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Virtual Screening and Experimental Verification to Identify Potential Inhibitors of the ErmC Methyltransferase Responsible for Bacterial Resistance against Macrolide Antibiotics

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Methyltransferases from the Erm family catalyze S*-adenosyl-*L*-methionine-dependent modification of a specific adenine residue in bacterial 23S rRNA, thereby conferring resistance to clinically important macrolide, lincosamide, and streptogramin B antibiotics. Thus far, no inhibitors of these enzymes have been identified or designed that would effectively abolish the resistance in vivo. We used the crystal structure of ErmC′ methyltransferase as a target for structure-based virtual screening of a database composed of 58679 lead-like compounds. Among 77 compounds selected for experimental validation (63 predicted to bind to the catalytic pocket and 14 compounds predicted to bind to the putative RNA binding site), we found several novel inhibitors that decrease the minimal inhibitory concentration of a macrolide antibiotic erythromycin toward an* Escherichia coli *strain that constitutively expresses ErmC′. Eight of them have IC_{50} values in the micromolar range. Analysis of docking models of the identified inhibitors suggests a novel strategy to develop potent and clinically useful inhibitors.*

Introduction

The majority of clinically useful antibiotics function by inhibition of protein synthesis by bacterial ribosomes. Despite the considerable structural diversity of antibiotics, they target the ribosome at very few locations, which results in overlap between many of their binding sites.[1] Most ribosome-targeting antibiotics are produced by species within the actinomycetes group of Gram-positive bacteria. To protect their own ribosomes from inhibition during antibiotic production, actinomycetes have developed various defense mechanisms, including enzymatic methylation of key rRNA nucleosides at the drug target site, drug modification, and active efflux of the drug from the cell. Unfortunately, extensive use of antibiotics in medicine and animal husbandry has led to the acquisition of these defense mechanisms by pathogenic bacteria, including streptococcal and staphylococcal species, and the rapid spread of drug-resistant pathogens. Currently, resistance to all major groups of antibiotics is on the rise, and the efficacy of many antibiotics in the clinical treatment of infections is severely compromised. This situation is particularly disturbing with respect to resistance mechanisms that involve rRNA methylation because the binding sites of many antibiotics overlap. Therefore, a single resistance determinant can confer cross-resistance to many chemically unrelated drugs.[2]

The best studied and commonly observed antibiotic resistance caused by rRNA methylation concerns macrolide antibiotics, natural polyketide products of secondary metabolism in many actinomycete species. Shortly after the introduction of erythromycin into therapeutic use in the 1950s, resistance to this antibiotic was observed in bacterial pathogens.[3] Disturbingly, these erythromycin-resistant strains were cross-resistant not only to all other macrolides but also to chemically unrelated lincosamide and streptogramin B drugs. This phenomenon was first observed in *Staphylococcus aureus* and came to be termed the macrolide–lincosamide–streptogramin B (MLS) antibiotic resistance phenotype. The resistance to all these antibiotics is caused by mono- or dimethylation of 23S rRNA at the N6 position of adenosine 2058 (*E. coli* numbering), which is a pivotal nucleoside for the binding of MLS antibiotics. The methylation of A2058 to m^6A and subsequently to $m^6{}_2A$ is introduced by enzymes encoded by a group of genes termed *erm* (erythromycin resistance methyltransferases).[2] Horizontal transfer of *erm* genes to numerous pathogenic strains and global dissemination of resistant clones and their descendants are major components of the present-day macrolide resistance

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problem.[4] Currently, it is the most prevalent resistance mechanism in clinical isolates of *Streptococcus* in Europe and the Far East, whereas drug efflux is the more common mechanism in North America. Disturbingly, A2058 dimethylation by constitutively expressed Erm enzymes can lead to resistance even against the new-generation semi-synthetic ketolide antibiotics, such as telithromycin, that are effective against other mechanisms of resistance (such as drug efflux, A2058 monomethylation, or mutation in ribosomal components).[5] Thus, Erm methyltransferases remain the most dangerous factor responsible not only for compromising the utility of "old" MLS antibiotics, but also the newest generation of macrolide derivatives.

Not surprisingly, Erm and other resistance methyltransferases (MTases) were proposed as potential targets for drugs that would reverse antibiotic resistance and which could therefore be co-administered with the corresponding antibiotics. A high-throughput screening analysis has detected compounds that selectively inhibit the ErmC′ enzyme in the nano- to micromolar range in vitro, but which failed, however, to inhibit macrolide resistance in an in vivo mouse model.[6] Although these synthetic leads were regarded as a promising starting point for the design of more potent inhibitors, without the knowledge of the protein binding mode it has been difficult to optimize their activity. To our knowledge, no follow-up study has appeared since the initial report, either as a patent or publication. Independently, using an NMR-based screen and parallel synthesis, lead compounds have been generated that bind to the ErmAM and ErmC′ MTases and inhibit their RNA MTase activities.[7] A crystal structure of one of these compounds has revealed that it acts by blocking the *S*-adenosyl-L-methionine (AdoMet) binding pocket. Another study reported the construction of a targeted ligand library by virtual screening against the ErmC′ structure and experimental confirmation that some of these compounds inhibit a closely related Erm enzyme from *Chlamydia pneumoniae*.[8,9] However, all these candidate leads bind in the AdoMet binding site that is strongly conserved among all Rossman-fold MTases. Nonselective inhibitors that target a pocket common to many enzymes can inhibit multiple MTases involved in many aspects of cellular metabolism and are therefore dangerous for human cells.

We proposed that another possible means of combating Erm-mediated macrolide resistance would be to develop drugs that block the unique RNA binding site of these enzymes.[10–12] Unfortunately, a crystal structure of Erm–RNA complex is not available (our own attempts to crystallize the complex have also been unsuccessful). Thus, we have used extensive alanine-scanning mutagenesis of the predicted RNA binding site on the surface of ErmC′ to identify the site of essential protein–RNA interactions.[10] We have also analyzed by mutagenesis the roles of individual amino acids in the catalytic pocket,[11] and of the basic N terminus,[12] which is disordered in the X-ray crystal structure.[13] Based on our results, we constructed a model of Erm–RNA interactions and proposed that the universally conserved small C-terminal domain is not required for binding and catalysis per se, but only for maintaining the structural integrity of the large N-terminal domain.[10] Our prediction was confirmed by the identification of an Erm family member that lacks the C-terminal domain, yet exhibits MTase activity, albeit with relaxed substrate specificity.[14,15] In the work reported herein, we used our model of ErmC′ activity as a platform for the structure-based design of Erm inhibitors that would block the Erm-specific substrate binding site rather than the ubiquitous AdoMet binding site.

Results and Discussion

Identification of the optimal receptor conformation

The active center of MTases that modify exocyclic nucleic acid bases is characterized by two well-defined clefts: a deep groove for binding of the common methyl group donor AdoMet, connected with a somewhat more exposed pocket, where the substrate base binds.[16] The residues that line up the cofactor binding site are conserved among different MTase families, while the substrate base binding site differs depending on the type of substrate and the type of methylation catalyzed.[17,18] For instance, nearly all MTases that methylate exocyclic amino groups in RNA or DNA to yield m^6A, m^2G, or m^4C, exhibit a characteristic motif: (N/D/S)-(I/P)-P-(Y/F/W/H).[19] In the protein–substrate co-crystal structure of DNA:m^6A–MTase M.TaqI, a relatively close homolog of Erm MTases, the aromatic residue at the terminal position stabilizes the methylation substrate base by face-to-face stacking interactions, while the main-chain carboxyl group of the conserved Pro residue and the side chain of the semi-conserved Asn hydrogen bond the target NH_2 group and stabilize it with respect to the methyl group donor.[20] Mutagenesis of the corresponding residues in ErmC′ has shown the aromatic Tyr 104 residue to be absolutely essential for ErmC′ activity, while the semi-conserved Asn 101 was found to be important but not absolutely essential.[11]

The RCSB Protein Data Bank (PDB) contains five crystal structures of the ErmC′ MTase: 2ERC (apo form, resolution 3.03 Å), 1QAM (apo form, 2.20 Å), 1QAO (complexed with AdoMet, 2.70 Å), 1QAN (complexed with *S*-adenosyl-L-homocysteine, 2.40 Å), and 1QAQ (complexed with adenosylornithine, 2.80 Å). The 2ERC structure is the lowest resolution, and its base binding pocket is completely blocked by the loop with the catalytic NIPY motif, which also exhibits large B-factor values, indicating high uncertainty. The remaining ErmC′ structures are very similar to each other (full atom root-mean-square deviation (RMSD) < 0.5 Å), with the only significant differences in the conformation of some exposed side chains that are most likely irrelevant to protein–ligand interactions. In all these structures the essential Tyr 104 residue, which is thought to stack with the target base, exhibits a rotamer with the side chain "flipped out" outside of the binding pocket, thereby precluding any interactions with the methylation substrate adenosine. We therefore generated a model of ErmC′ structure based on 1QAO coordinates, but with the Tyr 104 conformation resembling that of the homologous Tyr 108 in the M.TaqI–DNA complex.[20]

To validate the utility of the "M.TaqI-like" conformation of ErmC′ as a receptor for virtual screening, we compared it with the original crystal structures with respect to the ability to generate an energetically favorable complex with the substrate an-

alogues adenosine monophosphate (AMP) and methyladenosine monophosphate (mAMP). Thus, the structures of mAMP and AMP were docked into the original 1QAO, 1QAM, 1QAN, and 1QAQ structures, and to the receptor with Tyr 104 in the M.TaqI-like conformation. We found that docking poses for the M.TaqI-like model were scored much higher (Surflex affinity value 6.25 for AMP, 6.79 for mAMP) than for any of the original crystal structures (the highest Surflex affinity value was 4.57 for AMP docked to 1QAO). Moreover, the highest-scoring AMP poses generated for the M.TaqI-like conformation presented the N6 group in an optimal position to be methylated by the bound cofactor. In contrast, docking poses generated in the presence of the original crystal structures failed to bring the N6 group close to the methyl group donor (data not shown). Thus, the structural model of the ErmC′ MTase with an M.TaqI-like rotamer of Tyr 104 was regarded as catalytically competent and used in all further docking experiments as a receptor.

Virtual screening

In the virtual screening procedure we used the Surflex program to dock all compounds from the Maybridge Screening Collection to three separate receptors: 1) the adenosine binding pocket in the presence of bound AdoMet (conformation as in the 1QAO crystal structure), 2) the adenosine binding pocket in the absence of AdoMet, and 3) the predicted rRNA binding surface. All poses were rescored using a consensus function based on a series of scoring functions implemented in the CScore and X-Score programs (see Experimental Section for details). Based on the visual analysis of 200 top-scoring poses from each ranking list (for all three receptors), 77 structurally diverse compounds were selected for experimental validation and purchased.

In vivo and in vitro activity of test compounds

Candidates for leads against ErmC′ should be able to inhibit rRNA methylation in vivo, but at the same time not interfere with other methylation reactions. The simplest test for these properties is to assess the toxicity of selected compounds against *E. coli* with and without the antibiotic that is sensitive to rRNA methylation. The assay was performed on *E. coli* expressing recombinant ErmC. The MIC of the inhibitor combined with erythromycin was compared with the MIC values of the inhibitor alone and erythromycin alone, as summarized in Table 1. We found that the MICs of 17 of the 77 compounds tested were lower if used with non-inhibitive concentrations of erythromycin relative to the activity of the compound alone. Under these conditions the MICs of eight compounds were decreased from >100 to 50 mg L^{-1}. The test compound RF00667 exhibited the most effective in vivo activity; its MIC in the presence of erythromycin decreased from >100 to 6.25 mg L^{-1}.

All compounds that were able to lower the MIC of erythromycin in vivo at concentrations lower than their MIC value alone, were examined for their ability to inhibit the ErmC′ MTase in vitro. The potency of selected compounds in ErmC′ inhibition was determined at a concentration range of 5–1000 μM. The inhibitory effect inferred from in vivo analyses was confirmed for nine of the above-mentioned compounds (Table 1). Six of them (RF00667, PD00556, JFD03032, KM07948, RH01110, and BTB05276) exhibit IC_{50} values in the range of 180–500 μM. Compounds HTS12471 and HTS12610 were also found to inhibit ErmC′ MTase with approximate IC_{50} values lower than 500 μM, but we were unable to accurately measure their activity owing to their poor solubility and the limited DMSO concentration in the reaction mixture.

Table 1. Summary of in vitro and in vivo assays: MIC values for erythromycin in combination with a test compound compared with the MICs of individual test compounds.[a]

Test Compd	$MIC_{Erythromycin}/MIC_{Compd}$ [mg L^{-1}]	MIC_{Compd} [mg L^{-1}]	IC_{50} [μM][b]
BTB05276	2560/100	>100	250
DSHS00654	2000/50	100	–
CD08110	2000/50	>100	–
BTB12340	2560/100	>100	p
HTS00972	2560/100	>100	–
JFD03032	1280/50	>100	500
HTS12610	2000/50	>100	250*
HTS12471	2000/100	>100	300–400*
PD00206	1280/100	>100	p
RJC02873	2000/100	>100	>500
RH01110	2000/50	>100	500
RF00667	2560/6.25	>100	180
PHG00883	1600/50	>100	p
PD00556	2000/50	>100	300
SPB04836	2560/100	>100	p
SPB02743	2000/100	>100	p
KM07948	2000/50	>100	500

[a] MICs determined with *E. coli* DH5α cells expressing recombinant ErmC MTase, the MIC for erythromycin alone was >2560 mg L^{-1}. [b] Compounds with solubility problems are marked as "p", compounds with no inhibitory activity are marked with "–". The approximate IC_{50} values of clear inhibitors which were poorly soluble in the reaction buffer are indicated with "*".

Retrospective analysis of docking interactions

Five of the eight most potent inhibitors had been predicted to dock into the adenine binding pocket and not into the cofactor binding pocket. Figure 1a and b shows the structures of the two most potent compounds from this group, and Figure 2a and b illustrates their mode of interaction with ErmC′ predicted by the docking studies. Although their structures are dissimilar, they exhibit a comparable binding mode with a single or double ring stacked against the essential Tyr 104 residue, mirroring the predicted orientation of the substrate adenosine. Additional functional groups of all these compounds, linked to the stacked ring by flexible bonds, protrude out of the pocket and form a number of interactions with conserved and semi-conserved residues that have been implicated in binding the target nucleoside and substrate RNA.[10,11] This predicted mode of protein–ligand interaction suggests that the test compounds reported herein are likely to be specific against Erm MTases in general, but should not interfere with the activity of other MTases that exhibit different substrate

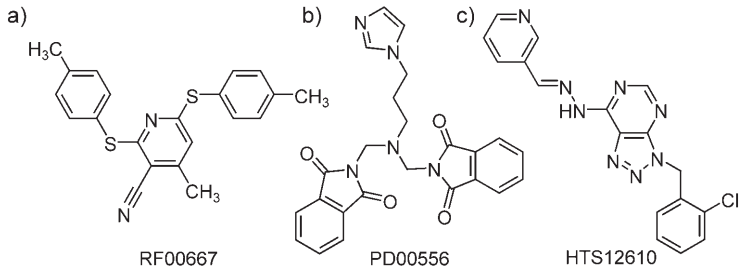

Figure 1. Structures of selected most potent ErmC′ MTase inhibitors identified herein: a) 4-methyl-2,6-di[(4-methylphenyl)thio]nicotinonitrile (Maybridge code RF00667), b) 2-({[(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)methyl][3-(1*H*-imidazol-1-yl)propyl]amino}methyl)-1*H*-isoindole-1,3(2*H*)-dione (PD00556) and c) nicotinaldehyde-*N*-[3-(2-chlorobenzyl)-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-7-yl]hydrazone (HTS12610).

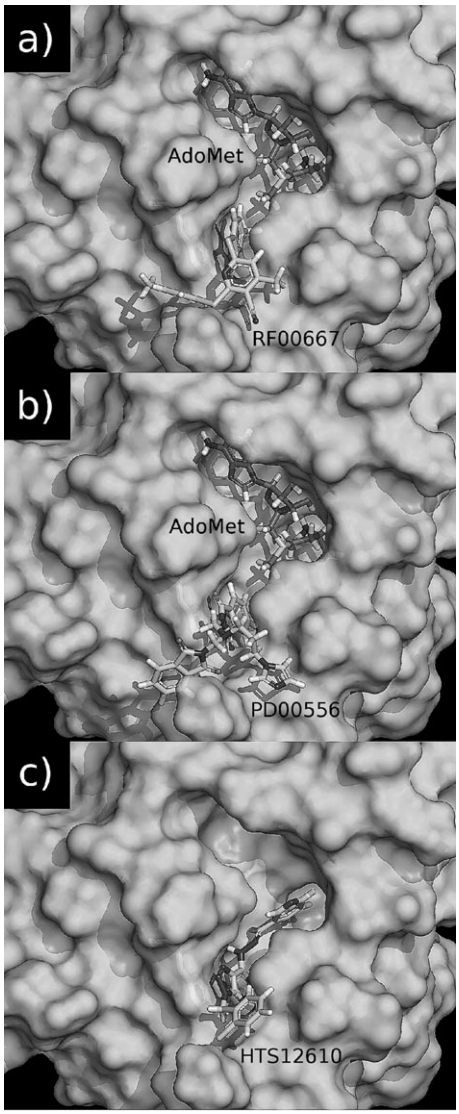

Figure 2. Docking models of the most potent ErmC′ MTase inhibitors interacting with the adenine binding pocket identified herein. The noncompetitive inhibitors a) RF00667 and b) PD00556 interact with the adenine binding pocket exclusively, and are shown in the presence of the AdoMet cofactor. c) HTS12610 protrudes to the AdoMet binding pocket, and as predicted its inhibition is competitive with the cofactor. The essential Tyr 104 residue is shown in stick representation. The terminal residues Ser 9 and Gln 10 are omitted for clarity.

binding pockets and that do not share the same residues at the protein surface surrounding the active site. This, however, remains to be verified by testing these compounds against other members of the Erm family as well against other RNA MTases, in particular, the closest homologs of Erm, such as Dim1 methyltransferases that play important roles in eukaryotic cells.[21]

Two other inhibitors were identified among the compounds predicted to interact with the two other receptors. Compound HTS12610 was predicted to interact with the active sites of Erm MTases in the absence of the cofactor. HTS12610 is composed of three rings. The central adenine-like ring, in a manner similar to previous compounds, stacks against Tyr 104, and the second chlorobenzyl group is directed outside the pocket, but the third pyridine ring is located in the cofactor binding pocket. It is connected to N6 of the adenine-like part by a hydrazone linker that threads the space normally occupied by the transferred methyl group. The last compound, BTB05276, has been predicted to interact with the positively charged region of ErmC′ responsible for the interaction with the substrate rRNA hairpin loop structure.[10]

To tentatively verify the predicted binding modes, we determined if the increasing concentration of AdoMet in the Erm MTase assay affects the IC_{50} value of any of the most promising inhibitors. Among the four compounds we tested, the inhibition of HTS12610 was competitive with AdoMet, and the inhibition caused by compounds RF00667, PD00556, and BTB05276 was noncompetitive with AdoMet (data not shown). These results are in perfect agreement with docking poses proposed in virtual screening. Only HTS12610 was predicted to partially occupy the AdoMet site on the enzyme, whereas the other three compounds were predicted to interact exclusively with the substrate binding area. If the predicted binding mode of HTS12610 is correct, it suggests that potent inhibitors could be obtained by chemically linking compounds docked into the adenine binding pocket with AdoMet analogues, which should result in increased binding affinity without the loss of specificity.

Comparison with a previously obtained ErmC′ inhibitor

We attempted to compare the potency and binding mode of our test compounds with those obtained earlier using high-throughput screening. However, we could obtain only one (UK 80882) of several compounds reported to inhibit ErmC′,[6] because others were not available commercially. In our hands, UK 80882 exhibits an IC_{50} value of 80 μM in vitro, which is better than our test compounds, and the inhibition was competitive with AdoMet. The docking analysis (Figure 3) suggests that UK 80882 binds to ErmC′ in a manner similar to our test compounds, but exhibits an additional feature, namely an extension of the aromatic ring that is predicted to protrude into the cofactor binding pocket which may thereby improve its binding affinity. This analysis, albeit preliminary, also supports our conclusions that the best inhibitors against ErmC′ mimic

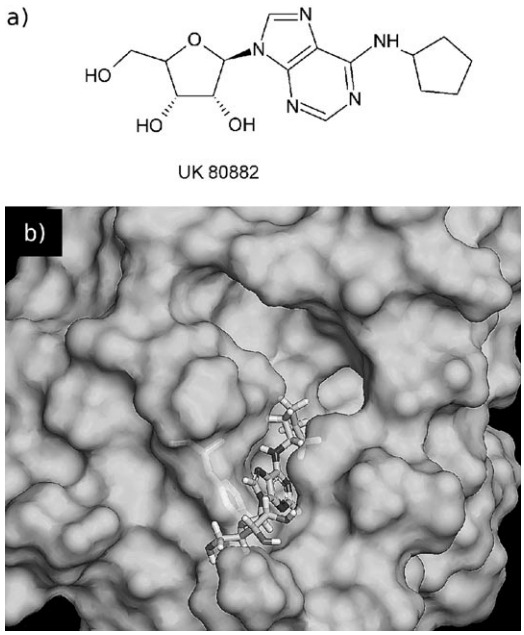

Figure 3. a) Structure of the previously identified ErmC′ inhibitor UK 80882 and b) its docking model. The essential Tyr 104 residue is shown in stick representation. The terminal residues Ser 9 and Gln 10 are omitted for clarity.

the binding mode of the target adenosine and that their potency can be increased by extending them into the cofactor binding pocket. These ideas will be tested in the future by designing new ligands and determining their activity in vitro and in vivo.

Conclusions

We have carried out virtual screening of compounds from the Maybridge Screening Collection against the ErmC′ methyltransferase structure, in which the conformation of residues in the active site was adjusted to resemble the active site of M.TaqI complexed with its substrate. The virtual screen successfully generated a small subset of ligands with compounds that exhibit an inhibitory effect on ErmC′. In the group of experimentally validated 77 compounds, 17 decrease the MIC erythromycin in the ErmC′-expressing strain of *E. coli*, and eight inhibit the MTase activity of ErmC′ in vitro, with IC_{50} values in the range of 180–500 μM. The analysis of docking models of the ErmC′ inhibitors identified herein and comparison with a compound previously identified by high-throughput screening suggests a strategy to generate potent leads.

Experimental Section

Ligand database preparation

We used the Maybridge Screening Collection (Cornwall, UK: http://www.maybridge.com) as a source of compounds for virtual screening. The three-dimensional conformers were generated and protonated using the Concord program from the Sybyl 7.1 package (Tripos Inc.) and stored in the mol2 format.

Protein preparation

The alternate rotamer of Tyr 104 in the active site of ErmC′ was modeled using Scwrl3.[22] The substrate binding pocket was minimized in vacuo in the presence of docked AMP using the CHARMM force field[23] as implemented in HyperChem 7 (Hypercube Inc.). The structure was processed using Sybyl 7.1 (Tripos Inc.). The protonation states of residues in the binding site were adjusted to the dominant ionic forms at pH 7.4. The crystallographic waters and the bound ligands were removed. The bound cofactor structure was converted into the mol2 format and included as a part of the receptor in one of the docking experiments (see the *Virtual screening* section).

Docking protocol

The virtual screening procedure was performed using Surflex 1.33.[24] The residues localized in the proximity of the substrate binding pocket (Gln 10, Asn 11, Asn 101, Ile 102, Pro 103, Tyr 104, Asn 105, Thr 108, Ile 126, Val 127, Glu 128, Tyr 129, Gly 130, Lys 133, Arg 134, Val 158, Phe 163, Pro 167, Lys 168, Val 169, Asn 170, and Ser 171) were used to generate a Surflex 'protomol' that represents the ligand binding cleft, while residues Thr 108, Arg 112, Arg 134, Arg 140, Ser 141, and Leu 142 were used to generate a protomol for the putative rRNA binding surface. All compounds from our ligand database were docked to the receptor structure. For each compound up to 10 best-scoring poses were retained for further analysis.

Post-processing and compound selection

All docked poses were re-scored using several external scoring functions: D_SCORE, CHEMSCORE, G_SCORE and PMF from the CScore program (Tripos Inc.), as well as HMCORE, HSCORE, and HPCORE from the X-Score program.[25] The final ranking of the screened compounds was generated according to the consensus of three scoring functions that were most successful in the discrimination of known ErmC′ ligands from random, drug-like compounds: Surflex affinity value, G_SCORE, and D_SCORE. The consensus value was computed as the sum of Z-score values for each component score. The final score of a given compound corresponded to the value of the consensus score of its highest-scoring pose.

Protein expression and purification

For expression and purification, we used the construct from our previous studies,[10–12] in which the *ermC′* gene with a C-terminal His tag was cloned into the pET-25b(+) vector. *E. coli* ER2566 cells transformed with the recombinant pET-25b(+) vector were grown in Luria Bertani (LB) medium supplemented with ampicillin (100 μg mL^{-1}). Cells were grown at 37 °C until the culture reached $OD_{600} = 1$. At this point, isopropyl-β-D-thiogalactopyranoside (IPTG, 1 mM) was added to induce expression of ErmC′, and the culture temperature was shifted to 30 °C. Growth was continued for an additional 5 h. The cells were harvested, and ErmC′ was purified as described previously.[10]

Determination of erythromycin MIC values

Erythromycin MICs were determined in *E. coli* DH5α using the dilution method.[26] The test was performed in microtiter plates on

DH5α cells transformed with the pUC18 vector carrying the *ermC′* gene, a construct from our previous studies,[10–12] according to the modified protocol of Clancy and co-workers.[6]

Preparation of microtiter plates

All test compounds were dissolved in dimethyl sulfoxide (DMSO) at concentrations of 2–2.5 g L^{-1}. Serial dilutions of test compounds were carried out by dilution of the stock solutions with LB medium supplemented with ampicillin (100 mg L^{-1}). For each compound, 50 μL of the first dilution was applied to a microtiter plate (from well A1 to well A8) and this process was continued with the remaining dilutions, finishing with well H8. The final concentrations of the test compounds after adding all components were 100 (wells A), 50 (wells B), 25 (wells C), 12.5 (wells D), 6.25 (wells E), 3.1 (wells F), 1.56 (wells G), and 0.78 mg L^{-1} (wells H). The dilutions of erythromycin were prepared similarly and applied in 50 μL volume from well A1 to well H1 for the highest concentration and so on, until reaching wells A8 to H8. The final concentrations of erythromycin after adding all components were 2.56 (wells 1), 2 (wells 2), 1.6 (wells 3), 1.28 (wells 4), 0.64 (wells 5), 0.32 (wells 6), 0.16 (wells 7), and 0.1 g L^{-1} (wells 8). Prepared plates were either used immediately or stored at −80 °C until use.

Inoculation of microtiter plates with bacterial culture

The overnight bacterial culture was diluted 1:50 in fresh LB medium supplemented with ampicillin (100 mg L^{-1}) and grown until $OD_{600} = 0.8–1$. The culture was diluted to approximately 5×10^5 cells mL^{-1}, and 100 μL of the suspension was added to the wells starting from A1 through H8. For each compound, specific controls were applied. Wells A10 through H10 contained dilutions of test compounds with bacterial inoculum and erythromycin substituted with LB medium supplemented with ampicillin. This control served as an indicator for possible toxicity of the test compound. Wells A11 through H11 contained only corresponding dilutions of test compounds in growth medium with ampicillin, which served as a control for the compounds that were colored or that showed absorbance in the absence of bacteria. Each microtiter plate contained a sterility control (200 μL LB medium), a bacterial growth control (100 μL LB medium with ampicillin and 100 μL bacterial inoculum), a control for compound sterility (150 μL LB medium with ampicillin and 50 μL highest concentration of test compound), and a control for erythromycin sterility (150 μL LB medium with ampicillin and 50 μL highest concentration of erythromycin). The microtiter plates were incubated for 18 h at 37 °C, and synergistic MIC was determined as a combination of concentrations of erythromycin and a test compound that completely inhibited bacterial growth. The individual MIC for the test compound was also determined in the same way, whereas the erythromycin MIC for *E. coli* DH5α expressing ErmC MTase was > 2.56 g L^{-1}.

Inhibition assays

Methylation of RNA in vitro was done using a synthetic 32-nt RNA oligonucleotide (5′-CGCGACGGACGGA^{2085}AAGACCCCUAUCCGUCGCG-3′, hairpin structure) designed to mimic the adenine loop in domain V of *B. subtilis* 23S rRNA (residues 2073–2090 and 2638–2651) which was used previously in studies of the wild-type and mutant variants of the enzyme. The RNA oligonucleotide was denatured at 90 °C for 1 min and renatured by cooling slowly (1 °C min^{-1}) to room temperature.

Inhibitory activity of selected compounds was determined in a reaction mixture consisting of Tris-HCl pH 7.5 (50 mM), KCl (40 mM), $MgCl_2$ (4 mM), 1,4-dithiothreitol (10 mM), RNA (1 μM), ErmC′ MTase (0.2 μM), [*methyl*-^{3}H]AdoMet (0.13 μM), and RiboLock (Fermentas) in a total reaction volume of 50 μL. Reaction mixtures were pre-incubated at 25 °C for 10 min prior to the addition of [*methyl*-^{3}H]-AdoMet and RNA. Test compounds were added in duplicate in the concentration range of 5–1000 μM. The serial dilutions of each compound were made to keep the volume of DMSO added to the reaction mixture constant. The reaction mixtures were incubated at 25 °C for 1 h. Reactions were terminated by the addition of trichloroacetate (TCA, 10%, 0.5 mL), and glycogen (Fermentas) was added to facilitate precipitation. Samples were chilled to 4 °C for 30 min, and RNA was pelleted by centrifugation; the pellets were then washed with 1 mL 10% TCA, dried, and counted for radioactivity. IC_{50} values were obtained by plotting percent inhibition relative to the control sample without inhibitor as a function of inhibitor concentration. In the Erm MTase assay with increasing concentrations of AdoMet, the [*methyl*-^{3}H]AdoMet concentration was in the range of 0.05–0.5 μM.

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