Poster F1-1869

In Vitro Activity of Nine Developmental Cationic Steroid Compounds (Ceragenins) against Clinical Isolates of *Clostridium difficile*

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AMMENDED ABSTRACT

Background: Increasing rates and severity of *C. difficile* associated disease (CDAD) in the USA are associated with an outbreak strain (NAP1) expressing increased virulence and resistance. We examined activity of nine developmental cationic steroid agents (CSAs or ceragenins; small molecule animonsterols mimicking endogenous antimicrobial peptides) against clinical isolates of *C. difficile*, including NAP1.

Methods: MIC values were determined for nine CSAs and metronidazole (control agent) using a reference (NCCLS M11-A6) agar dilution method against 30 *C. difficile* isolates submitted as part of international surveillance programs, including one NAP1 isolate. Quality control (QC) strains included *C. difficile* (ATCC 70057), *E. lentum* (ATCC 3055), *B. fragilis* (ATCC 25285), and *B. thetaiotaomicron* (ATCC 29741).

Results: All QC results were within specified CLSI ranges. Metronidazole was the most potent agent tested (MIC 50/90 results 0.5/1 µg/ml, range 0.25 to 1 µg/ml). Ceragenins with the greatest potency included CSA-13, CSA-22 and CSA-46 (MIC 50/90 results 8 µg/ml). MIC 50/90 results for other CSAs varied from 16 to 256 / 16 to >256 µg/ml, respectively.

Conclusions: CSA-13, CSA-22 and CSA-46 displayed consistent potent activity against the challenge isolates of *C. difficile*, including the virulent NAP1 strain (no difference). Based upon these results, development of CSAs as potential therapy for CDAD appears promising and warrants further consideration pending favorable toxicologic studies, documentation of drug availability at the intended anatomic site (gastrointestinal tract), and favorable pharmacokinetic/pharmacodynamic parameters.

INTRODUCTION

Clostridium difficile-associated diseases (e.g. antimicrobic-associated diarrhea and pseudomembranous colitis) are usually nosocomial in origin and result in excessive morbidity and mortality among hospitalized patients. There has been a significant increase in the incidence and severity of *C. difficile*-associated diarrhea (CDAD) in the past several years.[1] Current guidelines recommend treatment with metronidazole.[2] Recently, a new, highly virulent strain of *C. difficile* has appeared that is less responsive to standard therapy and is associated with a high rate of recurrence.[3]

Ceragenins represent a new class of antibiotics that mimic the bactericidal activities of endogenous antimicrobial peptides (e.g., cathelicidins, cecropins, magainins, etc.).[4] Unlike peptide antibiotics, ceragenins are relatively simple to prepare and purify on a large scale.[5] Like many endogenous antimicrobial peptides, ceragenin activity is believed to follow the 'carpet' model, wherein the compounds associate with charged molecules on the bacterial membrane, forming high local concentrations. The antimicrobial aggregate eventually results in a depolarization of the membrane and the bacterial cell is compromised. Because ceragenins mimic the behavior of endogenous antimicrobial peptides, they are not expected to readily engender resistance.

This study evaluated the *in vitro* efficacy of nine different cationic steroid compounds (ceragenins) against 30 clinical isolates of *C. difficile*, including the highly virulent strain, NAP1.

Figure 1. Structures of the CSAs test.

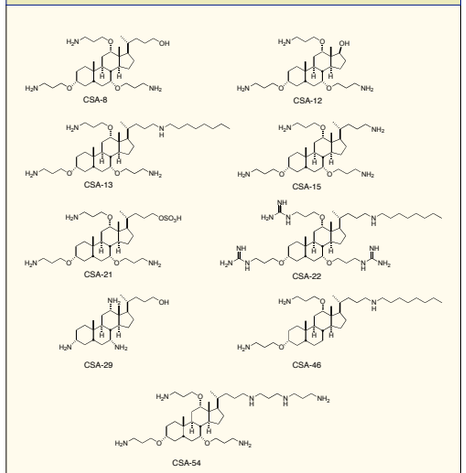

Table 1. Susceptibilities of *C. difficile* isolates to ceragenins (values in µg/ml)

Antimicrobial Agent	MIC (µg/mL) 50%	90%	Range
Metronidazole (control agent)	0.5	1	0.25-1
CSA-8	64	64	32-128
CSA-12	>32	>32	16->32
CSA-13	8	8	1-8
CSA-15	32	32	1-64
CSA-21	16	16	≤0.12-64
CSA-22	8	8	2-16
CSA-29	256	>256	64->256
CSA-46	8	8	1-8
CSA-54	64	128	≤0.12-128

Table 2. MICs of CSAs against various *C. difficile* isolates (values in µg/ml)

Isolate	CSA-8	CSA-12	CSA-13	CSA-15	CSA-21	CSA-22	CSA-29	CSA-46	CSA-54	Metronidazole
ATCC 29741	128	>32	32	128	64	32	>256	8	256	2
ATCC 25285	128	>32	64	256	128	64	>256	16	>256	1
ATCC 43055	64	>32	8	64	128	8	>256	4	256	1
ATCC 70057	64	>32	16	32	8	4	256	2	128	1
1479	64	>32	8	16	8	8	>256	8	32	0.5
1482	64	32	8	16	≤0.125	8	256	8	128	0.5
1484	64	>32	8	64	16	8	>256	8	128	1
1485	64	>32	8	32	16	8	256	8	128	0.5
1491	64	>32	8	32	16	16	>256	8	128	1
1493	64	32	1	2	4	2	128	2	2	0.25
1499	128	16	4	1	16	2	>256	8	≤0.125	0.5
1500	64	16	8	32	≤0.125	2	256	8	4	0.25
1501	64	16	8	1	≤0.125	2	64	2	4	0.5
1502	32	16	8	1	≤0.125	2	64	1	2	0.5
1505	32	>32	8	16	16	4	256	4	32	1
1508	32	>32	8	4	16	4	128	2	0.25	0.25
1509	32	>32	4	32	4	4	>256	8	64	0.5
1510	32	>32	8	32	16	8	>256	8	128	1
1511	64	>32	8	16	16	4	128	4	64	1
1512	64	32	8	32	≤0.125	8	256	8	128	0.5
1513	64	>32	8	32	16	8	>256	8	128	0.5
1514	64	>32	8	32	16	8	>256	8	128	0.5
1515	64	>32	8	32	16	8	128	4	64	1
1516	64	>32	8	32	16	8	>256	8	128	0.5
1517	64	>32	8	32	16	8	256	8	128	0.5
1518	64	>32	4	16	16	8	>256	8	32	0.5
1521	32	32	8	32	4	8	>256	8	32	0.5
1523	64	>32	8	32	32	16	>256	8	64	0.5
1528	64	>32	8	32	16	8	>256	8	64	0.5
1530	64	>32	8	32	16	8	>256	8	2	0.5
1532	64	>32	4	32	0.25	2	128	1	2	1
1533	32	32	4	32	0.25	4	256	4	2	0.5
1535	64	>32	8	32	16	8	128	8	128	0.5
1444	64	>32	8	32	64	8	>256	8	64	1

MATERIALS AND METHODS

Bacterial Strains: Strains were clinical isolates selected from the JMI Laboratories' collection. 30 *C. difficile* isolates were selected from international surveillance programs, including one toxigenic strain representing the clonal complex currently being described from numerous USA medical centers (strain #1444, isolated in Akron, OH; NAP1). The following CLSI-recommended strains were used for quality control (QC): *C. difficile* (ATCC 70057), *E. lentum* (ATCC 43055), *B. fragilis* (ATCC 25285), and *B. thetaiotaomicron* (ATCC 29741). **Compound Preparation:** CSA compounds were obtained from the Savage Laboratory (Brigham Young University). **MIC Determination:** MICs were determined by agar dilution methodology according to NCCLS guidelines.[6] All bacterial strains (including QC strains) were challenged with varying concentrations of ceragenins and metronidazole and incubated at 37ºC for 24 hours. Samples were read visually to determine the MIC after 24 hours exposure. MICs were taken to be the lowest concentration (µg/mL) at which noticeable growth was inhibited.

RESULTS

Ceragenins share a rigid sterol backbone; variations among the compounds are due to differing side chain attachments. Of the 9 CSAs tested, 3 compounds (CSA-13, CSA-22 and CSA-46) displayed consistent potent activity against the challenge isolates of *C. difficile* (see **Tables 1** and **2**), including the virulent NAP1 strain (no difference—MIC 50/90 of 8/8 for each compound for all strains tested). These compounds have in common a lipid chain extending from the steroid nucleus. While CSA-21 and CSA-54 were generally ineffective against most of the strains (MIC 50/90 of 16/16 and 64/128, respectively), both compounds did have MIC values against certain strains of 0.12 µg/ml, even lower than the lowest reported value for metronidazole (0.25 µg/ml). These compounds have higher charge density than many of the other ceragenins.

Ceragenins are cationic amphiphilic compounds that are electrostatically attracted to anionic phospholipids (especially lipid A) found on the surface of bacterial membranes. These compounds are thought to exert their bactericidal action by creating pores in bacterial membranes that lead to rapid ion efflux and cell death.[7,8] This wide variation in activity of the less-potent ceragenins (CSA-21 and CSA-54) suggests a corresponding variation in the lipid membrane structure of *C. difficile* strains and the possibility that structure-activity analysis might lead to modification of CSA-21 and CSA-54 to impart broad spectrum activity as displayed by CSA-13, CSA-22 and CSA-46. CSA-8, which has been shown to have good activity against *S. aureus* strains, including MRSA strains, is not active against any of the tested isolates (lowest MIC of 32 µg/ml and MIC 50/90 of 64/64). Metronidazole was the most potent agent tested, yielding MICs below 1 µg/mL for all strains tested.

CONCLUSIONS

CSA-13, CSA-22 and CSA-46 displayed consistent potent activity against the challenge isolates of *C. difficile*, including the virulent NAP1 strain (no difference). Based upon these results, development of ceragenins as potential therapy for CDAD appears promising and warrants further consideration pending favorable toxicologic studies, documentation of drug availability at the intended anatomic site (gastrointestinal tract), and favorable pharmacokinetic/pharmacodynamic parameters—especially target attainment. CSA-21 and CSA-54 showed a wide variation in activity against *C. difficile* isolates. This may be due to a variation in the lipid membrane structure of different strains. Structure-activity analysis should reveal whether there are significant differences in the lipid moieties of the different strains that would account for the variation in susceptibility to these two ceragenins.

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