F-1597

Analysis of disk testing methods and variables for GSK1322322 and Comparators against *Staphylococcus aureus, Haemophilus influenzae, Streptococcus pneumoniae* and *Streptococcus pyogenes*

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Abstract

Background: GSK1322322 is an agent that represents a new antibiotic class with a novel mode of action (inhibition of peptide deformylase function) and antibacterial activity against multi-drug resistant respiratory and skin pathogens. This study was conducted to determine the effect of various disk testing parameters on *in vitro* activity of GSK1322322 against relevant pathogens. **Methods:** Inhibition zone diameters were determined for 14 *S. pneumoniae,* 30 *S. aureus* 12 *H. influenzae*, 15 *S. pyogenes* and QC organisms by disk diffusion (20 µg GSK1322322, 5 µg levofloxacin and 10 and 30 µg linezolid disks) using 3 different methods: the CLSI method using 3 separate manufacturers: Becton Dickinson (BD), Hardy Diagnostics (HDY), and Oxoid, Ltd. (OX), the European Committee on Antimicrobial Susceptibility Testing (EUCAST) method and the British Society for Antimicrobial Chemotherapy (BSAC) method. The following significant variables were studied with the CLSI method only: temperature, incubation time, atmospheric conditions, inoculum concentration, pH, calcium, magnesium, zinc, potassium, thymidine, and polysorbate 80. **Results:** GSK1322322 and comparator agent zones, on all media against all organisms, were comparable with the exception of BSAC (IsoSensitest agar [ISA] with BSAC inoculum method). For *S. aureus* most variables, with exception of low inoculum, had only a minor effect or no affect at all. Variables that most affected GSK1322322 disk zones are shown in the table. For the most part, GSK1322322 and the comparator agent's disk zones acted similarly with variations against study isolates.

Testing Variable	GSK1322322 20 µg zone sizes compared to reference method zone sizes (average zone difference)			
	S. pneumoniae	*S. aureus*	*H. influenzae*	*S. pyogenes*
Inoculum (10^6 CFU/mL)	↑ 5.8 mm	↑ 7.2 mm	↑ 6.7 mm	↑ 6.1 mm
Inoculum (10^7 CFU/mL)	↑ 3.1 mm	↑ 4.6 mm	↑ 4.3 mm	↑ 3.9 mm
Potassium (+25 & 50 mmol/L)	↓.5 mm	-	↓ 4.9 mm	↓ 6.4 mm
Magnesium (25 µg/mL)	↑ 3.7 mm	-	-	-
Zinc (+2 & 5 mmol/L)	-	-	↑ 5.3 mm	-
pH 5.5	↑ 10.2	↑ 2.1	No growth	↑ 8.8
Thymidine (+1 µg/mL)			-	↑ 3.9 mm
40°C	-		-	↓ 2.7 mm
30°C	-		-	-
14 hours	-		-	↑ 4.5 mm
- Average difference <2 mm				

Conclusions: When performing disk diffusion testing with GSK1322322 it is important to control the inoculum concentration. Incubation temperature and time, thymidine and cation concentration may also affect GSK1322322 disk zones.

Introduction

Disk diffusion susceptibility testing of antimicrobial agents is typically performed according to standardized methods (1-3). 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 disk diffusion zones. This study was performed to determine the effect of various testing parameters on the in vitro activity of antimicrobial agent GSK1322322 and comparative agents, levofloxacin and linezolid, against *Staphylococcus aureus, Haemophilus influenzae, Streptococcus pneumoniae* and *Streptococcus pyogenes* using disk diffusion.

Methods

Antibiotics

Antibiotic	Disk Concentration (µg)	Disk Manufacturer
GSK1322322	20	Mast
Linezolid	30	BD
Linezolid	10	Oxoid
Levofloxacin	5	BD

Microorganisms

30 *Staphylococcus aureus*
12 *Haemophilus influenzae*
14 *Streptococcus pneumoniae*
15 *Streptococcus pyogenes*

Disk Diffusion Methods

The reference methodology used was the standard CLSI antimicrobial disk susceptibility testing method, a summary of which is shown for each bacterial species in the Disk Reference Method Table below (1). This standard method was utilized to compare Mueller Hinton Agar made from 3 different manufacturers, as well as IsoSensitest Agar (Media and Method Comparison Table). EUCAST and BSAC methods were also tested and compared (Media and Method Comparison Table). The variations to the standard CLSI method that were also tested in this study are shown in the Testing Variables Table.

Disk Reference Method

Organism	Media Used	Media Manufacturer	Incubation Time (hours)	Atmospheric Condition	QC Organism (ATCC No.)
S. aureus	Mueller Hinton Agar	BD	16-18	Ambient	29213, 25922
S. pneumoniae	Mueller Hinton Agar + 5% SB	BD	20-24	CO_2	49619
S. pyogens	Mueller Hinton Agar + 5% SB	BD	20-24	CO_2	49619
H. influenzae	Haemophilus Test Medium	BD	16-18	CO_2	49247, 49766, 8648

Media and Method Comparison

Abbreviation	Media Mfr.	*S. aureus*	Streptococcus	*H. influenzae*	Method
BD	BD	MHA	MHA +5% SB	HTM	CLSI (1)
OX	Oxoid	MHA	MHA +5% SB	HTM	CLSI (1)
HDY	Hardy	MHA	MHA +5% SB	HTM	CLSI (1)
BD-EU	BD	MHA	MHA+5% DHB + 20 mg/L NAD	MHA+5% DHB + 20 mg/L NAD	EUCAST (2)
ISA	Oxoid	ISA	ISA+5% DHB	ISA+5% DHB + 20 mg/L NAD	BSAC media with CLSI inoculum (0.5 McFarland)
BSAC	Oxoid	ISA	ISA+5% DHB	ISA+5% DHB + 20 mg/L NAD	BSAC media with BSAC inoculum* (3)
*Inoculum (dilution of 0.5 McFarland)	1:10		Beta Strep: 1:100 *S. pneumoniae*: 1:10	1:100 is the BSAC recommended dilution, however, due to large zones and poor growth inoculum was modified to 1:10.	

BD – Becton Dickinson, OX – Oxoid, HDY – Hardy diagnostics, BD-EU – BD media+EUCAST method, ISA – IsoSensitest Agar, BSAC – IsoSensitest Agar+BSAC method

Testing Variables

Variable Description	Reference Method Condition	Specific Variables Tested
Temperature	35±2°C	30, 35, and 40°C
Incubation time	S. aureus: 16-18 hours Streptococci: 20-24 hours	S. aureus: 14, 16-18, 24, 48 hours Streptococci: 16, 18, 20-24, 48 hours
Atmospheric conditions	Ambient	Ambient, 5% and 10% CO_2
Inoculum	10^8 CFU/mL	10^4, 10^5, 10^6, 10^7, 10^8 CFU/mL
Calcium	20-25 mg/L	25, 50 and 100 mg/L
Magnesium	10-12.5 mg/L (for broth)	12.5, 25 and 50 mg/L
pH	7.2-7.4	5.5, 6.5, 7.2-7.4 and 8.5
Polysorbate 80	none	0.002%
Thymidine	not defined	1 and 5 mg/L added
Zinc	not defined	2, 5 and 10 mmol/L added
Potassium	not defined	12.5, 25 and 50 mmol/L added

Results

Differences in disk diffusion zones for GSK1322322 and comparator agents, as a result of testing variables, were observed for *S. aureus, H. influenzae, S. pneumoniae* and *S. pyogenes*. Those method variations that resulted in less than 90% agreement with the reference method are shown below and in Tables 1-4. QC results are summarized in Tables 5-6.

GSK1322322 20 µg disk zones compared to reference method zones (average zone difference)				
Variable	30 *S. aureus* (mm)	12 *H. influenzae* (mm)	14 *S. pneumoniae* (mm)	15 *S. pyogenes* (mm)
HDY	↑ 1.8	-	-	-
ISA	↑ 2.3	-	-	-
BSAC	↑ 4.0	-	↑ 3.8	↑ 8.3
Ambient	-	-	↑ 1.7	-
pH 5.5	-	No Growth	↑ 10.2	↑ 8.8
pH 8.5	-	↓ 4.3	↑ 2.6	-
40°C	-	-	-	↓ 2.7
30°C	-	↑ 4.7	-	-
Inoculum 10^6 CFU/mL	↑ 7.2	↑ 6.7	↑ 5.8	↑ 6.1
Inoculum 10^7 CFU/mL	↑ 4.6	↑ 4.3	↑ 3.1	↑ 3.9
Inoculum 10^9 CFU/mL	-	↑↓* 1.3	-	↓ 3.1
14 hours Incubation	-	-	-	↑ 4.5
Calcium 100 µg/mL	↓ 2.6	-	-	-
Magnesium 25 µg/mL	-	-	↑ 3.7	↑ 2.1
Magnesium 50 µg/mL	-	-	↑ 1.9	↑ 1.5
Zinc 2 mmol/L	-	↑ 4.3	-	-
Zinc 5 mmol/L	-	↑ 6.1	-	-
Zinc 10 mmol/L	-	↑↓* 2.8	-	-
Potassium 12.5 mmol/L		↑ 4.2	-	↓ 4.9
Potassium 25 mmol/L	-	↑ 4.8	↓ 4.6	↓ 6.4
Potassium 50 mmol/L	-	↑↓* 5.8	↓ 4.4	↓ 6.5
Thymidine 1 µg/mL	-	-	↑ 5.2	↑ 3.9
Thymidine 5 µg/mL	-	-	↑ 3.5	-
Polysorbate 80 0.002%	-	↑ 2.2	-	-

*Varied (isolate specific smaller and larger zones) by enough strains to drop agreement rates below 90%

Table 1. *S. aureus* (n = 30) zone differences (compared to reference result on same day in mm) by variable

(a): GSK1322322 20 µg disk

Variable	≤ -10	-9	-8	-7	-6	-5	-4	-3	-2	-1	0	1	2	3	4	5	6	7	8	9	≥ 10	% ±4mm
Reference[1] Day 1								1		4	10	7	4	4								100.0%
Hardy MHA							1		1	2	6	6	2	4	3	3		2				83.3%
Oxoid ISA								1	1	2	4	3	6	6	1	1	3	1	1			80.0%
BSAC										1	2		6	5	6	1	3	2	3		1	66.7%
Reference[1] Day 3								5	7	6	5	5	1	1								100.0%
100 µg/mL Calcium	2				1	3	3	4		3	5	6	1		2							80.0%
Reference[1] Day 5								1	1	11	10	4	1	2								100.0%
10^6 CFU/mL [2]															1	5	5	11	1	2	4	3.4%
10^7 CFU/mL											2		2	3	9	6	3	1	2	1	1	53.3%

(b): Linezolid 10 µg disk

Variable	≤ -10	-9	-8	-7	-6	-5	-4	-3	-2	-1	0	1	2	3	4	5	6	7	8	9	≥ 10	% ±4mm
Reference[1] Day 1								2	2	7	7	7	4		1							100.0%
Hardy MHA	1							1		5	3	6	8	2	2	2						90.0%
Oxoid ISA										2	6	4	6	4	5		1		1	1		90.0%
BSAC											2	1	8	5	7	5					2	76.7%
Reference[1] Day 3										2	8	10	6	1			1	1				93.1%
100 µg/mL Calcium	1			2	2		2	1	3	5	8	3	1	1							1	80.0%
Reference[1] Day 5								2	3	9	6	4	3	2			1					96.7%
10^6 CFU/mL [2]														2	4	5	7	5	2		4	20.7%
10^7 CFU/mL [2]														3	6	5	5	4	3		3	31.0%

(c): Linezolid 30 µg disk

Variable	≤ -10	-9	-8	-7	-6	-5	-4	-3	-2	-1	0	1	2	3	4	5	6	7	8	9	≥ 10	% ±4mm
Reference[1] Day 1							2	2	5	4	7	8	1	1								100.0%
Hardy MHA								2	2	4	5	5	7	4		1						96.7%
Oxoid ISA										4	3	4	4	6	4	4	1					83.3%
BSAC										1	1	4	5	2	4	7	4	2				56.7%
Reference[1] Day 3									1	2	7	11	4	2	2		1					96.7%
100 µg/mL Calcium				1	3	2	3		3	4	4	4	2	3	1							80.0%
Reference[1] Day 5							1		2	3	8	5	5	3	1	1	1					93.3%
10^6 CFU/mL [3]														3	1	5	6	4	3	3	4	13.8%
10^7 CFU/mL												1	2	3	2	3	7	6	1		5	26.7%

[1]Reference for each day compared to the mean of all reference results (n=days 1-5), [2]One isolate had no growth (n=29)

Table 2. *S. pneumoniae* (n = 14) zone differences (compared to reference result on same day in mm) by variable

(a): GSK1322322 20 µg disk

Variable	≤ -10	-9	-8	-7	-6	-5	-4	-3	-2	-1	0	1	2	3	4	5	6	7	8	5	≥ 10	% ±4mm
Reference[1] Day 1											6	5	3									100.0%
BSAC												3		1	5	3	2					64.3%
Reference[1] Day 2									2		6	5	1									100.0%
Ambient								1	2	2	2	1	2	1			1		1		1	78.6%
Reference[1] Day 3									3	7	2	1	1									100.0%
10^6 CFU/mL													1	2	2	4	1	1	1		2	35.7%
10^7 CFU/mL												1	5	4	2		2					85.7%
Reference[1] Day 4									3	6	4					1						92.9%
5.5 pH [2]				1														1		1	8	0
8.5 pH										1	3	2	4	2		1					1	85.7%
1 µg/mL Thymidine								1					3		2	1	2	1	1		3	42.9%
5 µg/mL Thymidine							1				1	1		3	3	2		3				64.3%
25 µg/mL Magnesium					1					1	1	1		2	3	1			3		1	57.1%
50 µg/mL Magnesium				1						1	2	1	2	3	1	1	2					71.4%
Reference[1] Day 5										1	2	4	5	1	1							100.0%
25 mmol/L Potassium					4	4	3	2	1													42.9%
50 mmol/L Potassium				2		3	7	1	1													64.3%

(b): Linezolid 30 µg disk

Variable	≤ -10	-9	-8	-7	-6	-5	-4	-3	-2	-1	0	1	2	3	4	5	6	7	8	9	≥ 10	% ±4mm
Reference[1] Day 1								1	1	4	4	4										100.0%
BSAC											1	3		3	2	3	2					64.3%
Reference[1] Day 2										1	5	3	5									100.0%
Ambient											7	1	3				1		1	1		78.6%
Reference[1] Day 3										6	4	3	1									100.0%
10^6 CFU/mL															2	1	5	2	2	1	1	14.3%
10^7 CFU/mL												1	5	2	4	1		1				85.7%
Reference[1] Day 4									1	6	4	2	1									100.0%
5.5 pH [2]														1						1	9	9.1%
8.5 pH									1	3	5	2	1		1						1	92.9%
1 µg/mL Thymidine												1	3	1	3	1	2			1	2	57.1%
5 µg/mL Thymidine											1	2	2	5		1	1			1	1	71.4%
25 µg/mL Magnesium									1	3	2		5	1		1					1	85.7%
50 µg/mL Magnesium								1	1		3	2	2	1	3			1				92.9%
Reference[1] Day 5										2	6	3	3									100.0%
12.5 mmol/L Potassium									2	2	2	3	5									100.0%
25 mmol/L Potassium					1	2	3	5	1		1	1										78.6%
50 mmol/L Potassium					2	1	2	1	4	2				1	1							78.6%

[1]Reference for each day compared to the mean of all reference results (n=days 1-5), [2]Three isolates had no growth (n=11)

Table 3. *S. pyogenes* (n = 15) zone differences (compared to reference result on same day in mm) by variable

(a): GSK1322322 20 µg disk

Variable	≤ -10	-9	-8	-7	-6	-5	-4	-3	-2	-1	0	1	2	3	4	5	6	7	8	9	≥ 10	% ±4mm
Reference[1] Day 1									2	7	3	3										100.0%
BSAC																1	2	2	4	2	4	0
Reference[1] Day 3										2	6	4	2	1								100.0%
40°					1	3	1	3	3	2	1	1										73.3%
10^6 CFU/mL													1		1	4	2	4	2	1		13.3%
10^7 CFU/mL												1	3	3	3	1	3	1				66.7%
10^9 CFU/mL						3	5	4	2			1										80.0%
Reference[1] Day 4							1	5	5	4												100.0%
5.5 pH														1		1	3	2	2	2	4	6.7%
1 µg/mL Thymidine												2	2	1	2	6	2					46.7%
25 µg/mL Magnesium											3	3	5	1	1	1	1					86.7%
50 µg/mL Magnesium										1	4	3	4	1		2						86.7%
Reference[1] Day 5										1	4	7	2	1								100.0%
14 hours													1	3	4	3	2	2				53.3%
12.5 mmol/L Potassium			4	2		2		4	2	1												46.7%
25 mmol/L Potassium			3	6	2	2	2															13.3%
50 mmol/L Potassium			3	5	4	2	1															6.7%

(b): Linezolid 30 µg disk

Variable	≤ -10	-9	-8	-7	-6	-5	-4	-3	-2	-1	0	1	2	3	4	5	6	7	8	9	≥ 10	% ±4mm
Reference[1] Day 1									3	7	3	2										100.0%
BSAC																2	2	6	1	1	3	0
BSAC (Linezolid 10 µg)														1	1	4	3	2	3	1		13.3%
Reference[1] Day 3									1		3	5	3	2	1							100.0%
40°					1	2	6	3	2					1								80.0%
10^6 CFU/mL																4	3	7		1		0
10^7 CFU/mL												2	1	2	4	3	1	2				60.0%
10^9 CFU/mL				1		2	3		5	3			1									80.0%
Reference[1] Day 4								3	6	4	2											100.0%
5.5 pH													2		5	1	1	1	2	1	2	46.7%
1 µg/mL Thymidine											1	4	4	1	3	2						86.7%
25 µg/mL Magnesium											4	6	3		1	1						93.3%
50 µg/mL Magnesium										1	3	6	2	1		2						86.7%
Reference[1] Day 5									1	2	6	5	1									100.0%
14 hours											8	3	2	1	1							100.0%
12.5 mmol/L Potassium			1	3	2	1	2		1	5												53.3%
25 mmol/L Potassium			1	3	3	5	2	1														20.0%
50 mmol/L Potassium				2	4	5	3	1														26.7%

[1]Reference for each day compared to the mean of all reference results (n=days 1-5)

Table 4. *H. influenzae* (n = 12) disk zone differences (compared to reference result on same day in mm) by variable

GSK1322322 20 µg disk

Variable	≤ -10	-9	-8	-7	-6	-5	-4	-3	-2	-1	0	1	2	3	4	5	6	7	8	9	≥ 10	% ±4mm
Reference[1] Day 2								1	2	5	1	2		1								100%
0.002% P80										1	2	3	3	1				1	1			83.3%
Reference[1,5] Day 3								6	1	2		1	1									100%
2 mmol/L Zinc					1					1				1	2	2	3			1	1	33.3%
5 mmol/L Zinc [2]											1				3		1	2	1	1	1	40.0%
10 mmol/L Zinc [3]	3												1			1					3	12.5%
12.5 mmol/L Potassium		1								1	1				3	1		2	1	1	1	41.7%
25 mmol/L Potassium			1							1			1	1	2		2			2	2	41.7%
50 mmol/L Potassium		1										1	1			2	2	1	1	1	2	16.7%
10^6 CFU/mL [2]							1									2		1	2	2	2	10.0%
10^7 CFU/mL							2					1		2	1	1		2		1	2	50.0%
10^9 CFU/mL	1			1		1			1					4		1	1		1		1	41.7%
Reference[1] Day 4								1				2	1	4	1	1	1	1				75.0%
30° C										1	1	2	1	3						1	3	66.7%
Reference[1] Day 5							1	1	2	2	3		2	1								100%
5.5 pH [4]																						0
8.5 pH	1		1				3	3		3			1									83.3%

(b): Levofloxacin 5 µg disk

Variable	≤ -10	-9	-8	-7	-6	-5	-4	-3	-2	-1	0	1	2	3	4	5	6	7	8	9	≥ 10	% ±4mm
Reference[1] Day 2									3	3	1	2		2	1							100.0%
0.002% P80						2		1	1	2		2		2	1		1					75.0%
Reference[1] Day 3				1		1	3	3	1		1			1		1						75.0%
2 mmol/L Zinc							1		1				1		4		1	2	1		1	58.3%
5 mmol/L Zinc [2]											1			1	2	1	1	1	3			40.0%
10 mmol/L Zinc [3]	3												1	1		1		1			1	25.0%
12.5 mmol/L Potassium						1					1			2		1	2	2	2	1		25.0%
25 mmol/L Potassium								1		1				1	1	1	2	2	1	2		33.3%
50 mmol/L Potassium									1		1		1	1	2			2		1	3	50.0%
10^6 CFU/mL [2]							1									1	1	1		1	5	10.0%
10^7 CFU/mL								2						1	2	1			2	3	1	41.7%
10^9 CFU/mL	1				2					1			2	2	1		3					50.0%
Reference[1] Day 4										1				5	3	2		1				75.0%
30°C					1					1		1	3	1	2	1		1			1	66.7%
Reference[1] Day 5						1	1	2			1	3	4									91.7%
5.5 pH [4]																						0
8.5 pH						1	1	2		4	2		1		1							91.7%

[1]Reference for each day compared to the mean of all reference results (n=days 1-5, [2]Two isolates had no growth (n=10), [3]Four isolates had no growth (n=8), [4]No growth for all isolates (n=0)

Table 5. Quality control results for GSK1322322 20 µg disk (number of reference method results at each zone; established ranges are shown in blue shading)

QC Strain	Zone (mm)													
	17	18	19	20	21	22	23	24	25	26	27	28	29	30
SA25923				1	1	2		1						
SA29213						2	1		2					
SP49619									2	1	1		1	
HF49247					1	2		1	1					
HF49766	1				1	2		1						
HF8468				1		2	2							

Table 6. Quality control results for levofloxacin 5 µg, linezolid 10 and 30 µg disks (number of reference method results at each zone; established ranges are shown in blue shading)

QC Strain	Zone (mm)																					
	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41
Linezolid 30 µg disk zone (mm)																						
SA25923						1	1	1		1	1											
SA29213								4	1													
SP49619							2		1	2												
Linezolid 10 µg disk zone (mm)																						
SA25923	1		2		1	1																
SA29213			1	2	1																	
Levofloxacin 5 µg disk zone (mm)																						
HF49247														2	1			1	1			
HF49766												1	1	1					1			1
HF8468														3				1	1			

Conclusions

- ***Staphylococcus aureus*** **GSK1322322 and linezolid disk zones were affected by low inoculum concentration and some isolates were affected by higher calcium concentration.**
- ***Streptococcus pneumoniae*** **GSK1322322 and linezolid disk zones were affected by low inoculum concentration, 5.5 pH, ambient incubation, 40°C incubation temperature, and higher thymidine, magnesium (GSK322 only) and potassium concentrations.**
- ***Streptococcus pyogenes*** **GSK1322322 and linezolid disk zones were affected by low and high inoculum concentrations, 5.5 pH, 40°C incubation temperature, higher thymidine, magnesium, and potassium concentrations and 14-hour incubation time (GSK1322322 only).**
- ***Haemophilus influenzae*** **GSK1322322 and levofloxacin disk zones were affected by low and high inoculum concentrations, 5.5 pH (no growth), 30°C and 40°C incubation temperatures and magnesium, zinc, and potassium concentrations.**
- **When performing susceptibility testing with GSK1322322, therefore, it is important to be aware of and control these particular variables according to standardized methods.**

Acknowledgement

This study was funded by GlaxoSmithKline.

References:

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54th Annual Interscience Conference on Antimicrobial Agents and Chemotherapy Meeting, 5-9 September 2014, Washington, D.C., USA