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CAPABILITY OF CEM-101 TO SELECT FOR RESISTANT PNEUMOCOCCAL AND GROUP A STREPTOCOCCAL CLONES BY MULTISTEP RESISTANCE SELECTION

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Revised Abstract

Drug resistant (R) strains of streptococci occur worldwide. CEM-101 (CEM) is a new member of the macrolide-ketolide group which is 2 to 4-fold more active than telithromycin (TEL) against macrolide R pneumococci and is also more potent than TEL against *erm*(B) group A streptococci. We tested the ability of CEM to select for R clones of 8 *S. pneumoniae* and 5 *S. pyogenes* with varying resistotypes, compared to azithromycin (AZI), clarithromycin (CLA), TEL, and clindamycin (CLI). **Methods.** For pneumococci, one strain each was tested: macrolide S, *erm*(B), *mef*(A), *erm*(B) + *mef*(A), *erm*(A), with mutations in 23S rRNA, and L4, L22 ribosomal proteins. For *S. pyogenes*, one strain each was tested as follows: macrolide S, *erm*(B), *mef*(A), *erm*(A), and with L4 ribosomal protein mutations. CLSI macrodilution was used for MIC testing. Serial passages were daily in MHB + 5% lysed horse blood for each strain at subinhibitory drug concentrations, taking for each subsequent passage an inoculum from the tube 1-2 dilutions < MIC that matched turbidity of a growth control. Daily passages were continued until the MIC increased >4-fold (min. 14, unless MICs ≥32 were obtained, max. 50 passages). R clones were subcultured 10x in drug-free medium to test stability of selected R. Identity between parents and R clones was confirmed by PFGE and macrolide R determinates tested by PCR and sequencing. **Results.** For pneumococci, parental MICs (µg/ml) were: CEM, 0.004-1; AZI, 0.03-8; CLA, 0.016-16; TEL, 0.004-0.5; CLI, 0.016-1. Four strains with AZI, 2 CLA, 2 CLI MICs ≥64 µg/ml were not tested. CEM MICs increased after 14-43 days in all 8 strains tested. For 7 strains, MICs rose from 0.004-0.03 µg/ml (parents) → 0.06-0.5 µg/ml (R clones) in 14-43 days. For the eighth strain, containing *erm*(B) + *mef*(A), MICs rose from 1 µg/ml (parent) → 32 µg/ml (R clone) in 18 days. AZI had R clones after 14-29 days in 3/4 strains with MICs rising from 0.03-2 µg/ml (parents) → 0.5->64 µg/ml (R clones). CLA had R clones after 14-49 days in 5/6 strains with MICs rising from 0.03-16 µg/ml (parents) → 16->64 µg/ml (R clones). TEL had stable R clones after 14-38 days in 5 clones of 8 tested with MIC rising from 0.004-0.5 µg/ml (parents) to 0.06->64 µg/ml (R clones). CLI had R clones after 14-43 days in 2/5 strains with MICs rising from 0.03-0.06 µg/ml (parents) → 0.25->64 µg/ml (R clones). For *S. pyogenes*, parental MICs (µg/ml) were: CEM, 0.008-1; AZI, 0.06-4; CLA, 0.03-4; TEL, 0.008-8; CLI, 0.06. (1 strain with AZI, CLA, CLI MICs >64 µg/ml was not tested). CEM MICs increased after 18-43 days in 3/5 strains, rising from 0.03-1 µg/ml (parents) → 0.25-8 µg/ml (R clones). MICs for 2 of the clones did not go above 0.25 µg/ml when passages were continued for the maximum 50 days. AZI had R clones after 5-35 days in 3/4 strains tested, with MICs rising from 0.06-4 µg/ml (parents) → 1->64 µg/ml (R clones). CLA had R clones after 6 days in 1/4 strains tested, with MICs rising from 0.5 µg/ml (parent) → >64 µg/ml (R clone). TEL had R clones after 6-22 days in 2/5 strains tested, with MICs rising from 0.03-8 µg/ml (parents) → 0.25->64 µg/ml (R clones). CLI had R clones after 34-43 days in 2/4 strains tested with MICs rising from 0.06 µg/ml (parents) → 0.5->64 µg/ml (R clones). **Conclusions**. CEM yielded clones with higher MICs in all 8 pneumococcal strains, but 7 of 8 strains had clones with CEM MICs ≤0.5 µg/ml and in only 1 *erm*(B) + *mef*(A) strain with a parental MIC=1 µg/ml was a R clone found with an MIC=32 µg/ml and no changes in the ribosomal proteins (L4 or L22) or 23S rRNA. In 2 of the 3 R *S. pyogenes* clones with CEM [parents *erm*(A), L4], MICs were 0.25 µg/ml and only in the 1 strain with *erm*(B) did CEM MICs rise from 1-8 µg/ml with no changes in all macrolide R determinates tested.

Background

Strains of *Streptococcus pneumoniae* resistant to macrolides, ß-lactams, quinolones, and other agents occur worldwide. Macrolide resistance, which is now predominant in some countries such as Japan and Korea, is most likely due to overuse of azithromycin and clarithromycin during the past 15 years. Macrolide resistance also usually occurs (although genetically unlinked) together with penicillin G resistance (2, 3, 5, 6). Although all strains of group A streptococci are still ß-lactam susceptible, macrolide resistance does occur, especially in southern, central and eastern Europe and in Asia (11, 14).

Although the pediatric conjugate vaccine has dramatically decreased meningitis and bacteremia caused by most of the usual drug-resistant pneumococcal clones, recent papers have described the spread of pan-resistant pneumococcal strains with a serotype (19A) not included in the vaccine that causes otitis media not amenable to treatment with any currently FDA-approved antibiotic (15). Thus, the problem of drug-resistant pneumococci causing community-acquired respiratory infection, especially in children, is likely to worsen with the spread of this clone.

Introduction of telithromycin into the therapeutic armamentarium was, with the exception of *erm*(B) group A streptococci (which are naturally telithromycin resistant), intended to solve the problem of macrolide resistance in streptococci (5, 14). However, toxicities have limited the clinical utility of this drug. Additionally, when the free AUC/MIC of telithromycin against macrolide-resistant pneumococci even with low MICs was examined carefully it could be seen that the number was not significantly above 25: thus, resistance was predicted to occur and this has indeed been the case as evidenced by recent publications (16).

CEM-101 (Fig. 1) is an experimental macrolide/ketolide that is 2-4 fold more active than telithromycin (1, 7-10, 12, 13). In the current study we have performed multistep resistance studies to examine the capability of CEM-101 to select for resistant mutants of pneumococci and group A streptococci compared to telithromycin, azithromycin, clarithromycin, and clindamycin.

Materials and Methods

Bacteria and Antimicrobials. For resistance selection testing, one each of the following pneumococcal resistance phenotypes were tested: macrolide-susceptible, *erm*(B), *mef*(A), *erm*(B) + *mef*(A), *erm*(A), with mutations in L4 and L22 ribosomal protein, and 23S rRNA. Five strains of group A streptococci were tested with one each macrolide-susceptible, *erm*(B), *mef*(A), *erm*(A) and L4 ribosomal protein mutation. CEM-101 was obtained from Cempra Pharmaceuticals and other drugs from either their respective manufacturers or Sigma Chemical, Inc.

MIC Determination. CLSI macrodilution (4) was used for MIC testing.

Multistep Resistance Selection. Serial passages were performed daily for each strain in subinhibitory concentrations of all antimicrobials. In all cases, broth medium was 1 ml per tube of cation-adjusted Mueller-Hinton broth (BD Diagnostics, Sparks, MD) + 5% lysed horse blood. For each subsequent daily passage, an inoculum (10 µl) was taken from the tube one to two dilutions below the MIC that matched the turbidity of a growth control tube. The above inoculum was used to determine the next MIC. Daily passages were performed until a significant increase in MIC (≥8 times) was obtained. A minimum number of 14 passages was performed unless MICs ≥32µg/ml were obtained.

Materials and Methods (cont.)

The maximum number of passages was 50. Stability of the acquired resistance was determined by MIC determinations after 10 daily passages of the mutants on blood agar without antibiotics.

MICs of each resistant pneumococcal clone to each compound were determined by macrodilution MIC. Identity of the obtained mutants and their respective parents was confirmed by pulsed-field gel electrophoresis (PFGE) at the end of the study. PFGE of *Sma*I digested DNA was performed using a CHEF DR III apparatus (Bio-Rad, Hercules, CA, USA) with the following run parameters: switch time of 5 to 20 s and a run time of 16 h (5, 11). Selected resistant CEM-101 clones were examined for changes in resistance determinants, as described below.

Mechanism of Macrolide Resistance. Presence of *erm*(B), *mef*(A), *erm*(A) were detected by PCR amplification using primers and conditions described previously (2, 5, 11). Mutations in II and V domain of 23S rRNA, L4 and L22 ribosomal proteins were determined using sequencing analysis as described previously (2, 5, 11).

Results

Results of pneumococcal multistep resistance selection studies are presented in Table 1. As can be seen, for pneumococci, parental MICs (µg/ml) were: CEM-101, 0.004-1; azithromycin, 0.03-8; clarithromycin, 0.016-16; telithromycin, 0.004-0.5; clindamycin, 0.016-1. Four strains with azithromycin, two with clarithromycin, and two with clindamycin MICs ≥64 µg/ml were not tested. CEM-101 MICs increased after 14-43 days in all 8 strains tested. For 7 strains, MICs rose from 0.004-0.03 µg/ml (parents) → 0.06-0.5 µg/ml (resistant clones) in 14-43 days. For the eighth strain, containing *erm*(B) + *mef*(A), MICs rose from 1 µg/ml (parent) → 32 µg/ml (resistant clone) in 18 days. This CEM-101 resistant clone was subjected to sequencing analysis, which revealed no alterations in L4, L22 proteins and II and V domain of 23S rRNA compared to parental sequences. Azithromycin had resistant clones after 14-29 days in 3/4 strains with MICs rising from 0.03-2 µg/ml (parents) → 0.5->64 µg/ml (resistant clones). Clarithromycin had resistant clones after 14-49 days in 5/6 strains with MICs rising from 0.03-16 µg/ml (parents) → 16->64 µg/ml (resistant clones). Telithromycin had stable resistant clones after 14-38 days in 5 clones of 8 tested with MIC rising from 0.004-0.5 µg/ml (parents) to 0.06->64 µg/ml (resistant clones). Clindamycin had resistant clones after 14-43 days in 2/5 strains with MICs rising from 0.03-0.06 µg/ml (parents) → 0.25->64 µg/ml (resistant clones).

For *S. pyogenes* (Table 1), parental MICs (µg/ml) were: CEM-101, 0.008-1; azithromycin, 0.06-4; clarithromycin, 0.03-4; telithromycin, 0.008-8; clindamycin 0.06. One strain with azithromycin, clarithromycin and clindamycin MICs >64 µg/ml was not tested. CEM-101 MICs increased after 18-43 days in 3/5 strains, rising from 0.03-1 µg/ml (parents) → 0.25-8 µg/ml (resistant clones). The resistant clone with a CEM-101 MIC of 8 µg/ml was subjected to sequencing analysis, which showed no changes in all genes (L4, L22 and II and V domain of 23S rRNA) tested. MICs for 2 of the clones did not go above 0.25 µg/ml when passages were continued for the maximum 50 days. Azithromycin had resistant clones after 5-35 days in 3/4 strains tested, with MICs rising from 0.06-4 µg/ml (parents) → 1->64 µg/ml (resistant clones). Clarithromycin had resistant clones after 6 days in 1/4 strains tested, with MICs rising from 0.5 µg/ml (parent) → >64 µg/ml (resistant clone). Telithromycin had resistant clones after 6-22 days in 2/5 strains tested, with MICs rising from 0.03-8 µg/ml (parents) → 0.25->64 µg/ml (resistant clones). Clindamycin had resistant clones after 34-43 days in 2/4 strains tested with MICs rising from 0.06 µg/ml (parents) → 0.5->64 µg/ml (resistant clones).

Table 1. *Streptococcus pneumoniae* and *Streptococcus pyogenes* Multistep Selection Results

Strain	Phenotype [R-determ.]	Antibiotic	Initial MIC (ug/ml)	Selected Resistance		Retest MIC after 10 Antibiotic-free Subcultures				
				MIC	# Pass.	CEM-101	Azith	Clarith	Telithro	Clinda
S.pn	Macrolide-S	**CEM-101**	0.008	0.06	43	0.06	0.03	**0.06***	0.06	0.03
1077		**Azithromycin**	0.03	>64	29	0.03	>64	**>64**	**0.125**	**4**
		Clarithromycin	0.016	0.008	50					
		Telithromycin	0.004	0.25	15	**0.06**	**>64**	**>64**	0.25	**8**
		Clindamycin	0.016	4	49	**0.06**	**>64**	**>64**	**0.06**	8
S.pn	Macrolide-R	**CEM-101**	0.004	0.06	14	0.06	>64	>64	0.125	>64
24	[*erm*(B)]	**Azithromycin**	>64	NT	NT					
		Clarithromycin	>64	NT	NT					
		Telithromycin	0.25	32	14	**0.06**	>64	>64	16	>64
		Clindamycin	>64	NT	NT					
S.pn	Macrolide-R	**CEM-101**	0.03	0.5	14	0.5	16	4	0.25	0.03
3665	[*mef*(A)]	**Azithromycin**	8	16	50					
		Clarithromycin	2	16	26	0.06	8	8	0.06	0.03
		Telithromycin	0.125	2	14	0.03	4	2	0.25	0.03
		Clindamycin	0.016	0.03	50					
S.pn	Macrolide-R	**CEM-101**	1	32	18	32	>64	>64	**32**	>64
1076	[*erm*(B)&*mef*(A)]	**Azithromycin**	>64	NT	NT					
		Clarithromycin	>64	NT	NT					
		Telithromycin	0.5	>64	14	2	>64	>64	>64	>64
		Clindamycin	64	NT	NT					
S.pn	Macrolide-R	**CEM-101**	0.008	0.06	32	0.125	4	1	**0.03**	0.03
1635	[*erm*(A)]	**Azithromycin**	2	>64	14	0.004	>64	**>64**	0.004	0.03
		Clarithromycin	0.5	>64	49	0.008	**>64**	>64	0.016	0.25
		Telithromycin	0.004	0.008	50					
		Clindamycin	0.06	>64	14	0.004	4	0.5	0.008	>64
S.pn	Macrolide-R	**CEM-101**	0.03	0.5	22	1	>64	32	0.25	0.03
2686	[L4 mutation]	**Azithromycin**	>64	NT	NT					
		Clarithromycin	8	>64	14	0.03	>64	>64	0.06	0.03
		Telithromycin	0.06	0.5	25	0.016	>64	16	0.5	0.03
		Clindamycin	0.03	0.125	50					
S.pn	Macrolide-S	**CEM-101**	0.008	0.125	16	0.06	0.06	0.125	**0.06**	0.03
7127	[S20N in L4,	**Azithromycin**	0.06	0.5	29	0.016	1	**0.5**	0.016	0.06
	A105V in L22]	**Clarithromycin**	0.03	16	15	0.008	**>64**	16	0.008	**1**
		Telithromycin	0.008	0.06	38	0.008	0.06	0.06	0.03	0.03
		Clindamycin	0.03	0.25	43	0.004	0.03	0.016	0.008	0.25
S.pn	Macrolide-R	**CEM-101**	0.016	0.25	20	0.25	>64	**>64**	0.06	1
3009	[23SrRNA	**Azithromycin**	>64	NT	NT					
	mutation]	**Clarithromycin**	16	>64	25	0.03	>64	64	0.06	1
		Telithromycin	0.016	0.03	50					
		Clindamycin	1	2	50					
S.py	Macrolide-S	**CEM-101**	0.008	0.016	50					
2132		**Azithromycin**	0.06	1	28	0.016	1	**0.25**	0.03	0.03
		Clarithromycin	0.03	0.016	50					
		Telithromycin	0.008	0.03	50					
		Clindamycin	0.06	0.06	50					
S.py	Macrolide-R	**CEM-101**	1	8	18	8	>64	>64	**>64**	>64
2368	[*erm*(B)]	**Azithromycin**	>64	NT	NT					
		Clarithromycin	>64	NT	NT					
		Telithromycin	8	>64	6	0.5	>64	>64	>64	>64
		Clindamycin	>64	NT	NT					
S.py	Macrolide-R	**CEM-101**	0.03	0.25	43	0.25	4	**8**	**0.5**	0.06
2094	[*erm*(A)]	**Azithromycin**	4	>64	5	0.016	>64	1	0.03	0.06
		Clarithromycin	0.5	>64	6	0.016	**>64**	>64	0.03	0.06
		Telithromycin	0.03	0.25	22	0.03	**>64**	**8**	0.125	**>64**
		Clindamycin	0.06	>64	34	0.03	16	1	0.03	>64
S.py	Macrolide-R	**CEM-101**	0.125	0.125	50					
2011	[*mef*(A)]	**Azithromycin**	4	32	35	0.06	16	4	0.25	0.06
		Clarithromycin	4	8	50					
		Telithromycin	0.5	1	50					
		Clindamycin	0.06	0.06	50					
S.py	Macrolide-R	**CEM-101**	0.03	0.25	20	0.5	4	1	**1**	0.03
237	[L4 mutation]	**Azithromycin**	4	8	50					
		Clarithromycin	0.25	1	50					
		Telithromycin	0.06	0.125	50					
		Clindamycin	0.06	0.5	43	0.03	8	0.5	0.06	1

*Cross-reactivity denoted in bold

Conclusions

- CEM-101 yielded MICs which were usually a few dilutions lower than those of telithromycin, especially against *erm*(B), confirming previous findings (1,7-10,13).
- CEM-101 yielded clones with higher MICs in all 8 pneumococcal strains, but 7 of 8 strains had clones with CEM-101 MICs ≤0.5 µg/ml and in only 1 *erm*(B) + *mef*(A) strain with a parental MIC=1 µg/ml was a resistant clone found with an MIC=32 µg/ml and no changes in all macrolide resistance determinants.
- In 2 of the 3 R *S. pyogenes* clones selected with CEM-101 [parents with *erm*(A) and L4 mutation], MICs were 0.25 µg/ml and only in one strain with *erm*(B) did CEM-101 MICs rise from 1-8 µg/ml with no changes in the macrolide resistance determinants.
- The potent activity and low proclivity to select for resistant mutants of CEM-101 against all streptococcal strains tested irrespective of resistance phenotype points to a promising clinical future of this compound, subject to pharmacokinetic/pharmacodynamic, toxicity, and animal infection model studies.

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