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Simocyclinone D8 turns on against Gram-negative bacteria in a clinical setting

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ABSTRACT

Simocyclinone D8 (SD8) is known to affect Gram-positive bacteria only. By testing SD8 against several clinical isolates, we showed that SD8 resulted very active against Gram-negative bacteria from clinical specimens, while it was shown inactive against laboratory strains. The activity against the former was in part due to enhanced drug entry. In addition, SD8 appears to share chromosome- and plasmid-mediated resistance mechanisms with fluoroquinolones.

Simocyclinone D8 (SD8) belongs to a new class of angucyclinone antibiotics isolated from *Streptomyces antibioticus* Tü6040.[1,2] SD8 contains a halogenated aminocoumarin moiety, typical of the coumarin drugs, and an angucyclic polyketide core, a deoxyhexose, linked by a tetraene chain (Fig. 1).

SD8 was reported to be active only against Gram-positive bacteria, such as *Staphylococcus aureus*, *Bacillus brevis*, *Bacillus subtilis*, and *Streptomyces viridochromogenes*, while no activity has been ever shown against Gram-negative species, such as *Escherichia coli*, *Proteus mirabilis*, and *Pseudomonas fluorescens*.[2] However, SD8 acts as a potent inhibitor of DNA Gyrase from both Gram-positive and -negative bacteria (i.e., *E. coli* and *S. aureus*).[3] This apparent contradiction was explained by Oppegard et al. by the presence of the AcrB multidrug efflux pump which actively pumps off SD8 from *E. coli* cells.

However, all SD8 tests on *E. coli* were made using laboratory strains (e.g., MG1655[3]) derived from the *E. coli* K12 strain, from which almost all current laboratory strains derive.[4] A recent intriguing (and provocative) Letter[5] demonstrates that considering *E. coli* K12 as a model for *E. coli* bacteria is dramatically misleading. In fact, the advent of genomics has proven that genomes of singular strains are extremely different: for instance, the first two *E. coli* genomes completely sequenced (i.e., *E. coli* K12 MG 1655 and the enterohemorrhagic *E. coli* O157:H7) differed by an entire megabase DNA.[6] Moreover, the present version of *E. coli* K12 is likely to differ substantially from the original one having undergone not only repeated subculture and storage, but also deliberate mutagenesis and selection.[4]

Considering the reported drawbacks of laboratory *E. coli* strains,[5] we measured SD8 activity against two Gram-negative (i.e., *E. coli* and *Klebsiella pneumoniae*) and a Gram-positive species (*S. aureus*) from clinical isolates. Results were reported both as residual bacterial survival at 50 μg/mL SD8 concentration and as MIC_{50}.

As shown in Table 1, we found that some *E. coli* strains were highly (MIC_{50} = 3.12 and 0.78 mg/L for #24 and #41, respectively) or fairly (MIC_{50} = 25–50 mg/L, #47, #50, and #191) susceptible to SD8 treatment, while others were resistant (MIC_{50} >100 mg/L, #9, #85, #115, #95, #32, #21, #13, #7). Similarly, some *K. pneumoniae* strains were moderately susceptible to SD8 (MIC_{50} = 50–100 mg/L, #134, #92, #173, #97), while others were resistant (MIC_{50} >100, #5, #110, #172, #204, #16, #328, #370, #91). A Gram-positive clinical isolate of *S. aureus* and common K12-derived *E. coli* strains (TOP10, HB101, JM109, JM110, and XL-1 blue) were used as controls. Indeed, *S. aureus* was affected by SD8 treatment (MIC_{50} = 50 mg/L), though to a lower extent than the most susceptible *E. coli* clinical isolates. In contrast, all *E. coli* laboratory strains were confirmed to be totally resistant to SD8 treatment (MIC_{50} >100 mg/L) (Table 1).

Since SD8 was reported to inhibit bacterial topoisomerase II,[3] we analyzed the presence of mechanism(s) that could impair Gyrase/Topoisomerase IV activity in the tested bacterial strains.

Three *E. coli* isolates with MIC_{90} of ciprofloxacin (MIC_{CFX}) = 0.5–1 mg/L presented a Ser-83→Leu point mutation in the GyrA subunit (#85, #115, #95) and one sample with MIC_{CFX} >4 contained Ser-83→Leu and Asp-87→Asn mutations in GyrA and Ser-80→Ile in ParC (#9). In *K. pneumoniae*, six samples with MIC_{CFX} ranging between <0.25 and 4 mg/L values presented the Asp-87→Gly mutation in GyrA (#5, #110, #172, #16, #328, #370). Topoisomerase

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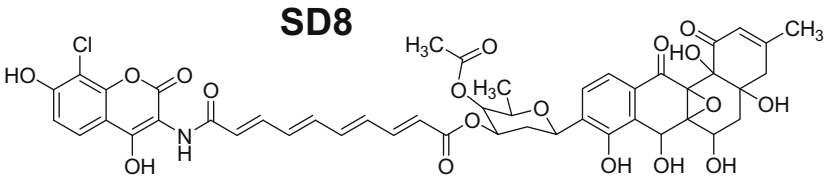

Figure 1. Chemical structure of simocyclinone D8.

Table 1
SD8 susceptibility and fluoroquinolone resistance mechanisms of the tested bacterial strains

Sample ID	Bacterial species	% Survival (SD8, 50 mg/L)	MIC_{50} (SD8, mg/L)	MIC_{90} (CFX,[a] mg/L)	GyrA mutations	ParC mutations	*qnr* Gene
5	*K. pneumoniae*	>90	>100	<0.25	D87G	—	B19
97	*K. pneumoniae*	68 ± 3	50	<0.25	—	—	B19
110	*K. pneumoniae*	>90	>100	<0.25	D87G	—	B19
172	*K. pneumoniae*	>90	>100	<0.25	D87G	—	B19
204	*K. pneumoniae*	>90	>100	0.75	—	—	B19
16	*K. pneumoniae*	>90	>100	2	D87G	—	B19
328	*K. pneumoniae*	>90	>100	>4	D87G	—	B19
370	*K. pneumoniae*	>90	>100	>4	D87G	—	B19
92	*K. pneumoniae*	77 ± 5	50	<0.25	—	—	—
134	*K. pneumoniae*	51 ± 4	25	<0.25	—	—	—
91	*K. pneumoniae*	>90	>100	>4	nd[b]	nd	—
173	*K. pneumoniae*	83 ± 7	100	>4	nd	nd	—
47	*E. coli*	44 ± 5	12	<0.25	—	—	B19
50	*E. coli*	41 ± 2	12	<0.25	—	—	B19
191	*E. coli*	56 ± 3	25	1	—	—	S1
9	*E. coli*	>90	>100	>4	S83L D87N	S80I	B19
24	*E. coli*	21 ± 3	3.12	<0.25	—	—	—
41	*E. coli*	9 ± 2	0.78	<0.25	—	—	—
85	*E. coli*	>90	>100	0.5	S83L	—	—
115	*E. coli*	>90	>100	0.5	S83L	—	—
95	*E. coli*	>90	>100	1	S83L	—	—
32	*E. coli*	>90	>100	2	nd	nd	—
21	*E. coli*	>90	>100	4	nd	nd	—
13	*E. coli*	>90	>100	>4	nd	nd	—
7	*E. coli*	>90	>100	>4	nd	nd	—
Top10	*E. coli* lab	>90	>100	<0.25	nd	nd	—
HB101	*E. coli* lab	>90	>100	<0.25	nd	nd	—
JM109	*E. coli* lab	>90	>100	>4	nd	nd	—
JM110	*E. coli* lab	>90	>100	>4	nd	nd	—
XL-1Blue	*E. coli* lab	>90	>100	>4	nd	nd	—
	S. aureus	66 ± 18	50	>4 Multires	nd	nd	—

[a] CFX = ciprofloxacin.
[b] nd = not determined.

mutations in samples displaying $MIC_{CFX} \geqslant 4$ mg/L were not assayed. Interestingly, in both bacterial species only isolates fully susceptible to ciprofloxacin (i.e., MIC_{CFX} <0.25 and no point mutations in topoisomerase subunits) were also susceptible to SD8. *K. pneumoniae* isolates, however, were generally more SD8-resistant than *E. coli* strains.

We next searched for the presence of the *qnr* gene, which is characteristic of a recently discovered plasmid-mediated mechanism of fluoroquinolone resistance.[8] Four *E. coli* strains were positive to *qnr*: specifically, samples #47, #50, and #9 exhibited the *qnrB19* type allele and sample #191 the *qnrS1* type. Eight *K. pneumoniae* strains contained *qnrB19*. When comparing susceptibility to SD8 in samples with the same MIC_{CFX} and no topoisomerase mutations, we found a trend where *qnr*-lacking samples were generally more susceptible to SD8 (i.e., #24, #41 versus #47, #50 in *E. coli* and #97 versus #134 in *K. pneumoniae*).

Finally, to test whether the lower susceptibility to SD8 of *K. pneumoniae* versus *E. coli* depended upon differences in drug permeability, we measured SD8 uptake in selected cultures by means of RP-HPLC analysis. Results were reported as absolute amount (pmol) calculated using a calibration curve and as relative % (ratio between the amount determined inside the cells and the total drug amount originally supplied). As shown in Table 2, there was a striking difference between the SD8 amounts found in *E. coli* versus *K. pneumoniae* clinical isolates, the former taking up 3–6 times the amount observed in the latter. In addition, *E. coli* laboratory strain K12 TOP10 showed a SD8 uptake only slightly larger than *K. pneumoniae*'s. These data indicate that *E. coli* clinical isolates are significantly more permeable to SD8 compared to both *K. pneumoniae* species and *E. coli* laboratory strains.

Table 2
SD8 cellular entry

Sample ID	Bacterial species	MIC_{50} (SD8, mg/L)	Intracellular SD8 (pmol)	Intracellular entry (%)
97	*K. pneumoniae*	50	40	1.30
92	*K. pneumoniae*	50	60	1.96
50	*E. coli*	12	180	5.87
24	*E. coli*	3.12	260	7.53
Top10	*E. coli* lab	>100	80	2.61

In contrast to current literature, our results indicate for the first time that SD8 can be highly active not only against Gram-positive species, but also against *E. coli* clinical isolates and also moderately effective against another frequent Gram-negative bacterial pathogen, such as, *K. pneumoniae*. This finding is in line with recent data showing that SD8 is equally active at the molecular level against Gyrase from both *E. coli* and *S. aureus* species.[3] One main difference between clinical and laboratory *E. coli* isolates rests on reduced SD8 cellular uptake which makes laboratory strains resistant. Reduced intracellular drug concentration also accounts for the diverse activity of SD8 among Gram-negative species, such as *E. coli* and *K. pneumoniae*, the latter being less sensitive than the former.

Additionally, we showed that mutations at aminoacids Ser-83 and Asp-87 at the *GyrA* level confer total resistance to SD8. These two mutations are frequently found in fluoroquinolone resistant bacterial strains and it was proposed that these two aminoacids represent important contacts in the Gyrase–quinolone–DNA complex.[10] Our data confirm SD8 mechanism of action at the Gyrase level, additionally suggesting a possible site of action on GyrA in common with fluoroquinolones. The increased SD8 resistance of *qnr*-positive strains additionally supports this indication. In fact, the *qnr* gene expresses the Qnr protein which belongs to the pentapeptide-repeat family and was suggested to protect DNA Gyrase from fluoroquinolone action by binding to the protein and so competing for Gyrase–DNA complex formation.[8] It is worth noting that *qnr* conferred only a minor resistance increase to SD8 and especially in *E. coli* strains (fourfold or higher in *E. coli* and maximum twofold in *K. pneumoniae*). This data is in line with *qnr*-mediated resistance to fluoroquinolones, which is reported to increase of 2–125-fold depending on the quinolone.[8]

In conclusion, our data strengthen the idea that laboratory bacterial strains cannot be safely used to assess in vivo drug susceptibility/resistance processes and suggest that SD8 represents an interesting lead structure to develop novel effective broad-spectrum antibacterial agents.

Experimental information: Gram-negative strains were chosen including all ranges of susceptibility to fluoroquinolones as preliminary measured by Vitek2 (BioMerieux, Hazelwood, MO, USA). Susceptibility to SD8 was measured after incubation of liquid bacterial cultures with 0.20–100 mg/L drug concentration (twofold serial dilutions) for 24 h in Luria–Bertani medium. Results were reported both as residual bacterial survival at 50 mg/L SD8 concentration and as MIC_{50}. Survival was calculated as the ratio between the turbidity (OD at 600 nm) of the bacterial culture after treatment with 50 mg/L drug concentration and that of a control culture in the absence of drug. MIC_{50} was the minimum inhibitory concentration required for 50% bacterial growth inhibition. Poor solubility of SD8 in the test medium prevented determination of MIC_{90} (minimum inhibitory concentration that inhibited 90% of bacterial growth). MIC_{90} of ciprofloxacin (MIC_{CFX}) was determined by disk diffusion test according to the CLSI criteria and the results were confirmed by means of E-test strips (AB Biodisk, Solna, Sweden). Mutations in the GyrA and ParC topoisomerase subunits were assessed by PCR amplification and sequencing of the QRDR of *gyrA* and *parC* genes using universal primers.[7] Screening of *qnrA*, *qnrB*, and *qnrS* genes was carried out by multiplex PCR amplification.[9] Positive-containing strains with known *qnr* genes, *qnrA1 E. cloacae* HM04-477, *qnrB4 E. cloacae* HM05-186, and *qnrS1 E. coli* HM5-184, and negative controls (without DNA template) were included in each run. SD8 uptake was measured in selected cultures by means of RP-HPLC analysis. After treatment with SD8, cells were pelleted, washed and lysed. SD8 was extracted from bacterial lysates by a double extraction with ethyl acetate. The organic phase was collected and dried under vacuum. The residual was resuspended in methanol and subjected to RP-HPLC analysis (Eclipse XDB C18 column, Agilent Technologies). Results were reported as absolute amount (pmol) calculated using a calibration curve and as relative % (ratio between the amount determined inside the cells and the total drug amount originally supplied).

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