

ABSTRACT

BACKGROUND: The spread of microbial drug resistance is a global public health challenge and has necessitated the development of new antibiotics. Dihydrofolate reductase (DHFR) is the target for the antimicrobial drug trimethoprim (TMP). However, bacterial resistance to TMP has diminished its use. Using structure-based drug design, we have discovered a novel series of DHFR antibacterials. A lead molecule from this series, Rx101005 was evaluated in advanced microbiological and *in vivo* efficacy studies.

METHODS: The *in vitro* activity of Rx101005 against *Staphylococcus aureus* (ATCC 29213) was investigated in constant concentration time-kill curve studies. Single step resistance mutation frequencies in *S. aureus* were determined at 4x the MIC of Rx101005 and TMP. *In vivo* efficacy of this compound was assessed against a *S. aureus* (ATCC 13709) intraperitoneal challenge model. Mice were infected with 7×10^5 CFU/mL IP and then dosed IV 5 minutes post infection with Rx101005. ED₅₀ was determined by survival 24 hrs post infection.

RESULTS: The MIC value for Rx101005 against *S. aureus* was 0.015 µg/mL. In a time-kill assay, Rx101005 at 2x, 4x, or 12x the MIC resulted in a 3 log drop in CFU by 6 hrs but was not cidal at 24hrs. The spontaneous mutation frequency for Rx101005 was $\sim 2 \times 10^{-8}$ at 4x the MIC. Rx101005 demonstrated a dose-dependent increase in survival in a lethal *S. aureus* systemic infection model with an ED₅₀ of ~ 2 mg/kg.

CONCLUSIONS: Rx101005 is a potent DHFR inhibitor with good antimicrobial activity and is effective *in vivo* against *S. aureus* infections. Kinetics of killing and resistance mutation frequency

BACKGROUND

Rx101005 is a novel dihydrofolate reductase (DHFR) inhibitor discovered in our structure-based design efforts. It was found to be a very potent inhibitor against Gram-positive organisms. Antimicrobial activity of trimethoprim (TMP), which acts through inhibition of DHFR, is known to be antagonized by thymidine, either present in media or *in vivo*. Resistance to TMP is due to amino acid substitutions creating structural changes that reduce TMP binding to the enzyme, such as F99Y. In our design of novel inhibitors, we have increased enzyme potency such that sufficient activity is maintained against resistant mutants both *in vitro* and *in vivo*. This improved potency may also increase bactericidal activity against *S. aureus* and decrease the incidence of resistance mutations.

TMP in combination with sulfamethoxazole (SMX) is highly synergistic, which enhances potency. However, SMX is known to cause adverse allergic reactions in patients. A more potent compound, effective in combination with a lower concentration of SMX, would be advantageous.

MATERIALS AND METHODS

MIC Determinations: Minimum inhibitory concentrations (MIC) values were determined for *S. aureus* ATCC 29213, 13709 and the F99Y TMP mutant (isogenic to 13709) using CLSI broth microdilution methods¹. Assays were conducted in Mueller Hinton cationic-adjusted (MH-CA) 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 µg/mL with a final DMSO concentration of 2% v/v.

Synergy: MIC Assays were conducted in MH-CA medium against *S. aureus* (ATCC 13709). Compound stocks were prepared in 100% DMSO at 10 mg/mL then serially diluted. Compounds were tested using standard checkerboard methodology². Pair wise interaction of the MIC was determined using Alamar Blue (Invitrogen) after 18 hrs of incubation³. Fractional inhibitory concentration index (FICI) values were determined for each compound².

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 OD₆₀₀ in MH-CA. A 1:400 dilution was made into MH-CA resulting in 5×10^5 CFU/mL. 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.

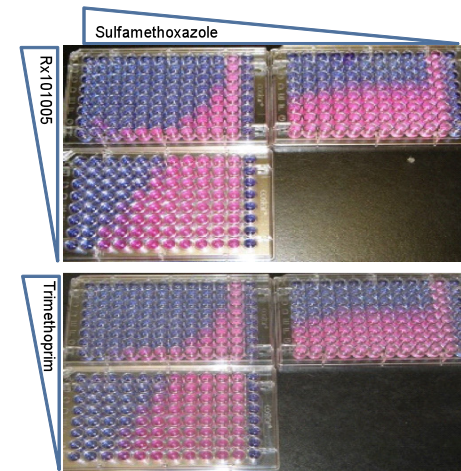
RESULTS

Minimum Inhibitory Concentrations (MICs)

Compound	<i>S. aureus</i> MIC µg/mL			
	ATCC 29213	ATCC 13709	ATCC 13709 + 20% mouse serum	F99Y mutant ⁴
Rx101005	.031	0.016	0.125	0.25
Trimethoprim	1	1	1	64
Vancomycin	1	1	1	1
Sulfamethoxazole	ND	32	64	64

- Rx101005 is potent against *S. aureus* wt and DHFR mutant strain F99Y.
- The mutant strain F99Y is resistant to trimethoprim.

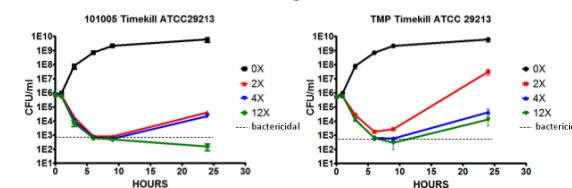
Antimicrobial Synergy Between Rx101005 or TMP and SMX



	FICI	FIC MIC potency µg/mL
Rx101005 x SMX	0.094 – 0.258	0.004 _{Rx101005} x 0.25 _{SMX}
Trimethoprim x SMX	0.094 – 0.515	0.063 _{TMP} x 1 _{SMX}

- Checkerboard studies resulted in equivalent FICI values but the Rx101005/SMX potency was greater than TMP/SMX.
- Rx101005, in combination with SMX is highly synergistic at concentrations lower than TMP/SMX combinations.

Time-Kill Curves for *S. aureus* Against Rx101005 and TMP



- Rx101005 exhibited bactericidal activity at 12x MIC, reducing the bacterial growth by >3 log₁₀ in 24 hrs. Lower concentrations exhibited cidalty by 6 hrs but experienced regrowth by 24hrs.
- All concentrations of TMP regrew by 24 hrs.

Resistance Incidence for Spontaneous Mutations

Drug Concentration	Frequency of Resistance	
	Rx101005	TMP
4x MIC	2.25x10 ⁻⁸ ± 9x10 ⁻¹⁰	2.28x10 ⁻⁸ ± 2x10 ⁻⁹
8x MIC	4.18x10 ⁻⁹ ± 6x10 ⁻¹⁰	1.63x10 ⁻⁹ ± 4x10 ⁻¹⁰

- Rx101005 mutation frequencies were calculated as the ratio of the number of resistant colonies at 48 hrs to the number of cells inoculated⁴.

Pharmacokinetics

Compound	PK Parameters				
	C _{5min} (µg/mL)	T _{1/2} (h)	AUC _{last} (h*µg/mL)	Cl (L/hr/kg)	V _Z (L/kg)
Rx101005	4.55	0.57	1.73	2.8	2.3

- Compound was dosed IV at 5 mg/kg.
- Noncompartmental plasma pharmacokinetic parameters following IV administration to female BALB/c mice.
- Rx101005 clearance is moderate with high tissue penetration.

Mouse Systemic Infection (ED₅₀)

Compound	<i>S. aureus</i> ATCC 13709 ED ₅₀ mg/kg
Rx101005	1.8
Trimethoprim	>50
Vancomycin	0.77

- Rx101005 provided significant protection against a lethal challenge with *S. aureus* wt compared to TMP treatment.
- Rx101005 was well tolerated to 50 mg/kg, the highest dose tested.
- TMP was ineffective in this model due to antagonism in the presence of thymidine and poor pharmacokinetics in mice.

MATERIALS AND METHODS (CONT.)

Resistance Incidence: The resistance incidence to *S. aureus* (ATCC 29213) was measured. Assay plates were prepared with 40 mL MH-CA agar containing compound at 4x or 8x MIC of *S. aureus*. Mid-log phase cultures (OD₆₀₀ ~0.8) were pelleted and resuspended in PBS to a concentration of $\sim 6 \times 10^8$ CFU/mL and 0.25 mL aliquots were spread onto MH-CA agar. Starting CFU were enumerated through triplicate plating of serial dilutions of the starting inocula in PBS. Plates were incubated at 37°C for 2 days. Putative resistant mutant colonies were confirmed by subculturing on MHA containing an equal amount of antibiotic. Mutation frequency experiments were performed using a total of 3 independent cultures. Spontaneous mutation frequencies were determined by dividing the number of resistant colonies on a given plate by the actual plated CFU⁴.

Pharmacokinetics: Single dose pharmacokinetics (PK) of Rx101005 were examined. 5 mg/kg Rx101005 was administered intravenous (IV). Blood samples were collected (3 mice per time point) at 0.083, 0.25, 0.5, 1, 2, 4, 8 hrs. Rx101005 plasma concentrations were determined by LC-MS/MS analysis.

In Vivo Efficacy in Mouse Septicemia Model: BALB/c female mice (17-20 grams) were acclimated for 6 days prior to the start of the study. All studies were performed under IACUC protocols. Mice (5 per group) received *S. aureus* (ATCC 13709) inoculum of 4×10^5 CFU/mouse via intraperitoneal (IP) injection. The mice were then treated IV with Rx101005 doses from 1.6 to 50 mg/kg. Vancomycin and trimethoprim were used as controls.

DISCUSSION

- Rx101005 shows potent antimicrobial activity against *S. aureus* including the TMP resistant strain, F99Y. TMP is not active against the F99Y mutant strain.
- Potent synergy was attained with Rx101005/SMX at lower concentrations than TMP/SMX.
- Time kill studies showed a 3-log₁₀ reduction in CFUs was apparent between 6-9 hrs of growth at all concentrations of Rx101005, but was maintained only at 12x the MIC by 24 hrs. TMP reduced CFUs by 3-log₁₀ only at 12x MIC. Regrowth was seen at all concentrations tested by 24 hrs.
- The spontaneous mutation rate of Rx101005 is equivalent to TMP.
- In mouse, Rx101005 has reasonable pharmacokinetic profile.
- Rx101005 exhibited *in vivo* dose dependent efficacy against *S. aureus* and was at least 25x more efficacious than TMP against this wt strain.

CONCLUSIONS

- ❖ Rx101005 represents a new antibacterial class of compounds that targets bacterial DHFR.
- ❖ Rx101005 has potent antibacterial activity against *S. aureus* including the TMP resistant mutant F99Y strain.
- ❖ Reduction in the dose of SMX could reduce side effect problems in combination therapy.
- ❖ Rx101005 is highly efficacious *in vivo* against lethal *S. aureus* infections suggesting that this class has the potential to yield interesting new therapeutic agents.

REFERENCES

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