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MECHANISMS OF RESISTANCE

New Topoisomerase Inhibitors: Evaluating the Potency of Gepotidacin and Zoliflodacin in Fluoroquinolone-Resistant *Escherichia coli* upon *tolC* Inactivation and Differentiating Their Efflux Pump Substrate Nature

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ABSTRACT Inactivating *tolC* in multidrug-resistant *Escherichia coli* with differing sequence types and quinolone resistance-determining mutations reveals remarkably potentiated activity of the first-in-class topoisomerase inhibitors gepotidacin and zoliflodacin. Differences between both structurally unrelated compounds in comparison to fluoroquinolones regarding the selectivity of *E. coli* RND (resistance-nodulation-cell division)-type transporters, efflux inhibitors, and AcrB porter domain mutations were demonstrated. The findings should reinforce efforts to develop efflux-bypassing drugs and provide AcrB targets with critical relevance for this purpose.

KEYWORDS gepotidacin, zoliflodacin, drug efflux, TolC, AcrB, YhiV (MdtF), RND-type transporter, fluoroquinolones, clinical *E. coli* isolates

The development of novel antibacterial drugs is essential in a world with continually increasing rates of multidrug-resistant (MDR) pathogens. Among promising candidates currently under clinical development are the topoisomerase type II inhibitors gepotidacin (GEP) (1) and zoliflodacin (ZOL) (2). Both GEP and ZOL belong to new drug classes, the triazaacenaphthylenes and the spiropyrimidinetriones, respectively, and both are active against *Neisseria gonorrhoeae* (3, 4). Like fluoroquinolones (FQs), they target the type II topoisomerases (DNA gyrase and topoisomerase IV), but by entirely different modes of action. Hence, mutations occurring in the quinolone resistance-determining regions (QRDRs) of the topoisomerase genes *gyrA*, *gyrB*, *parC*, and *parE* as a result of FQ treatment should not impair the efficacy of these drugs (4, 5). Activity of GEP against *Escherichia coli* and Gram-positive pathogens such as *Staphylococcus aureus* has also been shown (6), and activity of ZOL against fastidious Gram-negative pathogens has been demonstrated (2).

There has been evidence that the chemically unrelated compounds GEP and ZOL are substrates of the RND (resistance-nodulation-cell division)-type efflux pump MtrCDE in *N. gonorrhoeae* (7, 8). To our knowledge, nothing has been reported about the contribution of efflux to their resistance levels in *E. coli* and their putative substrate nature regarding the major *E. coli* RND-type MDR transporter AcrAB-TolC. To explore the role of efflux for the activities of the new topoisomerase inhibitors in MDR clinical *E. coli*, we selected highly FQ-resistant isolates from different sequence types and origins (Table 1) for *tolC* knockout experiments. Genetic engineering of MDR strains is challenging because of limited selection options (9, 10). We succeeded in constructing four *tolC* mutants from three different sequence types and with differing QRDR mutation patterns, including an isolate harboring the aminoglycoside/FQ resistance gene

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TABLE 1 *E. coli* strains and mutants used in this study

E. coli strain or mutant	Description, MLST[a] sequence type, and country	Mutation(s) in QRDR[b] and episomal FQ resistance genes[c]	Provider, source, or reference
KUN9180	Clinical MDR *E. coli* isolate, ST 131, Japan	GyrA: S83L, D87N; ParC: S80I, E84V; ParE: I529L	Department of Clinical Laboratory Medicine, Kyoto University Graduate School of Medicine, Kyoto, Japan
2012-0633	Clinical MDR *E. coli* isolate, ST 1193, Japan	GyrA: S83L, D87N; ParC: S80I; ParE: L416F	Department of Clinical Laboratory Medicine, Kyoto University Graduate School of Medicine, Kyoto, Japan
SA128	Clinical MDR *E. coli* isolate, ST 167, Sudan	GyrA: S83L, D87N; ParC: S80R, E84V; *aac(6′)Ib-cr*	Abha National Polyclinic, Abha, Saudi Arabia
FR11009	Clinical MDR *E. coli* isolate, ST 167, Germany	GyrA: S83L, D87N; ParC: S80I, E84G	Center for Microbiology, University Hospital Freiburg, Germany
KUN9180Δ*acrB*(::*PGK-gb2-neo*)	*acrB* knockout mutant	GyrA: S83L, D87N; ParC: S80I, E84V; ParE: I529L	9
KUN9180Δ*tolC*(::*rpsL-neo*)	*tolC* knockout mutant	GyrA: S83L, D87N; ParC: S80I, E84V; ParE: I529L	This study
2012-0633Δ*tolC*(::*rpsL-neo*)	*tolC* knockout mutant	GyrA: S83L, D87N; ParC: S80I; ParE: L416F	This study
SA128Δ*tolC*(::*rpsL-neo*)	*tolC* knockout mutant	GyrA: S83L, D87N; ParC: S80R, E84V; *aac(6′)Ib-cr*	This study
FR11009Δ*tolC*(::*rpsL-neo*)	*tolC* knockout mutant	GyrA: S83L, D87N; ParC: S80I, E84G	This study
ATCC 25922	Reference *E. coli* strain		DSMZ[d], no. DSM-1103.
AG100	K-12 derivative, wild-type (wt) *acrB*		22
3-AG100	AG100 derivative, wt *acrB* overexpressed	GyrA: D87G	11
3-AG100Δ*acrB*	*acrB* deletion mutant from 3-AG100	GyrA: D87G	14
3-AG100Δ*tolC*(::*rpsL-neo*)	*tolC* knockout mutant from 3-AG100	GyrA: D87G	This study
2-DC14PS	*acrAB* knockout mutant from AG100, *acrF* overexpressed	GyrA: S83L	23
DKO	*acrAB_acrF* double-knockout mutant from 2-DC14PS	GyrA: S83L	24
DKO20/1	*acrAB_acrF* double-knockout mutant from 2-DC14PS, *yhiV* overexpressed	GyrA: S83L, D87L; ParC: E84K	24
TKO	*acrAB acrF yhiV* triple-knockout mutant from DKO20/1	GyrA: S83L, D87L; ParC: E84K	24
F136A, F178A, F610A, F615A, F617A, and F628A	AcrB distal binding pocket mutants from 3-AG100	GyrA: D87G	15
V612F	AcrB distal binding pocket mutant from 3-AG100	GyrA: D87G	This study
G616N	AcrB distal binding pocket mutant from 3-AG100	GyrA: D87G	17
I38F/I671T	Entrance pathway mutant from 3-AG100	GyrA: D87G	18

[a]MLST, multilocus sequence typing.
[b]QRDR, quinolone resistance-determining region. Only known quinolone resistance-determining mutations are shown.
[c]FQ, fluoroquinolone. FQ resistance genes are given in italic.
[d]Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (https://www.dsmz.de).

aac(6′)Ib-cr (Tables 1 and 2), by applying a phage λ-based homologous recombination method. A neomycin/kanamycin resistance cassette was amplified with homology flanks for recombination in *tolC* by using oligonucleotides given in Table S1 in the supplemental material and a cassette template from the Red/ET counterselection BAC modification kit (Gene Bridges, Heidelberg, Germany). Homologous recombination was performed by using a curable Red/ET plasmid with a chloramphenicol cassette (supplemental material). Selection of knockout mutants was possible with 40 μg/ml paromomycin, because we had recognized that the neomycin/kanamycin cassette mediates cross-resistance to this aminoglycoside to which, in contrast to kanamycin (MICs >

TABLE 2 Susceptibilities of *E. coli* strains and mutants to selected fluoroquinolones and new topoisomerase inhibitors

	MIC (μg/ml) for[b]:				
	FQ drugs			New topoisomerase inhibitors	
***E. coli* strain or mutant**[a]	**LVX**	**MXF**	**NDX**	**GEP**	**ZOL**
A. Clinical MDR *E. coli* isolates and derived mutants					
2012-0633	>32	>8	>512	16	16
2012-0633Δ*tolC*	**4**	**2**	**2**	**0.06**	**0.03**
FR11009	32	>8	512	1	4
FR11009Δ*tolC*	**4**	**2**	**2**	**0.03**	**0.01**
SA128	>32	>8	>512	8	16
SA128Δ*tolC*	**4**	**4**	**8**	**0.06**	**0.03**
KUN9180	32	>8	>512	2	4
KUN9180Δ*acrB*	**4**	**2**	**32**	**0.06**	**0.25**
KUN9180Δ*tolC*	**4**	**2**	**8**	**0.03**	**0.06**
B. Laboratory strains and derived mutants					
ATCC 25922 (constitutive *acrB* expression)	**0.06**	**0.125**	**0.125**	4	2
AG100 (constitutive *acrB* expression)	**0.06**	**0.25**	**0.125**	**1**	4
3-AG100 (*acrB* overexpressed)	2	4	4	4	4
3-AG100Δ*acrB*	**0.06**	**0.06**	**0.03**	**0.06**	**0.06**
3-AG100Δ*tolC*	**0.06**	**0.06**	**0.06**	**0.06**	**0.01**
2-DC14PS (Δ*acrAB*, *acrF* overexpressed)	2	4	16	2	16
DKO (Δ*acrAB* Δ*acrF*)	**0.125**	**0.06**	**0.125**	**0.03**	**0.06**
DKO20/1 (Δ*acrAB* Δ*acrF*, *yhiV* overexpressed)	32	32	256	**0.06**	8
TKO (Δ*acrAB* Δ*acrF* Δ*yhiV*)	**8**	**4**	**8**	0.02	**0.03**
C. AcrB porter domain mutants derived from 3-AG100					
F136A	1	4	2	4	2
F178A	**0.5**	2	**1**	2	2
F610A	**0.125**	**0.5**	**0.5**	**1**	2
V612F	1	2	2	2	**0.5**
F615A	1	4	2	4	**1**
G616N	1	2	4	4	4
F617A	1	4	4	2	4
F628A	1	2	**1**	2	2
I38F/I671T	**0.06**	**0.25**	**1**	**0.06**	16

[a]For sections A and B, the parental *E. coli* strain or isolate is shown first and the mutants derived from the strain or isolate are indented. Strains are listed in Table 1.
[b]FQ, fluoroquinolone; LVX, levofloxacin; MXV, moxifloxacin; NDX, nadifloxacin; GEP, gepotidacin; ZOL, zoliflodacin. Values given in boldface type and underlined highlight MIC decreases by ≥4-fold and increases by ≥4-fold, respectively, with the following reference relationships: for section A, *tolC* and *acrB* mutants versus their respective parental clinical isolates; for section B, TKO versus DKO20/1, all other laboratory strains and mutants versus *acrB* overexpressing strain 3-AG100; for section C, AcrB mutants versus 3-AG100. >, MIC determination is limited due to drug precipitation at concentrations above the indicated values or to maximum concentrations in the commercial precast microdilution 96-well plates in the case of LVX and MXF.

50 μg/ml), our clinical isolates show relative susceptibility (MICs $\leq$ 4 μg/ml). We additionally knocked out *tolC* from laboratory strain 3-AG100, a derivative of *E. coli* AG100, which overexpresses AcrAB-TolC (11) (Tables 1 and 2 and Fig. 1A).

Efflux disruption in the *tolC* mutants was assessed by standard MIC testing (Table 2) and real-time efflux assays in which bacterial cells de-energized with 10 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were loaded with the piperazine arylideneimidazolone BM-27 to a final concentration of 10 μM. Efflux was started by reenergization with 1 mM glucose, and fluorescence of the intracellular remaining BM-27 was measured at an excitation and emission wavelength of 400 and 457 nm, respectively (Fig. 1A; see supplemental material also).

In an earlier study, we have shown that FQ susceptibilities significantly increase due to *acrB* inactivation in an *E. coli* isolate with QRDR mutations (9). As could be expected, we achieved similar results from the *tolC* mutants, including SA128Δ*tolC* harboring *aac(6′)Ib-cr*, but with an enhanced impact on the resistance to the more lipophilic nadifloxacin (NDX) (Table 2). The latter has been demonstrated to be least impaired by the outer membrane (OM) influx barrier (12), enabling the appearance of approximate net efflux. As reported previously, *tolC* disruption rarely increases FQ susceptibilities to

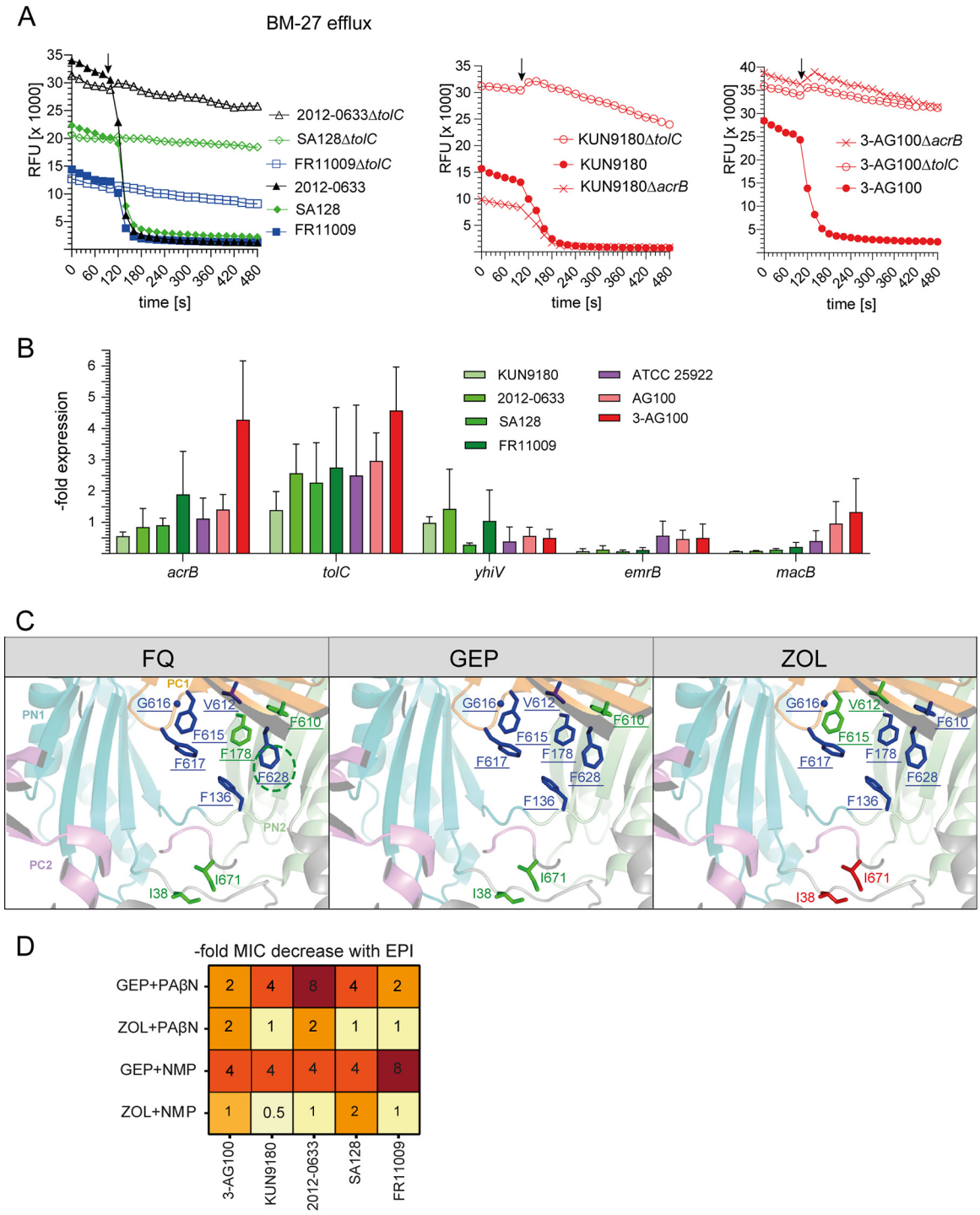

FIG 1 (A) BM-27 efflux initiated by the addition of glucose (black arrow). Assays were done at least in duplicate according to a published protocol (25). RFU, relative fluorescence units. (B) Expression of *tolC* and of TolC-dependent MDR transporter genes (shown if any expression levels of >0.4 were detected). Means plus standard deviations (SD) (error bars) from values normalized to the respective *gyrB* values ($n \geq 4$) are shown. (C) Part of the AcrB porter domain (PDB accession no. 2HRT, chain B), side view from the periplasmic cleft (PC1, PC2, PN1, and PN2, subdomains) visualized using PyMOL (version 2.1.1; Schrödinger, LLC). Amino acid side chains are depicted as sticks, glycine as a sphere, and distal binding pocket residues with underlined labels. Blue-, green-, and red-colored sticks indicate impact by mutation on susceptibilities (no significant change, a significant increase, and significant decrease, respectively) regarding FQs (dashed circle, increase of NDX susceptibility only), GEP, and ZOL. (D) Efficacies of EPIs with GEP and ZOL given as ratios of the MIC in the absence and presence of an EPI ($n \geq 3$).

clinical relevance in isolates with QRDR mutations and/or FQ resistance genes (10). However, with GEP and ZOL, MICs decreased by 32- to 512-fold, achieving values below 0.1 μg/ml (Table 2).

Even though AcrAB-TolC has been shown to play a major role for MDR in *E. coli* (13), the OM channel TolC could cooperate with further MDR efflux pumps, such as the RND-type transporters AcrF, YhiV (MdtF), and YegNO (MdtBC), the ABC (ATP-binding cassette)-type transporter MacAB, and the MFS (major facilitator superfamily) transporter EmrAB. To answer the question whether further TolC-dependent transporters contribute to efflux, we compared the *tolC* knockout mutants with the respective *acrB* knockout mutants available for *E. coli* 3-AG100 (14) and clinical isolate KUN9180 (9) from earlier studies. As just mentioned, remarkable differences between KUN9180Δ*acrB* and KUN9180Δ*tolC* were detected in susceptibilities to NDX but also to ZOL (Table 2) as well as in the BM-27 efflux of the KUN9180 mutants (Fig. 1A), indicating the involvement of at least one more TolC-dependent transporter. Interestingly, in the knockout mutants derived from laboratory strain 3-AG100, solely the ZOL susceptibilities were different (Table 2 and Fig. 1A). Results of gene expression studies suggested the relevance of YhiV within the clinical isolates KUN9180 (*yhiV* expression $>$ *acrB* expression, $P = 0.01$) and 2012-0633 and FR11009 (similar expression of *acrB* and *yhiV*). In contrast, AcrB appeared to be the dominant exporter in SA128 and the laboratory strains (*acrB* expression $>$ *yhiV* expression, P values < 0.01) (Fig. 1B).

In order to explore RND-type substrate pathway specificities of GEP and ZOL versus FQs, we examined the impact of AcrB porter domain mutations with known effects on the susceptibilities to other drugs. Among distal binding pocket (DB) mutations, the highest FQ susceptibility increases were achieved by F610A, which has been known to seriously impair resistance to almost all AcrB substrates except linezolid and sutezolid (14, 15). Remarkably, F610A also results in significant MIC decreases of GEP but not of ZOL. In contrast, DB mutation F615A exclusively affects resistance to ZOL among the drugs tested in this study, whereas DB mutation F178A showed significant effects with the FQs LVX and NDX, but none with the new topoisomerase inhibitors. No remarkable impact was found with G616N and F617A, which are substitutions at the so-called switch-loop (16, 17), as well as with F136A for all compounds tested, whereas F628A affects only resistance to the lipophilic FQ NDX (Fig. 1C). The most surprising effects were recognized from the double mutation I38F/I671T located in the lower porter domain and presumed to block a bottleneck in an entrance channel for predominantly smaller drug molecules (18). While this could also be demonstrated for GEP, ZOL appears more efficiently extruded in the I38F/I671T mutant than in the wild-type AcrB strain (Table 2 and Fig. 1C). Analogous results have been obtained with macrolides in an earlier study (18). The marginally larger size of ZOL (molecular weight [MW], 487.4) versus GEP (MW, 448.5) could explain pathway hindrance, but probably additional physicochemical factors play a role for the identified substrate route selectivity.

The experimental efflux pump inhibitors (EPIs) 1-(1-naphthylmethyl)-piperazine (NMP) and Phe-Arg-β-naphthylamide (PAβN) are known enhancers of FQ action (19, 20). Regarding the new topoisomerase inhibitors, both EPIs showed significant sensitizing with GEP but not with ZOL (Fig. 1D) when they were used as adjuvants in MIC assays (supplemental material). The efficacy of PAβN (used at 25 μg/ml) with GEP appeared more "strain dependent" than that of NMP (used at 100 μg/ml), presumably due to strain-specific cell envelope properties that might impair the permeabilizing activity of PAβN (21).

We additionally studied the substrate nature of GEP and ZOL regarding the AcrB homologs AcrF and YhiV by using mutants overexpressing these transporters while AcrAB and AcrAB_AcrF were knocked out, respectively. Both topoisomerase inhibitors appeared to be suitable substrates for AcrF, whereas MIC data suggest that ZOL, but not GEP, is extruded from YhiV (Table 2).

In conclusion, we have demonstrated a remarkable contribution of TolC-dependent efflux to resistance levels of the first-in-class topoisomerase inhibitors GEP and ZOL in

FQ-resistant *E. coli* isolates. In addition to AcrB, YhiV was shown to be a putatively relevant substrate-specific transporter contributing to efflux in a percentage of clinical isolates. Critical sites in the AcrB efflux pathway of both structurally unrelated topoisomerase inhibitors were discovered and could inform the design of efflux-bypassing drugs. To obtain deeper insight into the requirements of those agents, further exploration of differences in the substrate nature of GEP and ZOL regarding other homologous RND-type transporters, including the *Neisseria* efflux pump MtrD, are needed.

Data availability. ENA accession numbers of whole-genome sequencing data available from previous studies follow: for *E. coli* strain KUN9180, study accession no. PRJEB19331 and sample accession no. SAMEA100686418 (https://www.ebi.ac.uk/ena/browser/view/ERS1572363) (9); for *E. coli* strain 3-AG100, study accession no. PRJEB30347 and sample accession no. SAMEA5175928 (https://www.ebi.ac.uk/ena/data/view/ERS2983635) (21). ENA accession numbers of whole-genome sequencing data available from the present study follow: for *E. coli* strain 2012-0633, study accession no. PRJEB39933 and sample accession no. ERS4956175 (https://www.ebi.ac.uk/ena/browser/view/ERS4956175); for *E. coli* strain SA128, study accession no. PRJEB39933 and sample accession no. ERS4956177 (https://www.ebi.ac.uk/ena/browser/view/ERS4956177), and for *E. coli* strain FR11009, study accession no. PRJEB39933 and sample accession no. ERS4956176 (https://www.ebi.ac.uk/ena/browser/view/ERS4956176).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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We have no conflicts of interest to declare.

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