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ADVANCED MICROBIOLOGY STUDY OF RX100472, A NOVEL METHIONYL tRNA SYNTHETASE (MetRS) INHIBITOR

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ABSTRACT

Background: Antibiotic resistance in Gram-positive organisms is a growing concern to the medical community, leading to a need for the development of new antimicrobial agents active against novel targets. Investigation of a series of compounds targeting methionyl tRNA synthetase led to the discovery of RX100472, a novel inhibitor with potential for pharmaceutical applications. Extensive *in vitro* microbiology was assessed.

Methods: The *in vitro* activity of RX100472 against *S. aureus* was investigated in detail. First, this compound was evaluated using standard CLSI broth microdilution method in Mueller Hinton-CA broth with or without 20% serum. Constant concentration time-kill curves were performed using compound at 2x, 4x, 8x the MIC. Samples were taken at 1, 3, 6, 9 and 24 hours, dilutions plated and CFU per time point determined. Single-step resistance mutation frequencies were obtained for 4x and 8x MIC of RX100472 and ciprofloxacin towards *S. aureus*. K_i values were established for *S. aureus* MetRS, *S. pneumoniae* MetRS2 and human cytoplasmic MetS enzymes. Finally, the mechanism of action for RX100472 was confirmed by antisense hypersensitivity assay.

Results: The MIC value for RX100472 was 1 µg/mL with or without 20% serum. In a time-kill assay, addition of RX100472 resulted in bacterial growth inhibition by 24 hours. Resistance selection mutation frequencies for RX100472 ranged from 10^{-8} to 10^{-11} . The *S. aureus* MetRS enzyme K_i value was determined as 0.03nM but inactive against *S. pneumoniae* MetRS2 and human cytoplasmic MetS. Our mechanism of action assay showed that RX100472 was specific for MetRS with an 8.5 fold shift in antisense hypersensitivity.

Conclusions: RX100472 is shown to have a potent antimicrobial profile targeting the methionyl tRNA synthetase in *S. aureus*.

INTRODUCTION

RX100472 is a novel substituted pyrimidine class of compound under preclinical investigation. It is a very potent inhibitor of bacterial methionyl tRNA synthetase (MetRS) enzyme. It is active against *S. aureus* (MIC=1 µg/ml), *B. anthracis* (MIC=0.125 µg/ml), MRSA (MIC=1 µg/ml) and *E. faecium*-VRE (MIC=0.06 µg/ml). It selectively inhibits the Gram-positive bacterial enzyme and is inactive against the human MetS enzyme.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions: *Staphylococcus aureus* strain (ATCC 13709) was used to test the *in vitro* activity of RX100472. Cells were grown and tested in cation adjusted Muller-Hinton broth for MIC studies. 1.5% agar was used to prepare MH-agar plates. *E. coli* strains: DH5α was used for cloning and BL21-AI strain (Invitrogen) was used for protein expression. LB broth and agar plates with kanamycin were used for DNA manipulation and Terrific broth was used for protein expression.

MATERIALS AND METHOD CONT.

MIC Studies: MIC values were determined using the CLSI broth microdilution method, with two-fold dilutions of test compound solubilized in 100% DMSO and at 2% final DMSO concentration. Mouse serum was added to a final concentration of 20% to study the serum effect on MIC.

Resistance Studies: Resistance frequencies of RX100472 and ciprofloxacin were examined side by side. Freshly grown *Staphylococcus aureus* inoculums of 10^7 , 10^8 and 10^9 were plated on LB-agar plates with 4X and 8X of MIC of test compounds in duplicates.

Time Kill Studies: The bactericidal activity of RX100472 was studied at 2X, 4X, and 8X MIC levels. The starting inoculum was 5×10^5 cells/mL. Test compound concentrations were at 0X, 2X, 4X and 8X the MIC. Samples were collected at 0hr, 3hr, 6hr, 9 hr, and 24 hrs after treatment. The cells were serially diluted and 50µl of each dilution was plated on MH-agar plates. CFU/mL was determined after 24hr of incubation.

Mechanism of Action Studies: The antisense based hypersensitivity assay was used as described in reference (Ref. 1).

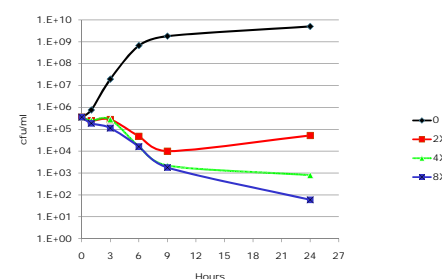
RESULTS

Table 1. In vitro Activity of RX100472 Tested Against Various Organisms

Compound	MIC (µg/mL)				
	<i>S. aureus</i>	<i>S. aureus</i> + 20% Serum	MRSA	<i>B. anthracis</i>	VR <i>E. faecium</i>
RX100472	1	1	1	0.125	0.062

- RX100472 is active against *S. aureus* including MRSA, as well as *B. anthracis* & VRE
- Unlike previous MetRS inhibitors, RX100472 does not lose potency in the presence of 20% mouse serum

Figure 2. RX100472 is bactericidal at 8X MIC vs *S. aureus* strain ATCC 13709



- RX100472 showed bactericidal activity at 8X MIC level, reducing the bacterial growth >3 logs in 24 hrs

Table 2. RX100472 Frequency of Resistance

Drug Concentration	Frequency of Mutation	
	RX100472	Ciprofloxacin
4X MIC	1.8×10^{-9} to 8.5×10^{-9}	$<7 \times 10^{-11}$ to $<2 \times 10^{-9}$
8X MIC	5.7×10^{-10} to 7.4×10^{-9}	$<7 \times 10^{-11}$ to $<2 \times 10^{-9}$

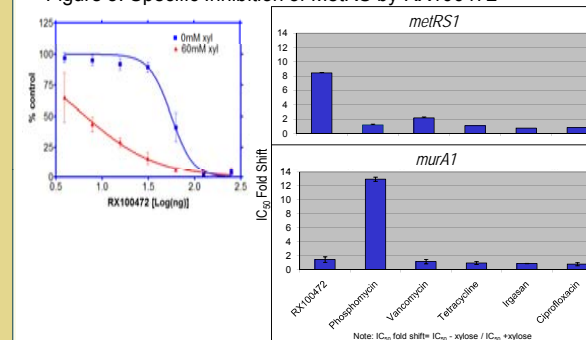
- RX100472 MIC= 1 µg/mL, ciprofloxacin MIC= 0.5µg/mL
- Mutation frequencies were calculated as the ratio of the number of resistant colonies at 48 hrs to the number of cells inoculated (Ref. 2)

Table 3. RX100472 is a specific inhibitor of *S. aureus* MetRS

RX100472	Enzyme activity of RX100472		
	<i>S. aureus</i> MetRS (K_i)	Human cytoplasmic MetS	<i>S. pneumoniae</i> MetRS2
	0.03nM	Inactive at 400,000nM	Inactive at 100,000nM

- RX100472 inhibits *S. aureus* MetRS enzyme (K_i =0.03nM)
- No inhibition of human cytoplasmic MetRS enzyme was detected up to 400µM compound
- RX100472 was inactive against *S. pneumoniae* MetRS2 enzyme

Figure 3. Specific Inhibition of MetRS by RX100472



- The *Bacillus anthracis* metRS antisense clone was hypersensitive to RX100472 (8 fold shift) but not phosphomycin
- A *murA1* clone was hypersensitive to phosphomycin, but not RX100472
- These data show that RX100472 is a specific inhibitor of MetRS

MATERIALS AND METHODS CONT.

Enzyme Assays: The *S. aureus* metRS gene was cloned into pET30 vector and enzyme was expressed as a 6x His-tagged protein in *E. coli* BL21-AI host and purified with nickel affinity chromatography. The histidine tag was cleaved with TEV protease. MetRS activity was monitored using a scintillation proximity assay (SPA) with 50 mM [³⁵S]methionine, *E. coli* methionyl specific tRNA (2.5µM; Sigma), 2.5nM MetRS, 5mM ATP, 100 mM Tris-HCl, pH 7.8, 28 mM MgCl₂, and 28 mM KCl. The Cheng-Prusoff equation was used to calculate K_i values from IC₅₀ measurements with inhibitor behaving competitively with respect to methionine and uncompetitively with respect to ATP.

RESULTS

- RX100472 is active against Gram positive organisms *S. aureus*, MRSA, *B. anthracis* and VRE (Table 1)
- Addition of 20% mouse serum did not alter MIC values
- RX100472 is bactericidal at 8X MIC, reducing CFU by 3 log cfu within 24 hrs of treatment (Fig. 2)
- The frequency of resistance against RX100472 ranged from 5.7×10^{-10} to 1.8×10^{-9} (Table 2)
- RX100472 showed good activity against *S. aureus* MetRS enzyme (K_i =0.03nM) but did not have any activity against human cytoplasmic MetRS enzyme tested as high as 400 µM and *S. pneumoniae* MetRS2 enzyme at 100 µM (Table 3)
- The antisense assay demonstrates the MOA of RX100472 is inhibition of MetRS (Fig.3)

CONCLUSIONS

- RX100472 is a novel compound targeting bacterial methionyl tRNA synthetase
- It is potentially useful in treating Gram-positive infections as well as the biodefense organism *B. anthracis*

REFERENCES

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