

# F1-837

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## Microbiological Profile of Novel 2,4-Diaminoquinazoline DHFR Inhibitors

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### ABSTRACT

**BACKGROUND:** Antibiotic resistance is a growing concern to the medical community, creating a need for rapid development of new antimicrobial agents. Investigation of a series of compounds targeting dihydrofolate reductase (DHFR) led to the discovery of novel inhibitors. Activity against resistant gram-positive organisms, including trimethoprim (TMP) resistant *Staphylococcus aureus* and multidrug resistant *Streptococcus pneumoniae* was of particular interest.

**METHODS:** MICs of new 2,4-diaminoquinazolines were determined for *S. aureus* (ATCC 13709) and *S. aureus* F99Y (TMP-resistant mutant isogenic to ATCC 13709) using CLSI guidelines. Synergy studies were performed on a subset of active compounds. Time-kill curves were determined for highly active compounds.  $K_i$  values were established for *S. aureus* DHFR enzyme activity. MIC values were determined for a set of *S. pneumoniae* strains, some with multi-drug resistance.

**RESULTS:** The 2,4-diaminoquinazolines were active against *S. aureus*, with MIC values from 0.008 to 1 mg/mL against the susceptible strain and 0.125 to 2 mg/mL against the F99Y TMP-resistant strain. The MIC<sub>50</sub> values for compounds tested on *S. pneumoniae* ranged from <math>\leq 0.5</math> to 32  $\mu\text{g/mL}$ . The enzyme  $K_i$  values from *S. aureus* ranged between 2.5 nM to sub nanomolar. TMP MIC values were 1 mg/mL against the susceptible strain and 64  $\mu\text{g/mL}$  against the F99Y TMP-resistant strain. The MIC<sub>50</sub> value for TMP tested on *S. pneumoniae* was 8  $\mu\text{g/mL}$ .

**CONCLUSIONS:** This novel class of 2,4-diaminoquinazolines has shown to have a potent Gram-positive antimicrobial profile with activity targeting DHFR. Compounds demonstrated good activity vs. a strain containing the commonly found F99Y mutation that leads to TMP resistance.

### BACKGROUND

Novel 2,4-Diaminoquinazoline dihydrofolate reductase (DHFR) inhibitors were discovered using our structure-based drug design. They have antibacterial activity against Gram + organisms such as *S. aureus* and multi drug resistant *S. pneumoniae*. Here we report *in vitro* potency and enzyme activity for 7 novel analogs, iclaprim and TMP. The collection of *S. pneumoniae* strains were biased toward highly drug resistant strains.

Antimicrobial activity of TMP, which acts through inhibition of DHFR, is known to be antagonized by thymidine present in the media<sup>1</sup>. Resistance to TMP is due to amino acid substitutions altering the enzyme structure and reducing TMP binding to the enzyme, the most significant of these being the F99Y in *S. aureus* and I100L in *S. pneumoniae*. Through our design of novel inhibitors, we have increased enzyme potency such that sufficient activity is maintained against resistant strains.

### MATERIALS AND METHODS

**Bacterial Strains:** *Staphylococcus aureus* strain (ATCC 13709) and the isogenic F99Y mutant were used to test *in vitro* activity of DHFR inhibitors. Ten isolates of *Streptococcus pneumoniae* were also tested in the MIC panel.

**Construction of F99Y Strain:** The DHFR F99Y mutant strain was isolated by selecting resistance to TMP. A *S. aureus* (ATCC 13709) mid-log phase culture was used to plate  $3 \times 10^7$ ,  $3 \times 10^8$  and  $3 \times 10^9$  CFU on Muller Hinton (MH) agar plates containing TMP at 4, 8, 16 and 32  $\mu\text{g/mL}$ , in duplicates. The plates were incubated at 37°C. The TMP resistant colonies that appeared after 24 & 48 hrs were selected. Genomic DNA from TMP resistant colonies were isolated and the *foIA* genes were PCR amplified & sequenced (Retrogen, San Diego, California) to verify the mutation in the gene.

**MIC Studies:** Minimum inhibitory concentrations (MIC) values of novel 2, 4-diaminoquinazolines were determined using CLSI broth microdilution methods<sup>2</sup>. Assays were conducted in Mueller Hinton cationic-adjusted medium with or without 20% mouse serum v/v to study the effect of serum on MIC. Compound stocks were prepared in 100% DMSO at 10 mg/mL. Serial dilutions were made for compound concentrations of 0.5-64  $\mu\text{g/mL}$  with a final DMSO concentration of 2% v/v.

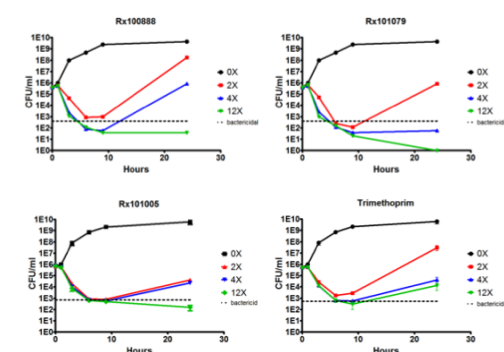
### RESULTS

#### Minimum Inhibitory Concentration (MIC) & Enzymatic Potency ( $K_i$ ) of Selected 2,4-Diaminoquinazolines

		Rx100880	Rx100888	Rx101005	Rx101079	Rx101127	Rx101205	Rx101250	Trimethoprim	Iclaprim
MIC ( $\mu\text{g/mL}$ )	<i>S. aureus</i>									
	ATCC 13709	2	0.5	0.016	0.125	0.02	0.03	0.03	1	0.25
	ATCC 13709 + 20% serum	2	1	0.125	1	0.5	0.25	1	1	0.125
	ATCC 13709 F99Y (TMP <sup>r</sup> )	>64	8	0.25	0.25	0.25	2	4	64	2
	ATCC 49619	16	2	0.25	0.125	$\leq 0.5$	0.25	0.5	4	0.125
	ATCC 700674	>64	32	8	4	16	>64	>64	>64	8
	ATCC 700669	>64	32	8	4	4	>64	2	>64	8
	ATCC 700671	32	4	32	4	32	>64	>64	>64	16
	ATCC 51916	32	8	$\leq 0.5$	$\leq 0.5$	4	2	1	>64	1
	ATCC 700676	4	$\leq 0.5$	$\leq 0.5$	$\leq 0.5$	1	2	1	8	$\leq 0.5$
ATCC 700677	16	4	$\leq 0.5$	$\leq 0.5$	0.25	0.25	0.25	4	$\leq 0.5$	
ATCC 700676	8	2	$\leq 0.5$	$\leq 0.5$	0.25	0.25	0.25	4	$\leq 0.5$	
ATCC 700904	64	16	4	1	4	8	4	>64	4	
ATCC BAA-295	4	$\leq 0.5$	$\leq 0.5$	$\leq 0.5$	$\leq 0.125$	$\leq 0.125$	$\leq 0.125$	0.5	$\leq 0.5$	
<i>S. pneumoniae</i> -MIC <sub>50</sub>	32	4	$\leq 0.5$	$\leq 0.5$	1	2	1	8	$\leq 0.5$	
<i>S. pneumoniae</i> -MIC <sub>90</sub>	>64	32	8	4	16	>64	>64	>64	8	
DHFR $K_i$ (nM)	Human	261	26.3	93.5	7.6	1026	663	2100	19070	578
	<i>S. aureus</i> WT ATCC 21709	2.5	0.48	0.002	0.011	0.005	0.012	0.01	1.24	0.046
	<i>S. aureus</i> F99Y (TMP <sup>r</sup> )	172	28.4	0.2	0.031	0.79	1.1	1.5	90.4	0.39
	<i>S. pneumoniae</i> WT ATCC 49619	16	4.9	0.02	0.018	0.27	0.032	0.051	6.4	0.009
	<i>S. pneumoniae</i> I100L (TMP <sup>r</sup> )	312	83	6.1	1.4	4.1	11.9	5.4	896	4.4

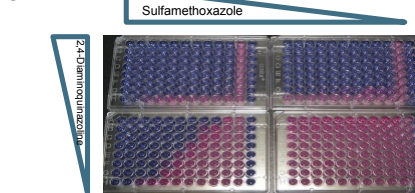
- The 2,4-diaminoquinazoline series showed activity against both wild-type *S. aureus* and the TMP resistant F99Y DHFR mutant.
- For some compounds the MIC values were shifted 1 - 32x by the addition of 20% mouse serum, while others were unaffected.
- This class of compounds show good activity against a select panel of *S. pneumoniae* strains, with MIC values as low as  $\leq 0.13 \mu\text{g/mL}$ .
- Replacing the 7-aryl group with substituted benzamidozoles improved potency and selectivity for the bacterial enzyme vs. *S. pneumoniae* and TMP resistant *S. aureus*.
- $K_m$  values for the H<sub>2</sub>F substrate were similar for WT and F99Y *S. aureus* DHFR enzymes (6.0 and 4.1  $\mu\text{M}$ , respectively). In contrast, the affinity for H<sub>2</sub>F substrate decreased in the TMP-resistant *S. pneumoniae* enzyme relative to the WT enzyme (16.4 and 4.9  $\mu\text{M}$ , respectively).
- Despite the fact that our *S. pneumoniae* panel included a number of drug resistant strains, MIC<sub>50/90</sub> and enzyme profiles of this class of compounds are very promising.

#### Time-Kill Curves for 2,4-Diaminoquinazolines vs. *S. aureus*



- 2,4-Diaminoquinazolines showed bactericidal activity at 12x MIC level (and 4x for Rx101079), reducing bacterial growth >3 log<sub>10</sub> in 24 hrs. Lower concentrations exhibited cidalty by 6 hrs but experienced regrowth by 24 hrs.

#### Antimicrobial Synergy with Selected 2,4-Diaminoquinazolines & SMX



	FICI	FIC MIC potency $\mu\text{g/mL}$
Rx100888 x SMX	0.125-0.502	0.125 <sub>100888</sub> x 0.5 <sub>SMX</sub>
Rx101005 x SMX	0.094-0.258	0.004 <sub>101005</sub> x 0.25 <sub>SMX</sub>
Rx101079 x SMX	0.094-0.266	0.004 <sub>101079</sub> x 0.25 <sub>SMX</sub>
TMP x SMX	0.094-0.515	0.063 <sub>TMP</sub> x 1 <sub>SMX</sub>

- Checkerboard studies resulted in equivalent FICI values of the tested 2,4-Diaminoquinazolines and TMP showing synergy with sulfamethoxazole.
- The potency of Rx101005/SMX and Rx101079/SMX was greater than TMP/SMX.

### MATERIALS AND METHODS CONT.

**Time-Kill:** MIC values were previously determined. Testing concentrations were calculated for 2x, 4x and 12x the MIC. *S. aureus* (ATCC 29213) was grown to 0.2 OD<sub>600</sub> in MH-CA. A 1:400 dilution was made into MH-CA resulting in 5x10<sup>5</sup> CFU/mL starting inoculum. Treatment was 2x, 4x and 12x MIC. Aliquots were sampled at the 0, 1, 3, 6, 9, 24 hrs. CFUs were enumerated through plating of 10-fold serial dilutions of the sampling at the given time points.

**Synergy:** MIC Assays were conducted in MH-CA medium against *S. aureus* (ATCC 13709). Compounds Rx100888, Rx101005 and Rx101079 or TMP were prepared in 100% DMSO at 10 mg/mL then serially diluted. Compounds were tested using standard checkerboard methodology for synergy with sulfamethoxazole<sup>1</sup>. Pair wise interaction of the MIC was determined using Alamar Blue (Invitrogen) after 18 hrs of incubation<sup>3</sup>. Fractional inhibitory concentration index (FICI) values were determined for each compound<sup>1</sup>.

**Enzyme Assays:** DHFR activity was determined spectrophotometrically by monitoring the decrease in absorbance at 340 nm resulting from the oxidation of NADPH coupled to reduction of substrate dihydrofolate (H<sub>2</sub>F). Enzymes (1-4 nM active site concentrations) were incubated with test compound in the presence of NADPH (100  $\mu\text{M}$ ) for 10 minutes prior to initiation of reaction by addition of H<sub>2</sub>F (100  $\mu\text{M}$ ). Assay buffer consists of 50 mM sodium phosphate pH 7.0, 25 mM potassium chloride, 1 mM EDTA, 10 mM  $\beta$ -mercaptoethanol and 20  $\mu\text{g/mL}$  BSA.  $K_m$  values for H<sub>2</sub>F were determined at fixed NADPH (100  $\mu\text{M}$ ) and varying H<sub>2</sub>F. Inhibition modalities were confirmed to be competitive with respect to H<sub>2</sub>F using standard procedures. Inhibition constants ( $K_i$ ) were determined using the Morrison tight-binding equation to account for ligand depletion. All analysis was carried out using GraphPad Prism 4.0.

### CONCLUSIONS

- 2,4-Diaminoquinazolines are a novel class of DHFR inhibitors with bactericidal activity against *S. aureus*.
- The 7-(benzamidozole-1-yl)-2,4-diaminoquinazoline series is highly active against TMP-resistant *S. aureus* and *S. pneumoniae*, including multidrug resistant strain ATCC 700904.
- Excellent selectivity is seen vs. human enzyme.
- $K_m$  values for H<sub>2</sub>F were similar for WT and F99Y *S. aureus* DHFR enzymes (6.0 and 4.1  $\mu\text{M}$ , respectively). In contrast, the affinity for H<sub>2</sub>F substrate decreased in the *S. pneumoniae* TMP resistance enzyme relative to the WT enzyme (16.4 and 4.9  $\mu\text{M}$ , respectively).
- This class of compounds are synergistic with sulfamethoxazole, thus potentially useful in combination therapy to treat Gram-positive infections.

### REFERENCES

- Pitell et al. 2005, Antimicrobial Combinations p.365-405, Antibiotics in Laboratory Medicine, 5<sup>th</sup> ed.
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- Barrow et al. 2006, Antimicrob. Agents Chemother. 27: 178-180