

The Effect of Testing Parameter Variations on the *In Vitro* Activity of GSK1322322 and Linezolid

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Abstract

Background: GSK1322322 is an antibacterial agent currently in development that inhibits peptide deformylase (PDF) function, a clinically unexploited target. GSK1322322 demonstrates targeted antibacterial activity against multi-drug resistant respiratory and skin pathogens, including methicillin-resistant *Staphylococcus aureus*, and represents a new antibiotic class with a novel mode of action. This study was undertaken in order to determine the effect of various testing parameters on the in vitro activity of GSK1322322 and a comparator agent, linezolid.

Method: 30 *S. aureus* (15 methicillin-susceptible, 15 methicillin-resistant) and quality control strain *S. aureus* ATCC 29213 were tested by three methods: CLSI broth microdilution (BMD), macrodilution and agar dilution. In addition, five different broths (cation adjusted Mueller Hinton, IsoSensitest, brain heart infusion (BHI), trypticase soy and brucella) were compared using CLSI BMD method. Three testing variables (temperature, incubation time, inoculum) were studied using BMD and agar dilution methods. Ten variables (CO₂, pH, calcium, magnesium, zinc, potassium, thymidine, polysorbate 80, albumin and serum) were also studied using BMD.

Results: There was good correlation of the three MIC methods. Variables that affected GSK1322322 BMD MICs the most were broth other than CAMHB, higher incubation temperature, longer incubation time, higher inoculum concentration and addition of serum.

Testing Variable	BMD MICs compared to reference BMD MICs	GSK1322322	Linezolid
Tryptic Soy Broth	Decreased 1.2 dilution	Decreased 0.6 dilution	Decreased 0.6 dilution
BHI Broth	Decreased 1.2 dilution	Decreased 0.5 dilution	Decreased 0.5 dilution
Brucella Broth	Decreased 1.6 dilution	Decreased 0.4 dilution	Decreased 0.4 dilution
40°C	Decreased 2.1 dilutions	Increased 0.5 dilution	Increased 0.5 dilution
48 hours	Increased 0.9 dilution	Increased 0.9 dilution	Increased 0.9 dilution
Inoculum 10 ⁶ CFU/mL	Increased 2.1 dilutions	Increased 1.6 dilution	Increased 1.6 dilution
Inoculum 10 ⁷ CFU/mL	Increased >5 dilutions	Increased >5 dilutions	Increased >5 dilutions
Serum 50%	Increased 1.2 dilution	Increased 0.6 dilution	Increased 0.6 dilution

Conclusion: When performing susceptibility testing with GSK1322322 and linezolid it is important to control the inoculum concentration according to standard methods. Other broth types, an increased incubation temperature and time, and serum will also affect GSK1322322 MICs.

Introduction

Susceptibility testing of antimicrobial agents is typically performed according to standardized methods. These guidelines provide standardized procedures for controlling important testing conditions such as inoculum concentration, incubation conditions, media, and pH and cation concentration of the media, which have been shown to have an effect on susceptibility results. This study was performed to determine effect of various testing parameters on the in vitro activity of a new peptide deformylase antimicrobial agent (GSK1322322) and a comparative agent, linezolid, against 30 *Staphylococcus aureus*.

Methods

Antibiotics

Antibiotic	Concentrations
GSK1322322	0.03-32 mg/L
Linezolid	0.03-32 mg/L

Microorganisms

- 30 *S. aureus* (15 MRSA and 15 MSSA)
- QC: *S. aureus* ATCC 29213

Media

Component Name	Manufacturer, City State
Brain Heart Infusion Broth (BHI)	Becton Dickinson, Sparks MD
Brucella Broth (BRU)	Becton Dickinson, Sparks MD
Cation Adjusted Mueller Hinton Broth (CAMHB)	Becton Dickinson, Sparks MD
Trek Diagnostics, Cleveland, OH	
IsoSensitest Agar (ISA) and IsoSensitest Broth (ISB)	Oxoid Ltd., Ogdensburg NY
Mueller Hinton Agar (MHA) and Mueller Hinton Broth (MHB)	Becton Dickinson (Difco), Sparks MD
Trypticase Soy Broth (TSB)	Becton Dickinson, Sparks MD

MIC Methods

An initial single MIC determination by broth microdilution using CAMHB was performed for each strain. Then triplicate testing was performed by all methods/media, utilizing the same initial inoculum for all methods. The following methods/media, were performed according to CLSI procedures (with exception of broth [other than CAMHB] and other variable changes).

- Broth Microdilution (BMD), CAMHB, ISB, BHI, TSB, BRU
- Broth Macrodilution (MD), CAMHB
- Agar Dilution (AD), MHA and ISA
- BMD – with following modification of test variables

Variables Studied

Variable Description	Method Tested	Specific Variables Tested
Temperature	BMD and Agar dilution	30, 35, and 40°C
Incubation time	BMD and Agar dilution	16, 24 and 48 hours
Atmospheric conditions	BMD	Ambient, 5% and 10% CO ₂
Broth Comparison	BMD	CAMHB, ISB, BHI, TSB and BRU
Inoculum	BMD and Agar dilution	10 ⁴ , 10 ⁵ , 10 ⁶ , 10 ⁷ CFU/mL
Calcium	BMD	2.35, 24.8, 49.5 and 102.0 mg/L
Magnesium	BMD	4.7, 11.5, 18.6 and 26.4 mg/L
pH	BMD	5.47, 6.62, 7.38 and 8.48
Serum	BMD	25% and 50%
Albumin	BMD	4 mcg/dL
Polysorbate 80	BMD	0.002%
Thymidine	BMD	1 and 5 mg/L
Zinc	BMD	2, 5 and 10 mmol/L
Potassium	BMD	12.5, 25 and 50 mmol/L

Results

GSK132322 MIC methods - Broth Microdilution, Broth Macrodilution and Agar Dilution

- BMD MICs did not vary by more than one doubling dilution during the course of the study (Figure 1)
- BMD MICs were lower with all broth media compared to CAMHB (Figure 2)
- Macrodilution MICs were slightly higher (0.6 dilution) compared to broth microdilution (BMD) MICs (Table 1)
- Agar dilution (MH-AD) MICs were slightly higher (0.13 dilution) compared to BMD MICs (Table 1)

Figure 1. Geometric Mean MICs (mcg/mL) of GSK1322322 and Linezolid for 30 *S. aureus* by CLSI Broth Microdilution Performed Over Study Testing Period (n=30 Per Day, Except for Day 1 n=90)

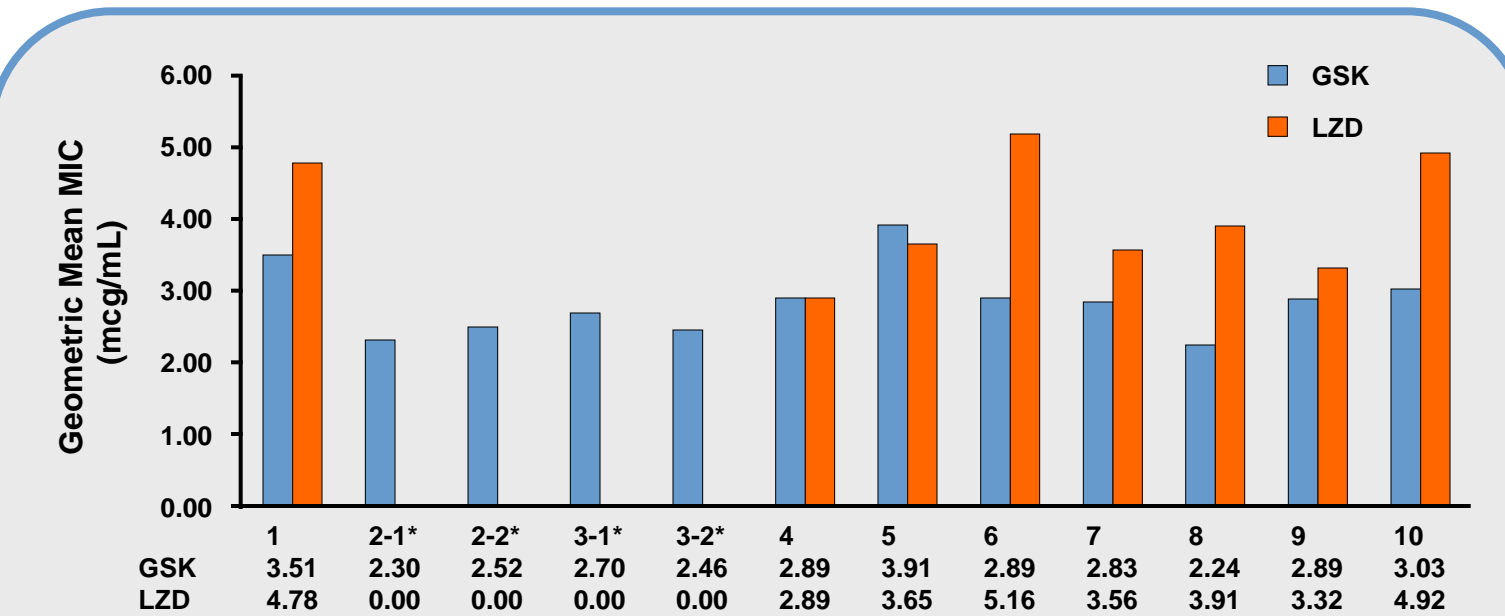


Figure 2. Geometric Mean MICs (mcg/mL) of GSK1322322 for 30 *S. aureus* by broth microdilution utilizing different broth media

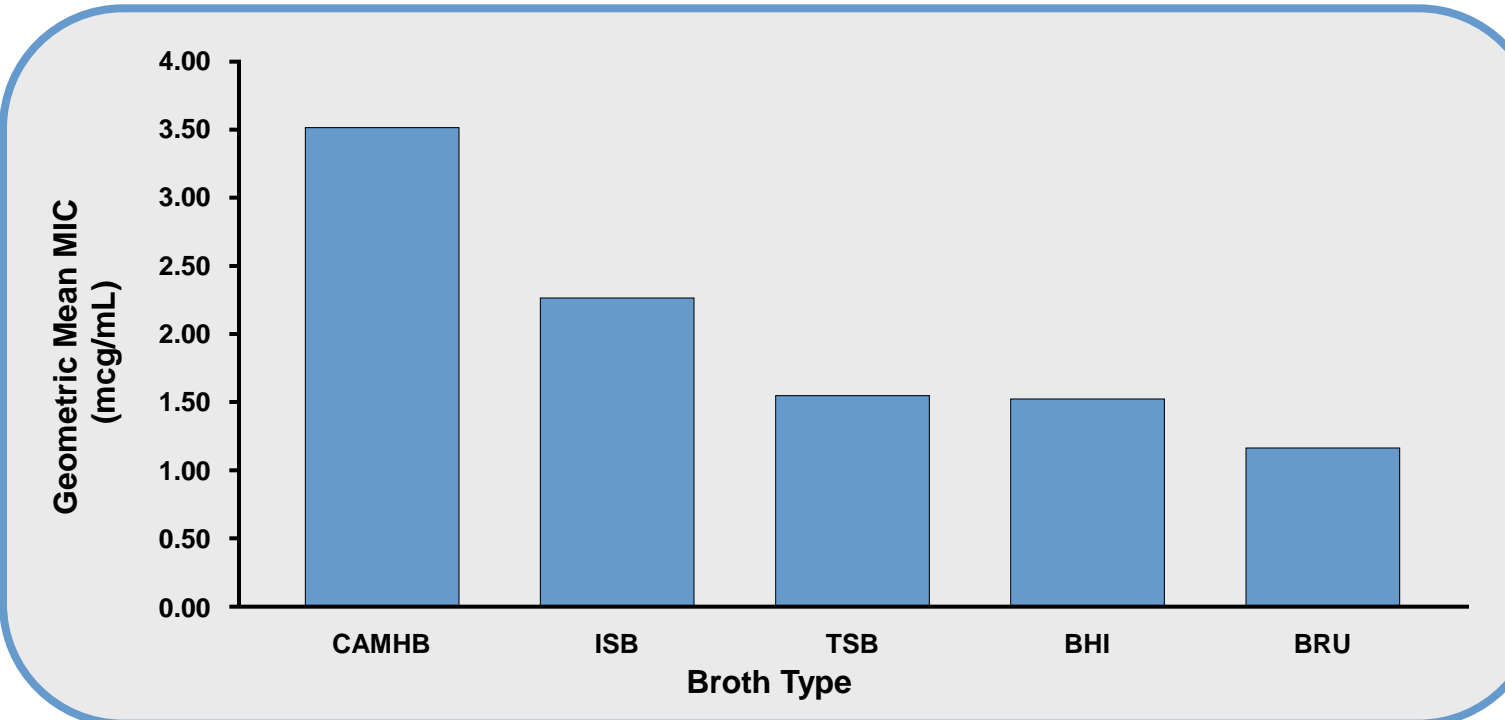


Table 1. *In vitro* Activity of GSK1322322 and Linezolid for 30 *S. aureus* as Determined by Broth Microdilution, Macrodilution and Agar Dilution Methodologies

Test (CLSI Reference) Method	Comparative Method	GSK1322322					Linezolid					
		Mean MICs (mcg/mL)	Mean MIC difference*	Mean Dilution	N(%) ≥1 dilution*	N(%) ≥2 dilution*	Mean MICs (mcg/mL)	Mean MIC difference*	Mean Dilution	N(%) ≥1 dilution*	N(%) ≥2 dilution*	
Broth Microdilution -CAMHB (BMD)	Macrodilution -CAMHB (MD)	90	BMD=3.51 MD=5.28	1.77	0.6	87 (74.4%)	87 (96.7%)	BDD=4.78 MD=3.70	-1.07	0.4	90 (100%)	90 (100%)
	Agar Dilution -MHA (MH-AD)	76	BMD=3.55 MH-AD=3.89	0.34	0.13	74 (97.4%)	76 (100%)	BMD=4.50 MH-AD=4.19	-0.32	-0.48	75 (98.7%)	76 (100%)
Agar Dilution -MHA (MH-AD)	MH (IS-AD)	48	AD=3.51 IS-AD=2.09	-1.42	-0.75	48 (100%)	48 (100%)	MHA=4.00 ISA=2.87	-1.13	-0.48	48 (100%)	48 (100%)

Broth Microdilution - Effect of Testing Variables (Table 2, Figures 2-4)

- The majority of all MICs were within one doubling dilution compared to the reference BMD MICs. The variables that impacted the MICs (as shown below) were media, incubation temperature, 48 hour incubation, inoculum concentration of 10⁶ and 10⁷ CFU/mL and addition of 50% serum.

Variable	GSK1322322	Linezolid
•Tryptic Soy broth	↓ 1.2 dilution	↓ 0.6 dilution
•BHI broth	↓ 1.2 dilution	↓ 0.5 dilution
•Brucella broth	↓ 1.6 dilution	↓ 0.4 dilution
•40°C temperature	↓ 2.1 dilutions	↓ 0.5 dilution
•48 hours incubation	↑ 0.9 dilution	↑ 0.9 dilution
•Inoculum 10 ⁶ CFU/mL	↑ 2.1 dilution	↑ 1.6 dilution
•Inoculum 10 ⁷ CFU/mL	↑ >5 dilutions	↑ >5 dilutions
•Serum 50%	↑ 1.2 dilutions	↑ 0.6 dilutions

Figure 3. Geometric Mean MICs (mcg/mL) of GSK1322322 for 30 *S. aureus* by Broth Microdilution at Different Bacterial Inoculum Concentrations

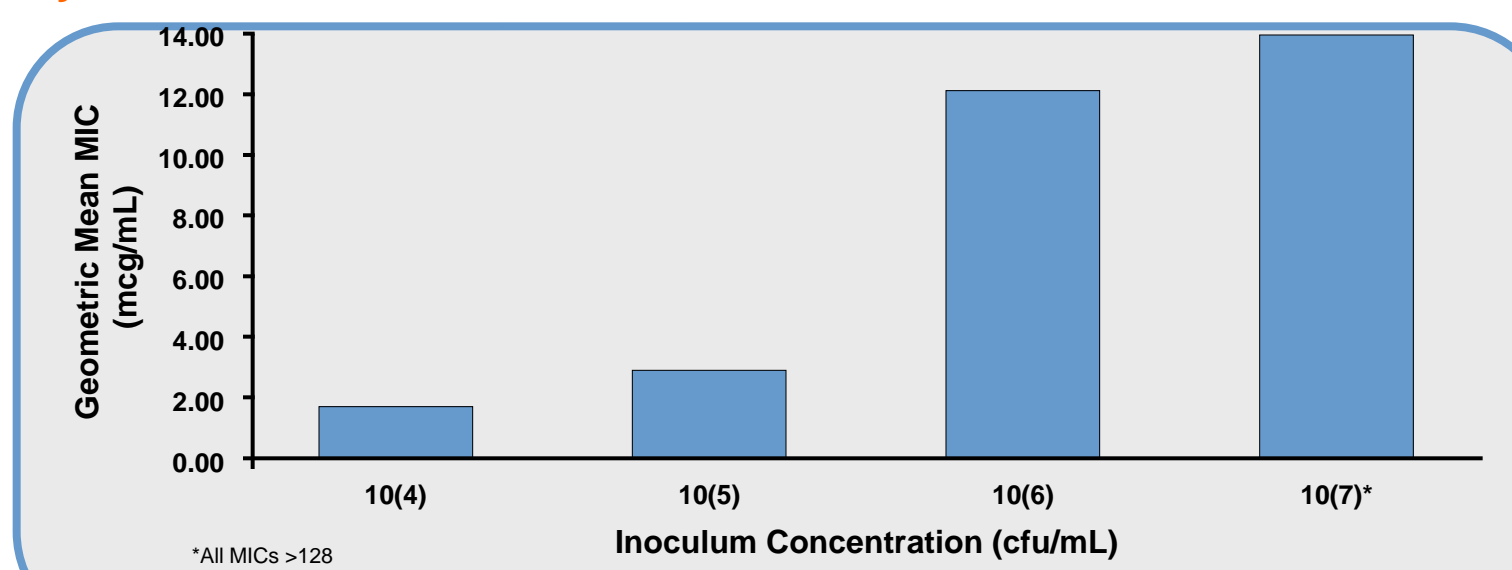


Figure 4. Geometric Mean MICs (mcg/mL) of GSK1322322 for 30 *S. aureus* by Broth Microdilution at Variable Serum and Albumin Concentrations

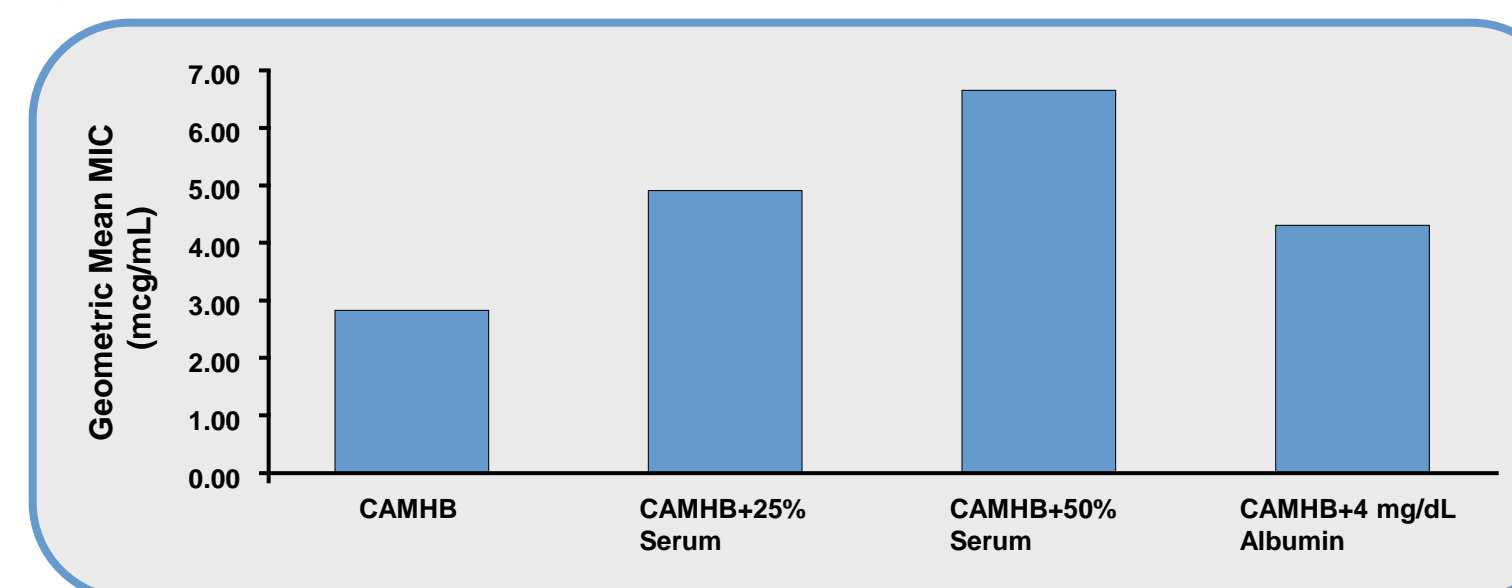


Table 2. *In vitro* activity of GSK1322322 and Linezolid Against 30 *S. aureus*: Comparison of the CLSI MIC Broth Microdilution Reference Condition to Other Testing Conditions

Test (CLSI Reference) Condition	Comparative Condition	GSK1322322 (MIC)					Linezolid (MIC)				
		Mean difference*	Mean Dilution	N(%) ≥1 dilution*	N(%) ≥2 dilution*	Mean Difference*	Mean Dilution	N(%) ≥1 dilution*	N(%) ≥2 dilution*		
Broth (CAMHB)	ISB	-1.25	-0.63	82 (91.1%)	90 (100%)	-0.45	-0.14	90 (100%)	90 (100%)		
	BHI	-1.98	-1.20	55 (61.1%)	86 (95.6%)	-1.29	-0.46	87 (96.7%)	90 (100%)		
	BRU	-2.34	-1.59	36 (40%)	84 (93.3%)	-1.13	-0.39	90 (100%)	90 (100%)		
	TSB	-1.96	-1.18	62 (68.9%)	88 (97.8%)	-1.58	-0.58	90 (100%)	90 (100%)		
Temperature (35°C)	30°C	0.00	0	29 (96.7%)	30 (100%)	-1.24	-0.60	28 (93.3%)	30 (100%)		
	40°C	-3.07	-2.10	7 (23.3%)	23 (76.7%)	1.63	0.53	30 (100%)	30 (100%)		
Incubation Time (16 hrs.)	24 hrs.	1.11	0.47	30 (100%)	30 (100%)	0.75	0.33	29 (96.7%)	30 (100%)		
	48 hrs.	2.63	0.93	23 (76.7%)	29 (97.7%)	2.63	0.93	27 (90.0%)	29 (96.7%)		
Inoculum (2 x 10 ⁶ cfu/mL)	3.2 x10 ⁶ cfu/mL	-1.19	-0.77	28 (93.3%)	30 (100%)	-0.75	-0.37	30 (100%)	30 (100%)		
	2.4 x10 ⁶ cfu/mL	12.13 ^b	2.07	12 (40%) ^c	22 (73.3%) ^c	10.31 ^b	1.63	20 (66.7%)	27 (90%)		
	3.3 x10 ⁶ cfu/mL	OS ^d	OS ^d	OS ^d	OS ^d	OS ^d	OS ^d	OS ^d	OS ^d		
Atmospheric (Ambient)	5% CO ₂	0.28	0.10	30 (100%)	30 (100%)	-0.32	-0.13	30 (100%)	30 (100%)		
	10% CO ₂	-0.66	-0.27	30 (100%)	30 (100%)	0.17	0.07	30 (100%)	30 (100%)		
Calcium (24.8 mcg/mL)	2.35 mcg/mL	-1.01	-0.87	26 (86.7%)	29 (96.7%)	-0.43	-0.17	29 (96.7%)	30 (100%)		
	49.5 mcg/mL	-0.29	-0.20	30 (100%)	30 (100%)	-0.26	-0.10	30 (100%)	30 (100%)		
	102 mcg/mL	0.33	0.20	30 (100%)	30 (100%)	-0.35	-0.13	30 (100%)	30 (100%)		
	4.7 mcg/mL	-1.01	-0.87	26 (86.7%)	29 (96.7%)	-0.43	-0.17	29 (96.7%)	30 (100%)		
Magnesium (11.5 mcg/mL)	18.6 mcg/mL	0.27	0.17	30 (100%)	30 (100%)	0.09	0.03	30 (100%)	30 (100%)		
	26.4 mcg/mL	-0.20	-0.13	30 (100%)	30 (100%)	0.00	0	30 (100%)	30 (100%)		
Potassium (No K added)	12.5 mmol/L	-0.39	-0.20	29 (96.7%)	29 (96.7%)	0.48	0.13	30 (100%)	30 (100%)		
	25 mmol/L	-0.63	-0.33	26 (86.7%)	29 (96.7%)	0.23	0.07	30 (100%)	30 (100%)		
	50 mmol/L	-0.89	-0.50	27 (90%)	29 (96.7%)	0.23	0.07	30 (100%)	30 (100%)		
	2 mmol/L	-0.45	-0.23	28 (93.3%)	30 (100%)	0.35	0.10	30 (100%)	30 (100%)		
Zinc (No Zn added)	5 mmol/L	-0.39	-0.20	28 (93.3%)	30 (100%)	-0.74	-0.23	30 (100%)	30 (100%)		
	10 mmol/L	-0.33	-0.17	28 (93.3%)	30 (100%)	0.23	0.07	30 (100%)	30 (100%)		
Thymidine (No Thy added)	1 mcg/mL	0.00	0	30 (100%)	30 (100%)	0.08	0.03	30 (100%)	30 (100%)		
	5 mcg/mL	-0.96	-0.60	28 (93.3%)	30 (100%)	0.08	0.03	30 (100%)	30 (100%)		
	5.47	-0.26	-0.13	24 (80%)	29 (96.7%)	-2.52	-0.97	23 (76.7%)	29 (96.7%)		
	6.62	0.07	0.03	29 (96.7%)	30 (100%)	-1.51	-0.50	30 (100%)	30 (100%)		
pH (7.38)	8.48	0.35	0.17	28 (93.3%)	30 (100%)	0.12	0.03	27 (90%)	30 (100%)		
	25%	2.10	0.80	22 (73.3%)	28 (93.3%)	2.09	0.67	28 (93.3%)	30 (100%)		
Serum (No Serum added)	50%	3.82	1.23	20 (66.7%)	25 (83.3%)	1.84	0.60	28 (93.3%)	30 (100%)		
	4 mg/dL	1.46	0.60	27 (90%)	30 (100%)	2.50	0.77	28 (93.3%)	30 (100%)		
Polysorbate 80 (No P80 added)	0.002%	1.62	0.33	28 (93.3%)	30 (100%)	0.64	0.23	30 (100%)	30 (100%)		
	0.005%	1.62	0.33	28 (93.3%)	30 (100%)	0.64	0.23	30 (100%)	30 (100%)		

a. Difference in log₂ Mean MICs, Comparative Condition - CLSI Reference
b. Dilution differences were calculated for each MIC by subtracting the log₂+10 test MIC from the log₂+10 reference MIC and mean dilution differences were determined for each method.
c. Number and percentage of MICs by the 2 methods within ±1 doubling dilution (essential agreement) or ±2 doubling dilutions of each other.
d. For the purpose of this analysis, MICs for 3 strains were 256 mcg/mL, but tested as >128 mcg/mL.
e. For the purpose of this analysis, MICs for 2 strains were 256 mcg/mL, but tested as >128 mcg/mL.
f. MICs for 29 strains were off scale >128 mcg/mL.

Agar Dilution - Effect of Testing Variables (Table 3, Figure 5)

- Of agar dilution variables tested (temperature, incubation time and inoculum concentration), those that differed the most were:

Variable	GSK1322322	Linezolid
•40°C temperature	↓ 2.2 dilutions	↑ 0.9 dilution
•48 hours incubation	↑ 1.4 dilution	↑ 1.7 dilution
•Inoculum 10 ⁶ CFU/spot	↓ 1.9 dilution	↓ 0.8 dilution
•Inoculum 10 ⁷ CFU/spot	↑ 1.1 dilution	↑ 0.8 dilutions
•Inoculum 10 ⁸ CFU/spot	↑ 3.0 dilutions	↑ 2.9 dilutions

Figure 5. Geometric Mean MICs (mcg/mL) of GSK1322322 for 30 *S. aureus* by Agar Dilution at Different Bacterial Inoculum Concentrations for Mueller Hinton and IsoSensitest Agar

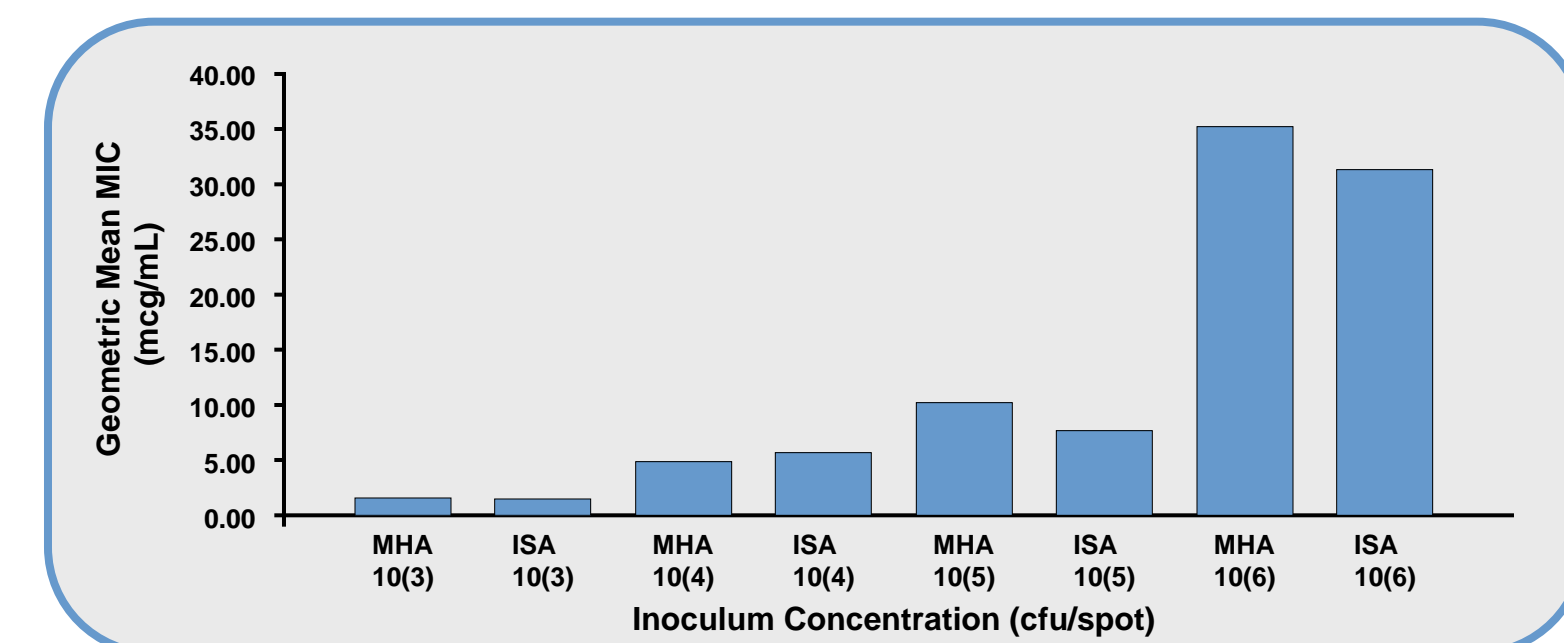


Table 3. *In vitro* Activity of GSK1322322 and Linezolid Against 30 *S. aureus*: Comparison of the CLSI MIC Agar Dilution Reference Condition to Other Testing Conditions

Test (CLSI Reference) Condition	Comparative Condition	GSK1322322					Linezolid				
		Mean difference*	Mean Dilution	N(%) ≥1 dilution*	N(%) ≥2 dilution*	Mean Difference*	Mean Dilution	N(%) ≥1 dilution*	N(%) ≥2 dilution*		
Temperature (35°C)	30°C	-0.77	-0.30	25 (83.3%)	30 (100%)	-0.74	-0.50	26 (86.7%)	29 (96.7%)		
	40°C	-3.20	-2.20	6 (20%)	23 (76.7%)	2.07	0.87	25 (83.3%)	30 (100%)		
Incubation Time (16hrs.)	24 hrs.	0.69	0.27	28 (93.3%)	30 (100%)	0.00	0	26 (86.7%)	30 (100%)		
	48 hrs.	5.57	1.37	20 (66.7%)	26 (86.7%)	5.48	1.67	19 (63.3%)	25 (83.3%)		
Inoculum MHA (10 ⁶ cfu/spot)	10 ⁶ cfu/spot	5.36	1.13	25 (83.3%)	28 (93.3%)	3.76	0.83	25 (83.3%)	30 (100%)		
	10 ⁷ cfu/spot	30.61	2.97	0 (0%)	9 (30%)	31.95	2.93	4 (13.3%)	10 (33.3%)		
	10 ⁸ cfu/spot	-3.34	-1.97	10 (33.3%)	22 (73.3%)	-2.72	-1.20	27 (90%)	28 (93.3%)		
	10 ⁹ cfu/spot	0.91	0.27	30 (100%)	30 (100%)	-2.17	-0.87	28 (93.3%)	28 (93.3%)		
Inoculum ISA (MHA 10 ⁶ cfu/spot)	10 ⁶ cfu/spot	2.97	0.73	25 (83.3%)	29 (96.7%)	0.11	0.03	28 (96.7%)	29 (96.7%)		
	10 ⁸ cfu/spot	26.78	2.80	1 (3.3%)	12 (40%)	50.90	3.53	2 (6.7%)	7 (23.3%)		

a. Difference in log₂ Mean MICs, Comparative Condition - CLSI Reference
b. Dilution differences were calculated for each MIC by subtracting the log₂+10 test MIC from the log₂+10 reference MIC and mean dilution differences were determined for each method.
c. Number and percentage of MICs by the 2 methods within ±1 doubling dilution (essential agreement) or ±2 doubling dilutions of each other.

Quality Control (*Staphylococcus aureus* ATCC 29213)

All GSK1322322 *S. aureus* ATCC 29213 MICs were within a three well range of 2-8 mcg/mL with exception of:

- Higher MICs (32 and >128 mcg/mL) with BMD using inoculum concentration of 10⁶ and 10⁷ cfu/mL and agar dilution using inoculum concentration of 10⁶ cfu/mL
- Lower MICs (0.5 mcg/mL) with BMD incubated at 40°C
- Lower MICs (1 mcg/mL) with BMD using four broths other than CAMHB, BMD with 5 mcg/mL thymidine and with ISA agar dilution using an inoculum concentration of 10⁷ cfu/mL.

Conclusions

- This study has shown that there were a number of variables that impacted GSK1322322 MICs, although most had no or only a minor effect. Those variables that were shown to impact GSK1322322 MICs the most were the use of broth other than CAMHB, incubation temperature of 40°C, longer incubation time, inoculum concentration and addition of serum.
- When performing susceptibility testing with GSK1322322, therefore, it is important to be aware of these differences and control these particular variables if possible according to