Rational prioritization strategy allows the design of macrolide derivatives that overcome antibiotic resistance

Gerhard König, Pandian Sokkar, Niclas Pryk, Sascha Heinrich, David Möller, Giuseppe Cimicata, Donna Matzov, Pascal Dietze, Walter Thielen, Anat Bashan, Julia Elisabeth Bandow, Johannes Zuegg, Ada Yonath, Frank Schulte, and Elsa Sanchez-Garcia

Abstract

Antibiotic resistance is a major threat to global health; this problem can be addressed by the development of new antibacterial agents to keep pace with the evolutionary adaptation of pathogens. Computational approaches are essential tools to this end since their application enables fast and early strategical decisions in the drug development process. We present a rational design approach, in which acylide antibiotics were screened based on computational predictions of solubility, membrane permeability, and binding affinity toward the ribosome. To assess our design strategy, we tested all candidates for in vitro inhibitory activity and then evaluated them in vivo with several antibiotic-resistant strains to determine minimal inhibitory concentrations. The predicted best candidate is synthetically more accessible, exhibits higher solubility and binding affinity to the ribosome, and is up to 56 times more active against resistant pathogens than telithromycin. Notably, the best compounds designed by us show activity, especially when combined with the membrane-weakening drug colistin, against Acinetobacter baumannii, Pseudomonas aeruginosa, and Escherichia coli, which are the three most critical targets from the priority list of pathogens of the World Health Organization.

Significance

Due to the development of resistance against commonly used antibiotics, new derivatives that avoid resistance mechanisms need to be developed. To address this problem, a rational prioritization strategy is outlined for macrolide antibiotics. Candidates are screened based on their solubility, membrane permeability, and binding affinity using a tiered optimization approach of free energy simulations and quantum mechanics/molecular mechanics calculations. After prioritization by computational methods, the best candidates are evaluated experimentally. The strategy creates a targeted library that is highly enriched in compounds with antibacterial activity. This allows for faster iterations in the development of new antibiotic derivatives.

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BIOPHYSICS AND COMPUTATIONAL BIOLOGY
solution (stage IIa), and exhibit sufficient membrane permeability (stage IIb) as well as high binding affinity toward the target (stages IIIa to IIIId). Moreover, candidates should avoid resistance mechanisms (stage IV) and must be nontoxic (stage V). The design process is simplified when the optimization starts from an approved drug since therapeutic agents generally fulfill most of these conditions. Thus, the focus lies on eliminating modifications that lead to a loss of essential properties and on favoring candidates for which these properties are improved or at least conserved. From one stage to the next, fewer molecules are evaluated but with increasing rigor. The rational design starts with a strategy for synthesis, followed by prioritization through a series of computational techniques. The prioritized molecules are then tested both in vitro and in vivo. The toxicity of the most active candidates is evaluated in the final stage.

In this work, we validate our design strategy with the study of 19 acylide replacements of the cladinose moiety of clarithromycin using four linear synthesis steps (Fig. 1). This is two steps shorter than that for telithromycin. The numbering of the library starts with the original clarithromycin molecule (1; CTY). Its derivatives include substituents with different polarities and electron densities, aliphatic analogs, or inflexible spacers between the macrolide core and the aryl substituent. The set was designed to evaluate the effects of nitro substituents (5, 6), an extended π system, an electron-donating substituent, and a cyclohexyl derivative (as a nonaromatic comparison), as well as halogenated and methylated derivatives. Both the spatial and electronic constraints on the scaffold are explored.

Results

All computational results are derived from a consistent set of relative free energy perturbation (FEP) simulations. The ligands were simulated both in the unbound state in aqueous solution and in the bound state within the bacterial ribosome. The bound states of clarithromycin and the acylide derivatives were modeled with the structure of the 50S ribosome of Deinococcus radiodurans together with explicit water and with an ionic concentration of 0.1 M. The clarithromycin–ribosome complex was equilibrated with molecular dynamics simulations (SI Appendix, section A and Fig. S1). In parallel, relative FEP protocols for all ligands were set up and tested in aqueous solution. The simulations decouple the side chains of the acylides as shown in Fig. 1, leaving only the common backbone as the alchemical intermediate state. This also allows the use of enhanced sampling methods for exploring the conformational flexibility of the side chains (19).

First, we estimated the relative affinities for water, in the absence of the ribosome, using implicit solvent calculations (stage IIa) (SI Appendix, section C) (20). These calculations represent an upper bound for their relative solubilities and neglect self-solvation effects (21), as well as the free energy of sublimation (22). Stage IIa was motivated by the relatively poor solubility of clarithromycin and the assumption that hydrophilic molecules might be less affected by promiscuous adenine triphosphate (ATP)–dependent efflux pumps (17, 23). Since all molecules were predicted to be more hydrophilic than clarithromycin (SI Appendix, Table S1), we used the five compounds with the highest affinity for water (6, 3, 8, 5, 4) for the next stage (stage III, where we calculated the binding free energy of the ligands toward the ribosome).

Likewise, we estimated membrane permeabilities using an implicit membrane model (stage IIb) (SI Appendix, section C) (24). The resulting transfer free energies gauge membrane permeability based on Fick’s First Law (25) but neglect self-solvation effects (26), as well as the nanostructure of the membrane. Since all candidates were considered to be membrane permeable

![Fig. 1. Synthesis of the acylide library. CTY denotes clarithromycin. (i) 6.1 eq. ArCH2CO2H, 6.0 eq. 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide·HCl, 1.1 eq. 4-dimethylaminopyridine (DMAP), dichloromethane (DCM), room temperature or 3.3 eq. ArCH2CO2H, 3.3 eq. trimethylacetyl chloride, 3.3 eq. Et3N, 1 eq. DMAP, DCM, −15°C → RT. (ii) MeOH, RT, 48 h. (iii) 2 eq. NiCl2 · 6H2O, 4 eq. NaBH4, MeOH, 0°C. Computed structures are highlighted in red. *Previously described compounds.](https://doi.org/10.1073/pnas.2113632118)
of computational free energy predictions by about 1 kcal mol\(^{-1}\) (30, 31). The QM/MM calculations indicate that compounds 6, 5, and 4 exhibit the most favorable interaction energies with the ribosome. They were, therefore, prioritized for the in vitro testing (stage IIIId). Molecules 7 and 8 were eliminated because of the predicted instability of their complexes with the ribosome (Table 1). The results for the other molecules are less clear, as stronger interactions with the ribosome are often offset by a simultaneous high affinity for the unbound state in water. Overall, the QM/MM interaction energies confirm the FEP results, indicating that 6 is a very favorable binder to the ribosome. In addition, ligands 5 and 4 emerge as potential effective inhibitors. A detailed discussion of the simulations of the ligand–ribosome complexes is provided in SI Appendix, section E.

To evaluate our rational design strategy, the inhibitory activities of all 19 candidates were determined by the half maximal inhibitory concentrations (IC\(_{50}\)) from an in vitro transcription–translation assay against the Escherichia coli ribosome (stage IIIId) (SI Appendix, part 2). As evidenced in Fig. 2, compounds 6 and 4 are indeed the most active in the whole set, and 5 is more active than clarithromycin. Molecule 7, which was predicted to be the worst binder in the prioritized set, also turns out to be the least active in this subset. Ligands 11, 13, 15, and 16 also show high affinity toward the ribosome but were not part of the prioritized set because of their solvation properties. Membrane permeability and hydrophobicity are expected to play a prominent role for biological activity.

In stage IV, we tested the acylides for in vitro antimicrobial activity by assessing minimal inhibitory concentrations (MICs) against gram-positive bacteria (Bacillus subtilis and Staphylococcus aureus) as well as against gram-negative bacteria (E. coli, Acinetobacter baumannii, Pseudomonas aeruginosa, and Klebsiella pneumoniae) and yeast strains (Candida albicans and Cryptococcus neoformans). The MIC results are listed in Fig. 3. We also tested the cytotoxicity and hemolytic activity by assessing cytotoxic concentration (CC\(_{50}\)) against the human kidney cell line HEK293 and hemolytic concentration (HC\(_{50}\)) against human red blood cells (stage V) (the last two rows of Fig. 3). For MIC values up to 32 \(\mu\)g mL\(^{-1}\), therapeutic indices are calculated (CC\(_{50}\)/MIC) and shown in parentheses below the respective MIC value in Fig. 3. As the ester bond in acylides might be prone to enzymatic breakdown, all acylides were tested to be stable in human blood serum (32).

All compounds are highly active against the methicillin-susceptible strain of S. aureus (MSSA, DSM 20231, ATCC 12600)
(33) with MIC values up to 0.1 µg mL⁻¹ while displaying no cytotoxicity or hemolytic activity at the same concentrations, as well as no activity against either of the yeast strains. Exceptions are only compounds 19 and 20, the two benzoic acid variants, which have no antibacterial activity. The high antibiotic activity of the other compounds is retained against S. subtilis (DSM 402, NCIB 10106) and mostly against two mutant strains of MSSA: S. aureus BAA-976 and BA-977. S. aureus BAA 977 features inducible macrolide–lincosamide–streptogramin B resistance due to the macrolide ribosomal methylase erm(A) gene. Both strains display resistance against the reference antibiotics erythromycin, clarithromycin, and telithromycin, and mostly against two mutant strains of MSSA:

### Table 2. Activity against the three top-ranked bacteria from the WHO priority pathogens list (33)

| Species            | Strain description | MIC (µg mL⁻¹)  |
|--------------------|-------------------|---------------|
|                    |                   | 4-8 | 1-2 | 0.1-0.5 | not determined |
| S. aureus          | DSM30027          | 0.125 | 0.5 | 0.125 |             |
|                    | P. aeruginosa     | 0.25 | >128 | 32.0 |             |
|                    | E. coli           | 0.25 | >128 | 32.0 |             |
| A. baumanii        | DSM 30007         | 0.125 | 0.5 | 0.125 |             |
|                    | P. aeruginosa     | 0.25 | >128 | 32.0 |             |
|                    | E. coli           | 0.25 | >128 | 32.0 |             |
| P. aeruginosa      | DSM 50071         | 0.125 | 0.5 | 0.125 |             |
|                    | P. aeruginosa     | 0.25 | >128 | 32.0 |             |
|                    | E. coli           | 0.25 | >128 | 32.0 |             |
| K. pneumoniae      | ATCC700603        | 0.125 | 0.5 | 0.125 |             |
|                    | P. aeruginosa     | 0.25 | >128 | 32.0 |             |
|                    | E. coli           | 0.25 | >128 | 32.0 |             |
| C. albicans        | ATCC 70028        | 0.125 | 0.5 | 0.125 |             |
|                    | P. aeruginosa     | 0.25 | >128 | 32.0 |             |
|                    | E. coli           | 0.25 | >128 | 32.0 |             |
| C. neoformans      | ATCC 208821; H90  | 0.125 | 0.5 | 0.125 |             |
|                    | P. aeruginosa     | 0.25 | >128 | 32.0 |             |
|                    | E. coli           | 0.25 | >128 | 32.0 |             |
| H. influenzae      | HEK293, ATCC CRL1573 | >32 | >32 | >32 | >32 |
|                    | P. aeruginosa     | >32 | >32 | >32 | >32 |
|                    | E. coli           | >32 | >32 | >32 | >32 |
| ATCC 25922         | 0.125 | 0.5 | 0.125 |             |
| K. pneumoniae      | ATCC 700603       | 0.125 | 0.5 | 0.125 |             |
|                    | P. aeruginosa     | 0.25 | >128 | 32.0 |             |
|                    | E. coli           | 0.25 | >128 | 32.0 |             |

*Best candidate.*

MICs are given for molecules 6, 11, and 16 in combination with subinhibitory concentrations of the membrane-weakening antibiotic colistin sulfate (COS). Erythromycin, clarithromycin, telithromycin, and all other acyclids were ineffective.

Next, antibacterial MIC data were determined against several gram-negative pathogens, including type strains from A. baumanii (DSM 30007, ATCC 19606), P. aeruginosa (DSM 50071, and ATCC 27835), E. coli (DSM 30083, ATCC 11775, and
coli Δ lpxC (variation of the lipopolysaccharide structure) and Δ tolC (without the major efflux pump) and P. aeruginosa Δ mexAB/CD/EF/XY (without five of the major efflux pumps).

Interestingly, all compounds regain significant activity (up to MIC values of 2 μg mL⁻¹) if the major drug efflux pump is eliminated in E. coli. This also includes compound 20, which is otherwise not even active against gram-positive bacteria. Variation of the outer membrane by Δ lpxC had a lesser effect, with only compounds 3, 5, 6, 11, 12, and 16 regaining activity to MIC values of 16 to 32 μg mL⁻¹. A similarly small effect was displayed for the P. aeruginosa strain that lacks five of the major drug efflux pumps. Only five compounds regain activity to MIC values of 32 μg mL⁻¹ (Fig. 3).

A similar outcome can be achieved by the coadministration of sub-MIC levels of colistin, an antibiotic that interacts with the outer membrane component Lipid A, thereby increasing the permeability of the membrane for other drugs. Colistin showed synergistic activity with each of the tested acylides 6, 11, and 16 against E. coli and A. baumanii, increasing the activity to MIC values of 0.25 to 1 μg mL⁻¹, at colistin concentrations of 10% of its MIC value (Table 2). Like the efflux pump mutants, activity with colistin against P. aeruginosa could not be reestablished to the same degree as for E. coli or A. baumanii, suggesting that P. aeruginosa still has effective efflux mechanisms. The activity against the efflux-deficient E. coli Δ tolC mutant suggests that the main cause of the resistance in gram-negative bacteria is the efficient elimination of the compounds via efflux pumps. In addition, the activities against E. coli Δ tolC agree with the IC₅₀ values, with most MIC values around 2 μg mL⁻¹ and IC₅₀ values < 0.5 μM. Exceptions are the weaker binders 7, 17, and 20, which also display IC₅₀ values above 1 μM and MIC values at 16 to 32 μg mL⁻¹. This suggests that the activity against S. aureus, which exhibits a wider range of MIC values, might be governed by permeability as well.

**Discussion and Conclusions**

Our rational design strategy allowed us to successfully reduce the library of 19 derivatives of clarithromycin first to 10 and then to 3 candidates (6, 4, 5) by calculating solubilities, membrane permeabilities, and binding affinities at three different levels of accuracy (end point methods with implicit solvent, FEP, and QM/MM calculations). In vitro experiments confirmed that molecules 6 and 4 exhibit the lowest IC₅₀ of the whole set. The heuristic prioritization process allows fast and early decisions based on several approximations. In theory, only three molecules had to be synthesized and tested to find the best candidate (6). An overview of the decision-making process is provided in Fig. 4.

Compounds 6, 16, and 11 exhibited some activity against the top three critical targets from the WHO priority pathogens list (32). Further experiments demonstrated that A. baumanii and E. coli can be successfully inhibited by these compounds in combination with the membrane-weakening compound colistin or by elimination of the major efflux pump system. This confirms that the uptake through the outer membrane is a limiting factor in gram-negative bacteria. In addition, the comparison with the membrane-deficient E. coli and MSSA S. aureus strain suggests that, even in gram-positive bacteria, permeability through the membrane affects the activity.

In vitro activity data for the high-priority pathogen S. aureus allowed us to identify molecules 6, 3, and 16 as the most promising candidates with high activity, no cytotoxicity, and the ability to overcome existing macrolide resistance. Compared with telithromycin, lead compound 6 exhibits a 56-fold higher activity against S. aureus BAA 976 and an 8-fold increased activity against S. aureus BAA 977. Both activities are below the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical break point of 1 μg mL⁻¹ (35), which makes the molecule...
a good candidate to break the macrolide resistance in *S. aureus*. The comparison between the biophysical IC₅₀ data (Fig. 2) and cell-based MIC data (Fig. 3) indicates a good correlation for gram-negative bacteria where the major efflux pump has been deactivated (E. coli Δ tolC). Nevertheless, several discrepancies can be observed for compounds with similar potencies in gram-positive wild-type strains, like *S. aureus*. This suggests different uptake rates in gram-positive bacteria. Compounds 6, 3, and 16 exhibit good MIC values.

The experimental data allow us to evaluate the design decisions, showing that an efficient strategy must account for more criteria than just binding affinity. Among the prioritized molecules in Table 1, the QM/MM calculations identified 80% of the active compounds in terms of IC₅₀, and 67% of the predictions were correct (SI Appendix, Fig. S11). Strikingly, not a single molecule that was prioritized because of its high membrane permeability (2, 7, 14, 18, and to a lesser degree, 9) turned out to be effective. In contrast, almost all molecules that were selected for their hydrophilicity (3, 4, 5, 6, and to a lesser degree, 8) were more active than clarithromycin. Thus, hydrophilicity turned out to be the better design criterion than high membrane permeability to reach low MIC values.

Some active compounds (in particular, molecules 16 and to some degree, also 11) were missed in the initial stages of the selection process. Molecule 16 almost progressed further, being among the six most hydrophilic molecules. However, MM-GBSA calculations predicted a low binding affinity for 16 (SI Appendix, Table S3). Compound 11 was given a low priority because it ranked low in terms of both hydrophilicity and membrane permeability. It also would have exhibited a poor binding affinity in a posteriori MM-GBSA calculations (SI Appendix, Table S3). Thus, it did not meet any of the considered requirements.

Any rational design scheme can only be as good as its design criteria. Future prioritization schemes could benefit from a better understanding of antibacterial activity against wild-type and efflux pump mutants, as well as other resistance mechanisms. A weighted scoring function that identifies the most important physicochemical traits and their contributions to uptake and activity could guide the rational design process. Theoretical bounds with improved convergence properties could speed up the computation, including prioritization schemes. Additional in silico steps to evaluate the binding affinity of the compounds against different ribosome strains should be the next stage in the development of rational design strategies.

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Materials and Methods

The simulations were carried out using the CHARMM (36, 37) and NAMD (38) programs with the CHARMM36 force field (39–41). The large ribosomal subunit (S50) was obtained from *D. radiodurans* (Protein Data Bank I.D. codes 7ZJR and 1SJA) (13, 14, 42) and all Mg²⁺ ions from the crystal structures were solvated in a cubic box with 0.1 M KCl. The box size was equilibrated with constant pressure simulations, and 20 ns of molecular dynamics simulations were performed. The free energy simulations and QM/MM calculations are based on 5ns simulations with Hamiltonian replica exchange (43, 44) of a truncated ribosome model. The free energy differences were analyzed with the FREN program of CHARMM (45, 46). The implicit solvent calculations of solubility (21, 30), membrane permeability (25), and MM-GBSA binding affinity (27, 28) were conducted with the explicit solvent model (20, 24). The QM/MM calculations were performed with the ChemShell package (47) using the MNDO program (48) for the semiempirical calculations with OM2-D3 (49–51), Turbomole (52) for the QM calculations with BP86-D3/Def2-SVP (53, 54), and DL_POLY (55) for the MM calculations (56).

For the experimental testing, the compounds were synthesized as indicated in Fig. 1. The compounds were subjected to an IC₅₀ determination assay against the *E. coli* ribosome. Cell lysates were prepared as described previously (57). Luminescence measurements were carried out on a Labtech Clarioskott microtiter plate reader. The stability of acylides in human blood plasma has been investigated as described before (33). The determination of the MICs and the cytotoxicity data was carried out as outlined in refs. 58 and 59.

A weighted computational (SI Appendix, part 1) and experimental methodologies (SI Appendix, part 2) (containing synthetic procedures, IC₅₀ determination, MIC determinations, cytotoxicity determination, KIT ComPat IDs for most of the final compounds, and further detailed information) are available in SI Appendix.

Data Availability. All study data are included in the article and/or SI Appendix.

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