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Efflux Pumps Are Involved in the Defense of Gram-Negative Bacteria against the Natural Products Isobavachalcone and Diospyrone[▽]

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The activities of two naturally occurring compounds, isobavachalcone and diospyrone, against documented strains and multidrug-resistant (MDR) Gram-negative bacterial isolates were evaluated. The results indicated that the two compounds exhibited intrinsic antibacterial activity against several Gram-negative bacteria, and their activities were significantly improved in the presence of an efflux pump inhibitor (MIC values decreased to below 10 μg/ml). In addition, the activities of isobavachalcone and diospyrone against various strains exhibiting deletions of the major efflux pump components (AcrAB, TolC) were significantly increased. The overall results indicate that isobavachalcone and diospyrone could be candidates for the development of new drugs against MDR strains and that their use in combination with efflux pump inhibitors reinforces their activity.

The continuous emergence of multidrug-resistant (MDR) bacteria drastically reduces the efficacy of our antibiotic armory and, consequently, increases the frequency of therapeutic failure (10, 27). Drug resistance is a consequence of the worldwide use of antibiotics, and the acute challenge for health care is to find measures that efficiently combat resistant organisms (10, 27, 31). This includes improved early infection control, the use of appropriate therapies, and the use of hospital measures to prevent the dissemination of MDR strains, as well as the development of new antibiotics (31). The resistance of bacteria to chemically unrelated antimicrobial agents (or MDR) may be associated with the overexpression of efflux pumps (15, 25). In Gram-negative bacteria, many of these efflux pumps belong to the resistance-nodulation-cell division (RND) family of tripartite efflux pumps. Among those efflux pumps, pumps belonging to the AcrAB-TolC family are detected in many clinical enterobacterial isolates and are reported to be a key factor in the expression of the MDR phenotype (16, 19, 28). Several RND efflux pumps have been identified in clinical isolates of *Pseudomonas aeruginosa*, another important nosocomial pathogen highly resistant to the commonly used antibiotics (2, 9, 15). This efflux pump mechanism can be blocked by various efflux pump inhibitors that restore the intracellular concentration as well as the activities of the antibiotics (23).

The scarcity of original synthetic antibiotics has stimulated the search for new antibacterial agents from medicinal plants (6, 12, 17). Isobavachalcone has been isolated from several medicinal plants (20, 21, 24). Isobavachalcone was reported to have very interesting activities against *Candida albicans* and *Cryptococcus neoformans* (9), and some preliminary results have indicated that this compound has activity against susceptible microorganisms (20). However, the activity of this compound against resistant bacteria and its mode of action were not elucidated. At present, *Diospyros canaliculata* is the only reported source of diospyrone (29). We have recently described the activity of diospyrone against *Neisseria gonorrhoeae* and *Mycobacterium tuberculosis* (14), but its activity against resistant bacteria and its target have not been reported.

In the study described here, we evaluated the activities of isobavachalcone and diospyrone against various Gram-negative bacteria, including MDR hospital isolates. The spectrum of action of these molecules regarding the role of efflux pumps in their activity was also investigated by using various documented strains and a previously described efflux pump inhibitor.

MATERIALS AND METHODS

Chemicals for antimicrobial assays. Isobavachalcone and diospyrone (Fig. 1) were obtained from the chemical stock bank of the Laboratory of Organic Chemistry, University of Yaoundé I, Yaoundé, Cameroon. We recently reported on the isolation and identification of isobavachalcone from *Dorstenia barteri* (20) and diospyrone from *Diospyros canaliculata* (29). Chloramphenicol and norfloxacin (Sigma-Aldrich, St. Quentin Fallavier, France), tetracycline hydrochloride (Merck KGaA, Darmstadt, Germany), imipenem-cilastatin (500/500 mg; Merck, Paris, France), and cefepime (Bristol-Myers, Reuil-Malmaison, France) were used as selected or reference antibiotics. *p*-Iodonitrotetrazolium chloride (INT), phenylalanine arginine ß-naphthylamide (PAßN), and 1,3,5-triphenyltetrazolium chloride (TTC) (Sigma-Aldrich) were also used in this study.

Bacterial strains and culture media. The microbial species used included MDR and reference strains of *Escherichia coli*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*. Their fea-

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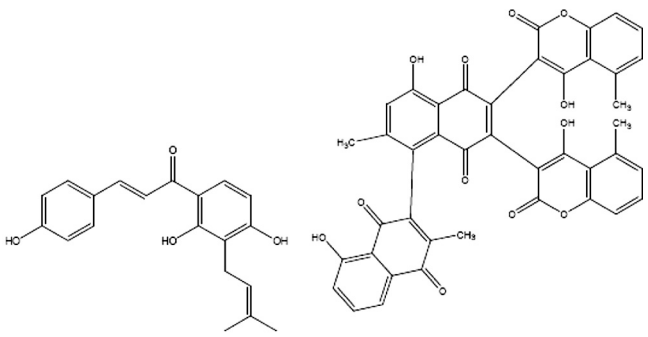

isobavachalcone diospyrone

FIG. 1. Chemical structures of isobavachalcone and diospyrone.

tures are summarized in Table 1. *E. cloacae* strains Ec0769 and Ec1194 were from the laboratory collection (UMR-MD1, Université de la Méditerranée, Marseille, France). All strains were precultured overnight on Mueller-Hinton agar, prior to any assay. Mueller-Hinton broth (MHB) was used as the liquid culture medium for susceptibility tests (13, 20).

Bacterial susceptibility determinations. The MICs of the two compounds and antibiotics were determined by a rapid INT colorimetric assay (8, 13). Briefly, the test sample and selected antibiotics were first dissolved in dimethyl sulfoxide (DMSO)-MHB. The solution obtained was then added to MHB and serially diluted twofold (in a 96-well microplate). One hundred microliters of inoculum (1.5×10^6 CFU/ml) prepared in MHB was then added. The plates were covered with a sterile plate sealer and then agitated with a shaker to mix the contents of the wells and incubated at 37°C for 18 h. The final concentration of DMSO was less than 2.5%, and DMSO did not affect the microbial growth. Wells containing MHB, 100 μl of inoculum, and DMSO at a final concentration of 2.5% served as the negative control (this internal control was systematically added). The MICs of samples were detected after 18 h of incubation at 37°C, following addition (40 μl) of 0.2 mg/ml INT and incubation at 37°C for 30 min. Viable bacteria reduced the yellow dye to pink. The MIC was defined as the lowest sample concentration that prevented this change and that resulted in the complete inhibition of microbial growth. The samples were tested alone and in the presence of PAβN at a final concentration of 20 μg/ml, as described previously (11). The MICs of PAβN were 64 μg/ml for *E. coli* AG100A, 256 μg/ml for *K. pneumoniae* ATCC 11296, and >256 μg/ml for all other strains and organisms. Each assay was repeated three times independently.

RESULTS AND DISCUSSION

Activities of isobavachalcone and diospyrone and role of efflux pumps in susceptibility of Gram-negative bacteria. The various strains and MDR isolates were tested for their susceptibilities to isobavachalcone, diospyrone, and reference antibiotics (norfloxacin, chloramphenicol) alone and then in the presence of PAβN, a well-known efflux pump inhibitor (4, 16, 19, 23). The results presented in Table 2 indicate that the two natural products exhibited activities against all strains. Interestingly, the activities of the two compounds against MDR isolates, e.g., strains EA5 and KP63, were better than those of the commonly used antibiotics (Table 2). The lowest MIC values for diospyrone (4 μg/ml) and isobavachalcone (8 μg/ml) were recorded for *E. coli* AG100A and *E. aerogenes* EA298, respectively. This result may indicate that the mechanisms involved in resistance to usual antibiotics are less efficient against these two compounds.

The antibacterial activities of the two compounds were sig-

TABLE 1. Bacterial strains and features

Bacterial strain	Relevant feature(s)[a]	Reference(s)
E. coli		
ATCC 8739 and ATCC 10536	Reference strains	
AG100	Wild-type *E. coli* K-12	30
AG100A	AG100 *acrAB*::Kan^r	22, 30
AG100A$_{Tet}$	Tet^r derivative of AG100A in which the *acrF* gene is markedly overexpressed	30
AG102	AG100 overexpressing the AcrAB pump	7
E. aerogenes		
ATCC 13048	Reference strain	
EA-CM64	Chl^r variant obtained from ATCC 13048 overexpressing the AcrAB pump	11
EA3	Clinical MDR isolate; Chl^r Nor^r Ofx^r Spx^r Mox^r Cft^r Atm^r Fep^r	18, 19
EA5	Clinical MDR isolate exhibiting energy-dependent norfloxacin and chloramphenicol efflux; Mox^r Cft^r Atm^r Fep^r	18, 19
EA27	Clinical MDR isolate exhibiting energy-dependent norfloxacin and chloramphenicol efflux with Kan^r Amp^r Nal^r Str^r Tet^r	18, 19
EA289	KAN-sensitive derivative of EA27	26
EA294	EA289 *acrA*::Kan^r	26
EA298	EA289 *tolC*::Kan^r	26
E. cloacae		
Ec0769 and Ec1194	Clinical isolates	This study
K. pneumoniae		
ATCC 12296	Reference strain	
KP55	Clinical MDR isolate; Tet^r Amp^r Atm^r Cef^r	3
KP63	Clinical MDR isolate; Tet^r Chl^r Amp^r Atm^r	3
P. aeruginosa		
PA01	Reference strain	
PA124	Clinical MDR isolate	17

[a] Smp^r, Atm^r, Cef, Cft^r, Chl^r, Fep^r, Kan^r, Mox^r, Str^r, and Tet^r, resistance to ampicillin, aztreonam, cephalothin, cefadroxil, chloramphenicol, cefepime, kanamycin, moxalactam, streptomycin, and tetracycline, respectively.

TABLE 2. MICs of the two natural compounds for reference and documented strains and clinical MDR isolates

Bacterium and strain	MIC (μg/ml)[b]							
	Isobavachalcone		Diospyrone		Chloramphenicol		Norfloxacin	
	−	+	−	+	−	+	−	+
E. coli								
ATCC 10536	128	2	32	2	1	0.5	0.06	0.03
ATCC 8739	256	8	128	4	4	1	0.12	0.12
AG100	64	0.5	64	1	4	0.25	0.12	0.12
AG100A	16	0.25	4	0.12	0.5	0.25	0.03	0.007
$AG100A_{Tet}$	64	8	16	0.24	32	2	1	0.25
AG102	64	8	64	2	32	2	1	0.25
E. aerogenes								
ATCC 13048	256	16	128	32	4	1	0.25	0.25
EA-CM64	>256	256	128	16	256	8	4	2
EA289	256	16	128	8	>256	128	128	128
EA294	32	0.5	128	8	64	16	64	32
EA298	8	0.5	32	16	64	16	8	8
EA27	256	8	128	16	>256	128	256	128
EA3	128	32	128	64	>256	128	128	64
EA5	64	16	128	64	>256	32	256	128
K. pneumoniae								
ATCC 11296	32	4	32	2	2	2	1	0.5
KP55	32	4	64	8	32	4	16	8
KP63	16	0.5	32	4	>256	128	16	4
P. aeruginosa								
PAO1	64	16	64	4	128	8	2	1
PA124	64	4	64	1	256	8	64	32
E. cloacae								
Ec07769	128	8	128	8	>256	256	>256	>256
Ec1194	64	1	32	2	2	1	32	32

[a] The drugs and compounds were tested in the absence (−) or in the presence (+) of PAβN at a final concentration of 20 μg/ml, as described previously (11). At this concentration, no intrinsic effect against the various bacterial strains (included as internal controls in each assay without antibiotic) was observed.

nificantly increased in the presence of the efflux pump inhibitor PAβN, with all MICs obtained decreasing to below 10 μg/ml for the *E. coli*, *K. pneumoniae*, and *E. cloacae* strains (Table 2). In addition, this enhanced activity was observed against various strains of *E. coli*, *E. aerogenes*, *K. pneumoniae*, *P. aeruginosa*, and *E. cloacae*. When norfloxacin was included with chloramphenicol and they were used as reference antibiotics, the presence of an additional resistance mechanism, such as the target mutation previously described in the clinical isolates tested, strongly modulates the level of restoration of susceptibility (19). It is important to mention that the susceptibilities of strains AG100A and EA294, with both strains being devoid of AcrA and with EA298 being devoid of TolC, were noticeably increased by PAβN (Table 2). A similar effect concerning the activities of macrolides-ketolides and chloramphenicol against the same strains has previously been reported, suggesting the presence of an additional efflux system (4, 11).

All *E. aerogenes* strains except EA298 were resistant to isobavachalcone and diospyrone (MICs, 8 and 32 μg/ml, respectively). Nevertheless, the activity of isobavachalcone was better than that of chloramphenicol against six of the eight *E. aerogenes* strains studied. Diospyrone was also more active than chloramphenicol against all *E. aerogenes* strains except strains ATCC 13048 and EA294. In the presence of PAβN, the activities of the two compounds against all *E. aerogenes* strains increased, with the isobavachalcone MIC values being below 1 μg/ml for strains EA294 and EA298 (Table 2).

In this study, the antimicrobial activities of isobavachalcone and diospyrone were significantly improved in the presence of an efflux pump inhibitor (Table 2), suggesting that efflux is likely one of the mechanisms that modulates the susceptibilities of *E. coli*, *E. aerogenes*, *K. pneumoniae*, *P. aeruginosa*, and *E. cloacae*. The significant increase in the levels of susceptibility to these compounds observed in strains with *acrB* and *tolC* deletions demonstrates the role of the major AcrAB-TolC efflux pump. However, the increase in the susceptibilities of strains EA298 and EA294 (*tolC* and *acrA* strains respectively) caused by PAβN indicated that an additional efflux system(s) is active in these strains, as reported previously (4, 11). This PAβN-susceptible efflux system also contributes to resistance to isobavachalcone and diospyrone. The efflux mechanisms clearly appear to be the first line of bacterial defense against these molecules, as has been demonstrated for other natural compounds (1, 5). This study supports the evidence of the antimicrobial potencies of these compounds and also highlights the activities of the compounds against MDR strains. The results obtained in the present study should be taken into consideration given the clinical relevance of the resistant bacteria studied. Both strain KP55 and strain KP63 were reported to be resistant to most of the commonly used antibiotics, show-

ing high levels of resistance to ampicillin, ceftazidime, and aztreonam (MIC values, up to 512 μg/ml) (3). In the present work, we observed that all those MDR bacteria were susceptible to the two compounds studied, especially in the presence of the efflux pump inhibitor. It appeared that isobavachalcone and diospyrone exhibited more significant effects against resistant strains compared with the activities of the reference drugs. In addition, Nishimura et al. (21) showed that isobavachalcone is nontoxic to healthy eukaryotic cells, supporting its possible clinical use.

The overall results of this investigation indicate that isobavachalcone and diospyrone may be interesting candidates for development as new antimicrobials with activity against MDR bacteria. The study also demonstrates that the drug efflux mechanism is one of the primary active defense mechanisms of bacterial cells against these molecules. This therefore indicates that the use of these types of natural products in combination with efflux pump inhibitors in future drug formulations should be considered.

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