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Bioorganic & Medicinal Chemistry Letters 14 (2004) 5257–5261
Bioorganic & Medicinal Chemistry Letters

Optimizing the antibacterial activity of a lead structure discovered by 'SAR by MS' technology

Elizabeth A. Jefferson,* Punit P. Seth, Dale E. Robinson, Dana K. Winter,† Alycia Miyaji, Lisa M. Risen, Stephen A. Osgood, Myra Bertrand† and Eric E. Swayze

Ibis Therapeutics, A Division of Isis Pharmaceuticals, Inc., 2292 Faraday Avenue, Carlsbad, CA 92008, USA

Received 15 July 2004; revised 16 August 2004; accepted 16 August 2004
Available online 15 September 2004

We dedicate this paper in memory of Professor Murray Goodman

Abstract—We report on lead optimization of a compound that was originally discovered to bind bacterial 23S rRNA near the L11 binding site and inhibit translation in vitro, but lacked detectible antibacterial activity. In this study, we were able to generate compounds with antibacterial activity against Gram-negative and Gram-positive pathogens, including a methicillin-resistant *S. aureus* strain.

In recent years, the emergence and spread of multi-drug resistant pathogens has become a serious problem in treating infectious disease.[1,2] As a result of this urgent health concern, there is a need to identify new antibacterials with unexploited modes of action.

Ribonucleic acids (RNAs) represent attractive antibacterial targets[3–5] since they are universally conserved in pathogens and critical to their life cycle.[6] We recently reported a strategy for lead discovery against RNA targets utilizing a mass spectrometry (MS) screening method.[7] Structure–activity relationships (SAR) are derived from identifying weak binding motifs and then elaborating them through chemical ligation to high affinity ligands. We applied this strategy to the 23S rRNA–L11 protein interaction, an underdeveloped target for small molecule drug intervention.[8–10] The rRNA subdomain is known to interact with the protein L11 and is the site of binding for thiostrepton, a poorly soluble drug useful only in topical veterinary medicine.[11] Using the 'SAR by MS' approach, weakly binding motifs **1** and **2** were ligated to provide biaryl **3** with improved affinity (6.5 μM) for the 23S rRNA U1061A subdomain (Fig. 1). Lead structure **3** inhibited bacterial protein synthesis

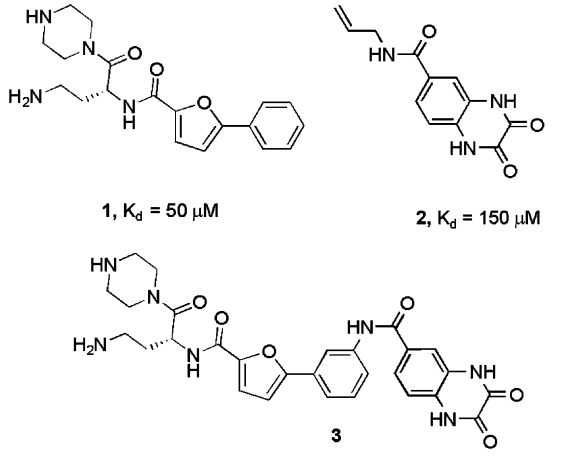

Figure 1. Weak binding motifs **1** and **2** and high affinity ligand **3** to the U1061A subdomain of 23S rRNA.

(IC_{50} = 14 μM) in a cell-free system,[12] but lacked antibacterial activity in MIC assays[13] against *E. coli* and *S. aureus* pathogens.

A group at RiboTargets recently reported a structure-based design strategy for targeting the 23S rRNA–L11 interaction.[14] The most potent compounds from this study are at least 5-fold less inhibitory than **3** in their

Keywords: RNA; Antibacterial; Biaryl; MIC.
* Corresponding author. Tel.: +1 760 603 2533; fax: +1 760 603 4654; e-mail: ejeffers@isisph.com
† Undergraduate Medicinal Chemistry COOP student (University of Ottawa, Canada).

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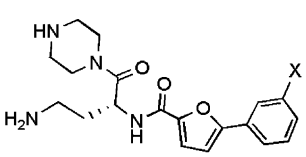
doi:10.1016/j.bmcl.2004.08.033

cell-free bacterial protein synthesis assay. The compounds also lacked detectible antibacterial activity in MIC assays.

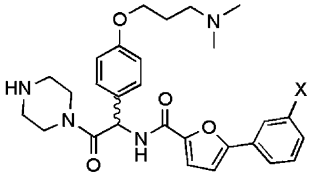
In this paper we report modifications to structure **3** that led to the identification of a set of compounds with antibacterial activity against Gram-positive and Gram-negative bacterial strains. Relatively few follow-up compounds to parent **3** were synthesized and evaluated, highlighting the effectiveness of our 'SAR by MS' drug discovery strategy for this antibacterial target.[7]

Biaryl **3** inhibits protein synthesis in a cell-free *E. coli* extract but fails to display antibacterial activity against *E. coli* and *S. aureus* strains at 100 μM. The lack of activity may be due to poor cell penetration and/or the action of efflux pumps, resulting in inadequate compound availability inside the cell.[15,16] As an initial attempt to obtain derivatives of compound **3** that are active in MIC assays, we decided to focus on the quinoxalinedione end-group (Table 1, compounds **4**–**11**). Analogs of parent **3** were conveniently prepared, following the solid phase route outlined in Scheme 1. In the synthesis, an Fmoc-protected amino acid is coupled to piperazine-derivatized ArgoGel™ Wang resin, deprotected, and then allowed to react with 5-bromofuroic acid. A key Suzuki cross coupling reaction with 3-nitrophenylboronic acid provides the biaryl core. After aryl nitro group reduction, the amine is reacted with either a carboxylic acid or a sulfonyl chloride. All compounds were cleaved from the resin in >80% purity and purified by reversed-phase HPLC to >95% homogeneity (isolated

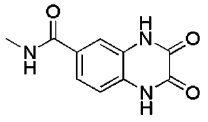
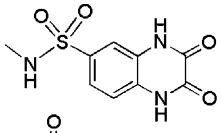
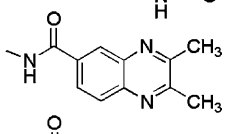
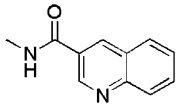
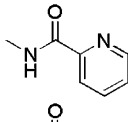
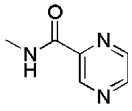
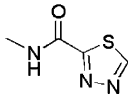
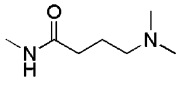
Table 1. Bacterial protein synthesis inhibitory activity of biaryls



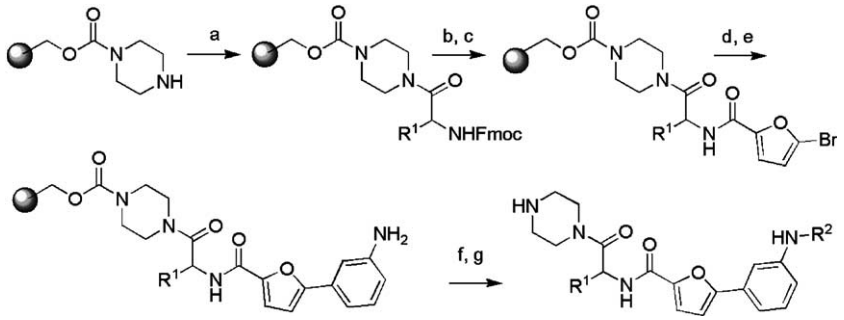
A



B

Compd	X	Structure	IC_{50}[a,b] (μM)	Compd	Structure	IC_{50}[a,b] (μM)
3[c]		A	14	**12**	B	3
4		A	>100	**13**	B	>100
5		A	>100	**14**	B	33
6		A	15	**15**	B	1
7		A	46	**16**	B	5
8		A	>100	**17**	B	20
9		A	>100	**18**	B	15
10		A	>100	**19**	B	13
11		A	>100	**20**	B	>100

[a] In vitro bacterial translation inhibition (*E. coli* extract).
[b] Compounds did not display antibacterial activity at 100 μM (<75% inhibition) against *E. coli* and *S. aureus* bacterial strains.
[c] IC_{50} = 14 μM for enantiomer.



Scheme 1. Reagents and conditions: (a) HATU, FmocNHCHR^{1}CO$_2$H, collidine, DMF; (b) piperidine, DMF; (c) 5-bromofuroic acid, HATU, collidine, DMF; (d) 3-nitrophenylboronic acid, Pd(PPh$_3$)$_4$, Na$_2$CO$_3$, EtOH, DME, 70 °C; (e) H$_2$, Pd/C, MeOH; (f) carboxylic acid (R^2 = COX), HATU, collidine or sulfonyl chloride (R^2 = SO$_2$X), DCM/NMP (10:1); (g) TFA/TIS (95:5).

as AcOH salts in most cases). Spectral data was obtained for all compounds (LC–MS and ^{1}H NMR) and was consistent with the proposed structures.

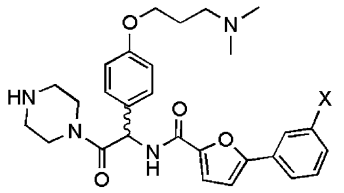
Biaryl **4**, lacking the quinoxalinedione functionality was inactive in the protein synthesis inhibition assay (<75% inhibition at 100 μM). Replacing the quinoxalinedione amide linkage with a sulfonylamide linkage (compound **5**) also afforded an inactive structure. Since we hypothesized that a reduction in hydrophilicity of biaryl **3** may improve cell penetration in antibacterial MIC assays, the quinoxalinedione moiety was replaced with more lipophilic groups.

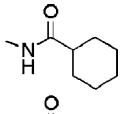
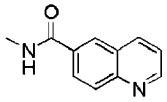
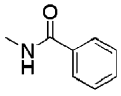
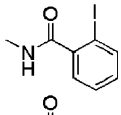
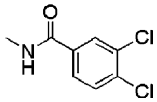
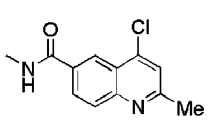
With the dimethylquinoxaline substitution (biaryl **6**) the antibacterial inhibitory activity was maintained. However, the quinoline substitution (biaryl **7**) led to ~3-fold reduction in activity compared to parent **3**. Biaryls **6** and **7** failed to display antibacterial activity against *E. coli* and *S. aureus* pathogens. The fused bicyclic end-group appears to be an important feature for activity as the quinoline, pyridine, piperazine, and thiadiazole derivatized structures (**8**–**10**) are noninhibitory in the protein synthesis assay at 100 μM. The larger bicyclic end-group may be advantageous at filling a hydrophobic pocket in the binding site. A cationic functionality was also incorporated in the end-group SAR and found to be noninhibitory at 100 μM (biaryl **11**). This finding also supports our hypothesis that the end-group is interacting with a hydrophobic site.

Since in vitro antibacterial activity was not found by replacing the quinoxalinedione functionality with a series of less hydrophilic substituents, we decided to modify an alternative structural region of biaryl **3**. From our 'SAR by MS' strategy, it was found that analogs of motif **1** lacking cationic side chains bound poorly to the A1061 23S rRNA MS screening construct.[7] Presumably, the cationic aminoethyl side chain interacts with a negatively charged phosphate in the binding site. Other RNA-targeted drug discovery programs identified dimethylaminoalkoxy as an RNA-recognition motif.[17,18] As a result of these reports, we decided to substitute the aminoethyl side chain of **3** with a similar type of motif. Since both enantiomers of biaryl **3** gave identical IC$_{50}$ values in the bacterial protein synthesis assay, we decided to incorporate a cationic tyrosine-derived side chain into our SAR plan via the commercially available racemic building block (Scheme 1).[19]

We were pleased to discover that the closest analog of our lead structure with the new modification was ~5-fold more potent (Table 2, compound **12**, IC$_{50}$ = 3 μM). Unfortunately, the compound still lacked antibacterial activity in our initial antibacterial screens at 100 μM.

Table 2. Bacterial protein synthesis inhibitory activity of biaryls



Compd	X	IC$_{50}$[a,b] (μM)
23		>100
24		>100
25		6
26		40
27		38
28		4
29		2

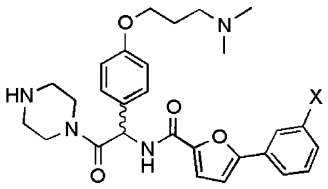
[a] In vitro bacterial translation inhibition (*E. coli* extract).
[b] Compounds did not display antibacterial activity at 100 μM (<75% inhibition) against *E. coli* and *S. aureus* bacterial strains.

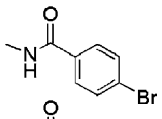
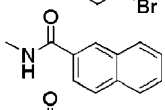
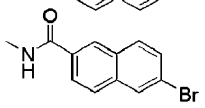
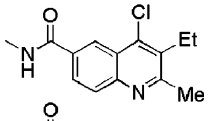
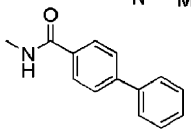
Incorporating the new side chain into our SAR study, we decided to re-explore the end-group modifications made to our parent lead structure **3**. As with biaryl **4**, removing the quinoxalinedione group abolished the antibacterial activity for biaryl **13**. However biaryl **14**, with the sulfonylamide link is >3-fold more potent than analog **5** and dimethylquinoxaline-substituted biaryl **15** is ~15-fold more potent than analog **6**. Furthermore, the activity of the quinoline-substituted biaryl **16** was improved ~10-fold relative to biaryl **7**. With the dimethylaminopropoxyphenyl side-chain modification, the monocyclic end-group analogs are tolerated (biaryls **17**–**19**). Some fragmented structures were also explored to assess the importance of the piperazine and the biaryl pharmacophores (Fig. 2). Biaryl **21**, lacking the piperazine functionality, is ~3-fold less potent than **12** while compound **22**, lacking the biaryl core, is inactive at 200 μM. Like the aminoethyl series, the cationic end-group of **20** was not tolerated. Compounds **13**–**22** were inactive in our initial MIC assays at 100 μM, presumably still suffering from poor cell permeability and/or intracellular efflux.

Since a wider variety of end-groups are tolerated in the new biaryl series, we decided to explore some additional substitutions, including more hydrophobic substitutions (Tables 2 and 3). The acetamide and cyclohexane end-group substitutions (compounds **23** and **24**) abolished the in vitro antibacterial activity. The quinoline-substituted analogs (**25** vs **16**) gave similar inhibitory activities. The phenyl-substituted biaryl gave similar inhibitory activity as the 2-iodophenyl analog (**26** vs **27**). The dichlorophenyl-substituted analog was ~10-fold superior with an IC_{50} of 4 μM. The more lipophilic quinoline end-group gave a 3-fold improvement in antibacterial activity compared to quinoline-substituted biaryl **25**. However, none of the compounds in Table 2 displayed antibacterial activity in our preliminary screens at 100 μM.

As we continued to explore additional end-group modifications, some biaryls were discovered to display antibacterial activity (Table 3). The bromophenyl-substituted analog displayed low μM inhibitory activity similar to its dichlorophenyl analog (**30** vs **28**). Unlike biaryl **28**, biaryl **30** gave good antibacterial activity against *E. coli* and *S. aureus* pathogens. The naphthalene substituted analog gave a 4-fold better IC_{50} than its phenyl analog in the cell-free assay (**31** vs **26**) as well as displaying some weak antibacterial activity. The aryl-bromo substitution also improves antibacterial activity

Table 3. Antibacterial activity of biaryls

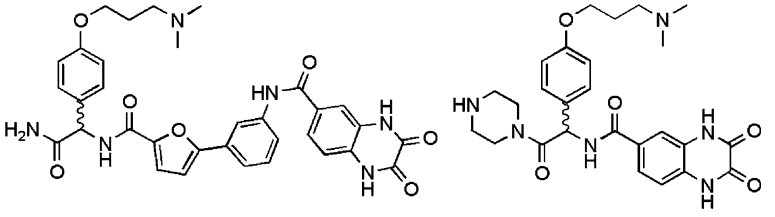


Compd	X	IC_{50}[a] (μM)	MIC (μM)[b]	
			E. coli	*S. aureus*
30		5	6–13	13–25
31		10	25–50	50–100
32		5	6–13	13–25
33		0.78	13–25	>100
34		9	6–13	3–6

[a] In vitro bacterial translation inhibition (*E. coli* extract).
[b] Bacterial strains: *E. coli* ATCC 25922, *S. aureus* ATCC 13709.

(**32** vs **31** and **30** vs **26**) and the incorporation of ethyl to the end-group of **29** led to analog **33** with antibacterial activity.

The biphenyl substitution improves in vitro antibacterial activity 4-fold compared to the phenyl substitution (**34** vs **26**). Furthermore, unlike **26**, biaryl **34** shows good antibacterial activity in our initial screens (MIC = 3–6 μM vs *S. aureus* and 6–13 μM vs *E. coli*). Biaryl **34** was evaluated against some additional bacterial strains. The analog shows antibacterial activity against Gram-positive *Enterococcus faecalis* and *Streptococcus pyogenes* (3–6 μM) as well as Gram-negative *Klebsiella pneumoniae* (13–25 μM) and *Proteus vulgaris* (50–100 μM).[20] Furthermore, biaryl **34** was found to be active against a methicillin-resistant *S. aureus* strain at 13–25 μM. This is an especially important finding as multi-drug resistant *S. aureus* is a serious clinical



21, IC_{50} = 10 μM (bacterial translation) **22**, IC_{50} > 200 μM

Figure 2. Fragmented analogs of biaryl **12**.

problem. We also confirmed that biaryl **34** is selective for bacterial protein synthesis inhibition over eukaryotic protein synthesis inhibition (<75% at 200 μM).[12]

In summary, we have taken a lead from an 'SAR by MS' strategy and uncovered new structures with antibacterial activity against Gram-negative and Gram-positive bacterial strains, including a methicillin-resistant *S. aureus* strain. This relatively quick optimization of the initial lead structure highlights the usefulness of our high-throughput 'SAR by MS' screening program for RNA-targeted therapeutics. We are continuing to explore the SAR of these promising antibacterial agents.

Acknowledgements

This work was supported in part by the NIH (SBIR grant R44 AI45210-03).

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12. The DNA template, pBestLuc™ (Promega), is a plasmid containing a reporter gene for firefly luciferase fused to a strong *tac* promoter and ribosome binding site. Messenger RNA from 1 μg pBestLuc™ is transcribed and translated in *E. coli* S30 bacterial extract (Lesley, S. A.; Brow, M. A.; Burgess, R. R. *J. Biol. Chem.* **1991**, *266*, 2632–2638) in the presence and absence of test compound. Compounds are tested in a black 96 well microtiter plate in an assay volume of 35 μL. Each test well contains: 5 μL test compound, 13 μL S30 premix (Promega), 4 μL 10× complete amino acid mix (1 mM each), 5 μL *E. coli* S30 extract and 8 μL of 0.125 μg/μL pBestLuc™. The transcription/translation reaction is incubated for 35 min at 37 °C followed by detection of functional luciferase with the addition of 30 μL LucLite™ (Packard). Light output is quantitated on a Packard TopCount. For IC_{50} determination, compounds are tested at 15 doses in duplicate wells. Signal generated from duplicate wells (blanks, no drug, test compound) are averaged. Averaged signal from test wells is compared to control wells containing no compound (no drug control). Percent inhibition of coupled transcription/translation is determined at all 15 doses and plotted with curve fitting in GraphPad™. IC_{50} values are generated by GraphPad™ as the concentration where signal is 50% of plateaus at 100% and 0% inhibition.
13. 'Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically. Approved Standard M7-A5.' National Committee for Clinical Laboratory Standards 2002, Suite 1400, Wayne, PA 19087. The MIC assays were carried out in a 150 μL volume in duplicate in 96-well clear flat-bottom plates. The bacterial suspension from an overnight culture growth in the appropriate medium was added to a solution of test compound in 0.5% DMSO in water. Final bacterial inoculum was approximate 10^2–10^3 CFU/well. The percentage growth of the bacteria in the test wells relative to that observed for a control well containing no compound was determined by measuring absorbance at 595 nm (A_{595}) after 20–24 h at 37 °C. The MIC was determined as a range of concentrations where complete inhibition of growth was observed at the higher concentration and the bacterial cells were viable at the lower concentration.
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