0.2 µM of fMet-tRNA$\text{Met}$ and 0.012 µM 70S ribosomes. The fragment reaction contained 0.24 µM of 70S ribosomes, 6 µM of CCA-Phe-X-Biotin fragment and 50 µM of puromycin (0.5 Ci/mmol). Incubation times for fragment reaction experiments were selected based on linearity of the corresponding time courses. KaleidaGraph software was used both for IC50 curves plotting, linear regression and non-linear regression analysis of the data. $K_i$ values were derived by fitting the [S] - V data into the corresponding rate equations.

For sparsomycin, a competitive inhibitor of A-site [13-15], equation 2 was used:

$$\frac{V_o}{V_{\text{max}}} = \frac{[A][B]}{K_A K_B} \frac{1}{1 + \frac{[A]}{K_A} \beta + \frac{[A][B]}{K_A K_B}} \quad \text{where } \beta = 1 + \frac{[I]}{K_i} \quad (2)$$

Based on competitive behavior towards both initiator tRNA (P-site substrate) and puromycin (A-site) [1] equation 3 was used for oxazolidinones:

$$\frac{V_o}{V_{\text{max}}} = \frac{[A][B]}{K_A K_B} \frac{1}{\beta + \frac{[A]}{K_A} + \frac{[A][B]}{K_A K_B}} \quad \text{where } \beta = 1 + \frac{[I]}{K_i} \quad (3)$$
It was shown previously that chloramphenicol binds to a different site on the ribosome than A-site inhibitor sparsomycin [16] and also inhibits sparsomycin-induced binding of the fragment P-site substrate to the ribosomes [17]. Mixed competitive or competitive behavior towards puromycin (A-site substrate) [18, 19] has also been reported for this inhibitor. Based on these results, it was assumed that chloramphenicol is a competitive inhibitor in respect to both sites and the data were fitted to the same equation (2) as for oxazolidinones.

**Modeling of oxazolidinones binding site.** Nucleotides in *T. maritima* 23S RNA for the oxazolidinone resistant mutations reported for *H. halobium* and *E. coli* in [2, 20, 21] have been assigned by alignment of the corresponding 23S sequences: G2032 (2073), C2057 (2098), G2058 (2099), C2062 (2104), G2447 (2482), C2453 (2487), U2499 (2535), C2500 (2536), A2502 (2538), and U2503 (2539), with *T. maritima* numbering given in brackets. Mutated nucleotides as well as A- and P-loop regions have been mapped onto the 50S crystal structure using WebLab ViewerLite 3.5 software. Oxazolidinone cross-linking positions reported by Matassova et al. [22] could not be visualized in the 2.4 Å electron density map [23], thus the docking procedure was predominantly based on steric considerations as well as on mutant inhibition data. The oxazolidinone inhibitor XA043 was modeled into a potential binding pocket on the ribosome using the X-ray coordinates with PDB entry code 1FFK and Sybyl molecular modeling software. The inhibitor was built and minimized in the gas phase before manually docking it into the pocket near residues where oxazolidinone-resistant mutations occur and the active site K⁺ resides.
RESULTS

Catalytic properties of 23S RNA mutants in puromycin reaction. $K_m$ and $V_{max}$ values for both A- and P-site substrates in the puromycin reaction for the drug resistant 23S RNA mutants were determined and compared with the wild type ribosomes. For mutant G2447A (position 2482 in \textit{T. maritima}), significant changes in both $K_m$ and $V_{max}$ values are observed for both substrates (Fig. 1, A and B). The values of $K_m$ for initiator tRNA and puromycin were increased by 5 and 2-fold, respectively, and $V_{max}$ decreased 100-fold (Table 1). Erythromycin resistant mutant A2058G (2099 in \textit{T. maritima}), which is weakly resistant to oxazolidinones \textit{in vivo} [2, 33], exhibited a 3-fold increase in $K_m$ value for initiator tRNA with no change for that of puromycin. Oxazolidinone and chloramphenicol-resistant and erythromycin hypersensitive mutant G2032A (2973) has $K_m$ and $V_{max}$ values that are similar to the wild type. Thus, two of the three studied oxazolidinone-resistant mutants (G2447A and A2058G) had decreased affinity for the initiator tRNA (P-site) substrate with none of the mutations affecting the interaction with the A-site substrate puromycin.

Effect of peptidyltransferase inhibitors upon activity of 23S RNA mutants in puromycin and fragment reactions. To define the oxazolidinones binding site we studied inhibition of oxazolidinone resistant mutants in two different substrate systems:

- In the puromycin reaction that employs initiator fMet- tRNA$_f^{Met}$ as a P-site substrate and puromycin as an A-site substrate;

- In the fragment reaction where truncated version of P-site substrate, CCA-Phe-Biotin, is utilized, while A-site substrate, puromycin, is the same as in the first system.
To compare inhibitory properties of oxazolidinones with those of known peptidyltransferase inhibitors chloramphenicol and sparsomycin, we determined corresponding IC50 values in both puromycin and fragment reactions performed by mutant ribosomes.

Inhibition profiles of mutant peptidyltransferases in the puromycin reaction with oxazolidinones, XA043 and linezolid, are shown in Fig. 2, panels A and B respectively. As expected based on their high levels of resistance in vivo [2, 33], oxazolidinone-resistant mutants G2447A and G2032A are not inhibited by oxazolidinones in vitro. The effect of chloramphenicol on peptidyltransferase mutants is shown in Figure 2, panel C. Oxazolidinone-resistant mutant G2447A is not inhibited by chloramphenicol either, even though it is highly sensitive to the A-site inhibitor sparsomycin. In contrast, oxazolidinone-resistant mutant G2032A shows rather a moderate in vitro resistance against chloramphenicol indicated by less than 4-fold decrease in affinity for this inhibitor. Inhibition of mutant peptidyltransferases with A-site inhibitor sparsomycin is shown in Figure 2, panel D. An inhibition constant of ~ 0.4 µM determined in this work for sparsomycin was in a good agreement with the $K_i$ value of ~ 1 µM previously reported for this antibiotic with a pentamer fragment substrate by Harris and Pestka [34]. Oxazolidinone-resistant mutants are either more sensitive to inhibition by sparsomycin than the wild type (A2058G and G2447A) or 2-fold less sensitive (G2032A), indicating little cross resistance.

When fragment P-site substrate was used in place of initiator fMet-tRNA, the cross-resistance to oxazolidinones (Fig. 3, panels A and B) and chloramphenicol (Figure 3, panel C) for mutant G2447A remains with somewhat lower level of resistance to
oxazolidinone XA043. It is noteworthy that an opposite effect – a stimulation of the fragment reaction - was observed for this mutant in the presence of linezolid. The effects of inhibitors on the reactions catalyzed by mutant G2032A were affected by which P-site substrate was used: initiator tRNA leads to less inhibition than the truncated P-site substrate. At the same time, mutations had insignificant, less than 2-fold, effect on the magnitude of inhibition with sparsomycin (Figure 3, panel D).

To compare inhibitors with different mechanisms of action, $K_i$ values were calculated (summarized in Table 2). Mutants A2058G and G2032A are ~3-4-fold less sensitive towards linezolid in comparison with XA043. Both of these mutants are less affected by chloramphenicol than wild type. Mutant G2447A was not inhibited by chloramphenicol at all, but its sensitivity to oxazolidinones decreased by more than 100-fold. A weakly resistant to oxazolidinones mutant A2058G [2, 33] is sensitive towards oxazolidinones in a fragment reaction, while displayed a moderate in vitro resistance when whole initiator tRNA was used as a substrate (compare panels A and B in Fig. 3 and Fig. 2 respectively). None of the mutations studied displayed a significant level of resistance to an A-site inhibitor sparsomycin.

**DISCUSSION**

Mutational studies of the ribosome by genetic means have been a fruitful approach to investigation of the functional role of the different regions of this remarkable ribozyme [24 - 26]. However, the biochemical effects of ribosomal mutations were hindered by an inability to isolate mutant ribosomes free of wild type counterparts, because of the presence of multiple copies of both 16S and 23S RNA in most eubacteria.
Development of the single plasmid-encoded rRNA operon system in *E.coli* [11] made possible engineering, isolation and enzymatic characterization of mutant ribosomes *in vitro*. Steady state experiments using purified drug resistant ribosomal mutants have yielded meaningful catalytic parameters. These, in turn, have allowed initial structure-function relationships at the peptidyltransferase center to be determined, as well as possible mechanisms of action and resistance to antibiotics at the level of the catalyst itself.

We find that 23S RNA G2032A and G2447A mutations result in decreased affinity for the P-site substrate initiator fMet-tRNA and insensitivity towards oxazolidinones and chloramphenicol. Nucleotide 2447 belongs to the peptidyltransferase region that has been shown to be involved in interactions with the acyl moiety of initiator tRNA (P-site) [27-30]. We also demonstrate that the affinity for the A-site substrate is not affected by the mutations that confer resistance to oxazolidinones. According to our inhibition results (Fig. 2 and 3), chloramphenicol, an inhibitor of both P- and A-sites [16, 17] displays partial cross-resistance with oxazolidinones, while a specific inhibitor of the A-site, sparsomycin, does not. Thus, this suggests that oxazolidinones inhibit the methanolic peptidyltransferase reaction primarily via interference with the P-site substrate binding. In addition we have shown previously that oxazolidinones are competitive with initiator tRNA (P-site substrate) inhibitors [1]. The accumulated evidence leads us to conclude that oxazolidinones are inhibitors of the peptidyltransferase P-site.

Truncation of the P-site substrate did not eliminate *in vitro* resistance towards oxazolidinones and chloramphenicol. This can be interpreted in terms of the binding site
for these drugs being located on the 50S part of the P-site in a close proximity to the CCA-fMet binding site. We suggest that mutations at positions G2447 and G2032 interfere with the binding of isoacceptor part of the initiator tRNA and at the same time contribute to formation of the oxazolidinone binding site. Mutant A2058G showed no resistance towards oxazolidinones in the fragment reaction, while a moderate decrease in affinity for oxazolidinone XA043 and chloramphenicol was observed. This can be interpreted in terms of the possible structural changes in the P-site induced by this mutation, in particular in binding of non-isoacceptor portion of the initiator tRNA. A remarkable observation that a catalysis of the fragment reaction by mutant G2447A is stimulated in the presence of linezolid, but inhibited by XA043, suggests that inhibition with oxazolidinones might occur via conformational change in the mutant active center that distorts the substrate(s) binding site leading to a dichotomous outcome depending on the structure of the inhibitory molecule bound.

To further define the oxazolidinones binding site, mutations that lead to oxazolidinones resistance [2, 20, 21, 33] were mapped onto the crystal structure [23] of the peptidyltransferase 50S subunit (Fig. 4). Although the mutated nucleotides are relatively dispersed in the primary structure of 23S rRNA, these mutations map into a confined space between the P- and A-loops in the three-dimensional structure, which partially overlaps with the P-site. As seen in Figure 4A, the mutations are clustered near the central channel of the ribosome that permits substrate entry to the P-site. There is a cavity of sufficient size to accept the inhibitor XA043 as shown in Figure 4C, which contains a potassium ion that might be required for catalytic activity [30]. Little reorganization of the nucleotide residues is necessary for inhibitor access to the binding site. Although the binding site model is preliminary and cannot predict the exact
conformations of the binding pocket and the inhibitor especially in light of potential structural differences between archaeal and eubacterial ribosomes, the model offers an explanation of the inhibitory action against P-site activity by oxazolidinones.

The functional role of the nucleotide G2447 is of a particular interest due to the ongoing controversy about the mechanism of catalysis by ribosomal peptidyltransferase. The low catalytic efficiency and markedly decreased affinity for the P-site substrate of mutant G2447A suggests an important contribution to the catalysis by this nucleotide. At the same time, it raises questions about the proposed general acid-base mechanism hypothesis [30-31]. If nucleotide G2447 plays an important role in the pKₐ shift of the catalytic residue A2051, one would expect this nucleotide to be essential both in vivo and in vitro, which has not been observed [3, 10, 32]. Thus, there is a clear need for further mechanistic studies of this essential ribozyme.

We conclude that oxazolidinones inhibit binding of the initiator tRNA to the peptidyltransferase P-site on 50S, preventing the formation of the first peptide bond. One would also predict that clinical resistance to this class of drugs would evolve by introducing mutations that would alter the fine structure of the P-site, as was observed in the case of two oxazolidinone resistant mutants, selected in the laboratory. One would also predict that frequency of resistance acquired through this target-based mechanism should be quite low due to redundancy of rRNA operons in bacteria. Thus, this key ribozyme, encoded by multiple rDNA genes, presents an excellent drug discovery target for the development of novel antibiotics, the clinical usefulness of which would not be eroded by single point mutations.

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FOOTNOTES

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The abbreviations used are: CAP, chloramphenicol; β-ME, β-mercaptoethanol; PMSF, phenylmethysulfonyl fluoride; and BH, benzamidine hydrochloride.
LEGENDS TO FIGURES

Figure 1. Determination of catalytic parameters for 23S RNA mutant peptidyltransferases in puromycin reaction. Determination of the $K_m$ values for the P-site substrate was performed at fixed concentrations of puromycin (150 µM) and 70S ribosomes (0.010 µM) under the concentration of initiator f-$[^{35}\text{S}]$Met- tRNA$_{\text{Met}}$ in 0.01 - 0.4 µM range. Time courses at each set of conditions were taken and resulting initial rates were determined.

Panel A – double-reciprocal plot of puromycin reaction in the presence of different concentrations of initiator tRNA for (○) - wild type, and (●) - A2058G, (▼) - G2032A, (■) - G2447A mutants.

Panel B – double-reciprocal plot of puromycin reaction in the presence of different concentrations of puromycin for (○) - wild type, and (●) - A2058G, (▼) - G2032A, (■) - G2447A mutants.

Figure 2. Inhibition of activity of oxazolidinone resistant peptidyltransferases in puromycin reaction with various antibiotics. Antibiotics at various concentrations were added to the 50 µL reaction mixtures containing 0.012 µM of the corresponding E. coli 70S ribosomes, 0.2 µM f-$[^{35}\text{S}]$Met-tRNA$_{\text{Met}}$ and 50 µM puromycin, where (○) - wild type, and (●) - A2058G, (▼) - G2032A, (■) - G2447A mutants:

Panel A – inhibition with oxazolidinone XA043
Panel B – inhibition with oxazolidinone linezolid
Panel C – inhibition with chloramphenicol
Panel D – inhibition with sparsomycin

Figure 3. Inhibition of activity of oxazolidinone resistant peptidyltransferases in fragment reaction with various antibiotics. Antibiotics at various concentrations were added to the 50 µL reaction mixtures containing 0.24 µM of the corresponding E. coli 70S ribosomes, 6 µM of CCA-Phe-X-Biotin fragment and 50 µM of puromycin (0.5 Ci/mmol), where (○) - wild type, and (●) - A2058G, (▼) - G2032A, (■) - G2447A mutants:
Panel A – inhibition with oxazolidinone XA043
Panel B – inhibition with oxazolidinone linezolid
Panel C – inhibition with chloramphenicol
Panel D – inhibition with sparsomycin

Figure 4. A Proposed Binding Site for Oxazolidinones on the 50S Ribosome.
Panel A – mapping of oxazolidinone resistant mutations onto 50S ribosomal subunit. A- and P-loop regions are shown in white and blue color, respectively, with oxazolidinone resistant mutations displayed in yellow. The yellow arrow points to the area occupied by residues, which upon mutation confer oxazolidinone resistance: G2032 (2073), C2057 (2098), G2058 (2099), C2062 (2104), G2447 (2482), C2453 (2487), U2499 (2535), C2500 (2536), A2502 (2538), and U2503 (2539), with T. maritima numbering given in brackets.
Panel B – oxazolidinones binding site on 50S ribosomal subunit. T. maritima residues are labeled with the corresponding E. coli residues in parentheses.
Panel C – docking of oxazolidinone XA043 structure into the binding pocket. After docking the inhibitor XA043 into the putative binding pocket on the ribosome, an 8 Å sphere around the inhibitor was extracted for display. The residues that upon mutation confer oxazolidinone resistance are colored in purple, the other residues are in green, XA043 is colored by atom type, and the K+ cation is in orange. T. maritima residues are labeled with the corresponding E. coli residues in parentheses. The view is from the center of the P-site helix (Figure 4 A) looking outward radially.
Table I
Catalytic properties of mutant peptidyltransferases in puromycin reaction.

| 23S Mutant | Initiator tRNA (P-site) | Puromycin (A-site) |
|------------|-------------------------|--------------------|
|            | $K_m$ (µM) | $K_m$ Ratio Mutant/WT | $V_{max}$ Ratio Mutant/WT | $V_{max}/K_m$ Ratio Mutant/WT | $K_m$ (µM) |
| Wild Type  | 0.054 ± 0.005 | 1.00 | 1.00 | 1.00 | 16.9 |
| G2032A     | 0.062 ± 0.003 | 1.16 ± 0.06 | 0.62 ± 0.05 | 0.54 ± 0.01 | 20.9 |
| A2058G     | 0.180 ± 0.059 | 3.41 ± 1.46 | 0.19 ± 0.04 | 0.06 ± 0.01 | 12.7 |
| G2447A     | 0.247 ± 0.109 | 4.57 ± 1.65 | 0.01 ± 0.02 | 0.02 | 35.1 |
Table 2.

\( K_i \) values for peptidyltransferase inhibitors in puromycin reaction.

| Inhibitor       | \( K_i \) (\( \mu M \))         |
|-----------------|---------------------------------|
|                 | Wild Type | A2058G | G2032A | G2447A |
| XA043           | 3.25 ± 0.19 | 11.79 ± 0.93 | 27.1 ± 5.7 | 385.8 ± 94.7 |
| Linezolid       | 5.04 ± 1.17 | 43.7 ± 4.2  | 141.8 ± 28.5 | 794.2 ± 184.6 |
| Chloramphenicol | 0.25 ± 0.03 | 0.74 ± 0.04  | 0.90 ± 0.10 | No Inhibition |
| Sparsomycin     | 0.44 ± 0.05 | 0.12 ± 0.01  | 0.87 ± 0.08 | 0.12 ± 0.02   |
Catalytic properties of mutant 23S ribosomes resistant to oxazolidinones
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