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MECHANISMS OF RESISTANCE

Acquisition of Extended-Spectrum β-Lactamase GES-6 Leading to Resistance to Ceftolozane-Tazobactam Combination in *Pseudomonas aeruginosa*

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ABSTRACT A clinical *Pseudomonas aeruginosa* isolate resistant to all β-lactams, including ceftolozane-tazobactam and carbapenems, was recovered. It belonged to sequence type 235 and produced the extended-spectrum β-lactamase (ESBL) GES-6 differing from GES-1 by two amino acid substitutions (E104K and G170S). GES-6 possessed an increased hydrolytic activity toward carbapenems and to ceftolozane and a decreased susceptibility to β-lactamase inhibitors compared to GES-1, except for avibactam. We show here that resistance to ceftolozane-tazobactam may occur through acquisition of a specific ESBL in *P. aeruginosa* but that ceftazidime-avibactam combination remains an effective alternative.

KEYWORDS ESBL, GES-6, *Pseudomonas aeruginosa*, ceftolozane-tazobactam

Multidrug resistance, including carbapenem molecules, is currently a common feature encountered among *Pseudomonas aeruginosa* isolates. This is the consequence of the accumulation of multiple resistance determinants within that species, in addition to its intrinsic reduced susceptibility to many drugs (1). Resistance to carbapenems is either due to porin deficiency or to the acquisition of carbapenemase genes. Acquired carbapenemases are increasingly reported in *P. aeruginosa*, and there is currently a variety of those enzymes that have been identified in that species. The most commonly identified carbapenemases encountered in that species are the metallo-β-lactamases (IMP, VIM, SPM, GIM, and NDM enzymes) belonging to the Ambler class B (1). In addition, class A (KPC) and class D (OXA-181) carbapenem-hydrolyzing β-lactamases have been rarely identified in *P. aeruginosa* (2–4). One specific group of β-lactamases quite frequently identified in *P. aeruginosa* corresponds to GES enzymes, with GES-1 being an extended-spectrum β-lactamase sparing carbapenems (5), while the GES-2 and GES-5 variants have been shown to possess significant carbapenemase activities (6). GES-6 was first identified in a *Klebsiella pneumoniae* isolate from Greece (7) and later detected in *Enterobacter cloacae* and *Pseudomonas aeruginosa* clinical isolates recovered in Belgium (8) and Portugal (9), respectively, but the hydrolytic properties of GES-6 have not been characterized.

The ceftolozane-tazobactam (C/T) association, combining a novel cephalosporin with an established β-lactamase inhibitor, has recently been launched and is particularly effective against multidrug-resistant (including carbapenem-resistant) *P. aerugi-*

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TABLE 1 β-Lactams MICs for various *P. aeruginosa* and *E. coli* strains examined in this study[a]

β-Lactam(s)[b]	MIC (μg/ml)						
	P. aeruginosa				*E. coli*		
	BOR (GES-6)	PAO1 (pUCp24-GES-6)	PAO1 (pUCp24-GES-1)	PAO1	TOP10 (pUCp24-GES-6)	TOP10 (pUCp24-GES-1)	TOP10
Amoxicillin	>512	>512	>512	>512	>512	>512	4
Amoxicillin + CLA	>512	>512	>512	>512	16	32	3
Ticarcillin	512	>512	256	32	>512	64	8
Ticarcillin + CLA	128	>128	32	4	>512	2	1
Piperacillin	512	128	64	4	256	16	2
Piperacillin + TZB	32	32	4	1	32	1	0.5
Cefoxitine	>512	>512	>512	>512	128	2	2
Ceftazidime	128	>128	>128	8	64	8	0.25
Ceftazidime + CLA	64	128	32	1	16	0.5	<0.5
Ceftazidime + TZB	128	128	16	2	32	0.5	<0.5
Ceftazidime + AVI	2	2	2	1	1	0.25	0.25
Cefotaxime	512	64	64	16	32	8	0.06
Cefotaxime + CLA	256	32	32	16	0.5	<0.25	<0.06
Cefotaxime + TZB	128	32	16	16	0.5	<0.25	<0.06
Cefepime	32	32	8	4	0.5	2	0.5
Ceftolozane	128	128	32	2	128	32	0.25
Ceftolozane + CLA	64	128	16	<0.5	64	16	<0.25
Ceftolozane + TZB	32	64	16	0.25	64	8	<0.25
Aztreonam	8	2	2	3	0.38	0.09	0.06
Imipenem	16	2	1	1	0.25	0.12	<0.06
Meropenem	64	32	0.5	0.5	0.12	<0.06	<0.06
Ertapenem	256	256	8	8	2	<0.06	<0.06

[a]*P. aeruginosa* clinical isolate BOR producing GES-6, *P. aeruginosa* transformants PAO1 producing GES-6 and GES-1, respectively, *E. coli* TOP10 transformants PAO1 producing GES-6 and GES-1, respectively, and *P. aeruginosa* and *E. coli* recipient strains.
[b]Clavulanic acid (CLA) was added at 2 μg/ml, tazobactam (TZB) was added at 4 μg/ml, and avibactam (AVI) was added at 4 μg/ml.

nosa isolates (10). Although not affected by efflux pumps or porin loss, C/T was shown to be ineffective against carbapenemase-producing isolates, including OXA-539-, KPC-, and metallo-β-lactamase producers (11–13). This drug combination is licensed for complicated intra-abdominal infections and complicated urinary tract infections, although there are reports of successful treatments of ventilator-associated pneumonia (14, 15). The EUCAST breakpoint for C/T has been established as resistant at >4 μg/ml for *P. aeruginosa* (16).

There have been several reports of successful clinical experiences with this new drug combination for treating infections caused by multidrug-resistant *P. aeruginosa*; however, the emergence of resistance on therapy has been reported (11). In different studies evaluating the efficacy of C/T against *P. aeruginosa* isolates, an overall excellent activity was reported, even though resistant isolates were observed, corresponding mainly to overproducers of peculiar AmpC derivatives. Some of these isolates actually accumulate a significant number of other narrow-spectrum β-lactamases (17–20).

Our study was initiated by the isolation of a clinical *P. aeruginosa* isolate that is multidrug resistant and that shows particular resistance to C/T. Our goal was to decipher the molecular mechanism(s) leading to this resistance phenotype.

RESULTS AND DISCUSSION

Features of the clinical isolate. *P. aeruginosa* strain BOR was recovered from a respiratory specimen obtained from a 26-year-old patient hospitalized at an intensive care unit at Hospital Pellegrin (Bordeaux, France) in November 2017. A patient with congestive heart failure was transferred from a hospital in Portugal. She developed fever and was diagnosed with a respiratory tract infection. *P. aeruginosa* BOR was resistant to all β-lactams, including carbapenems, except to aztreonam (Table 1). This isolate was additionally resistant to all tested aminoglycosides (with a plazomicin MIC of 16 μg/ml) and fluoroquinolones, but it remained susceptible to fosfomycin and colistin. Of note, this isolate was resistant to the C/T combination (MIC of 32 μg/ml) (Table 1), even though the patient did not receive this antibiotic previously. Interest-

ingly, this isolate remained susceptible to ceftazidime-avibactam (CZA) at an MIC at 2 μg/ml (EUCAST breakpoint being at $>$8 μg/ml for *P. aeruginosa*). The patient was successfully treated with a combination of aztreonam, fosfomycin, and colistin.

Identification of the GES-6 β-lactamase. The production of an extended-spectrum β-lactamase (ESBL) and a carbapenemase was assessed using Rapid ESBL NP and Rapid Carba NP tests, respectively (21, 22). Both tests yielded positive results. PCR amplification, followed by sequencing, did not identify any carbapenemase gene but did identify a bla_{GES}-type ESBL gene. Further sequencing identified the bla_{GES-6} gene. β-Lactamase GES-6 differs from GES-1 by two amino acid substitutions (Glu104Lys and Gly170Ser) (6). Position 170 of GES-1 was previously shown to be critical, since a Gly-to-Asn change observed in GES-2 was shown to slightly expand the hydrolysis spectrum toward cephamycins and carbapenems (23). Then, the Gly-to-Ser substitution identified in GES-5 showed that the latter enzyme might definitely be considered a carbapenemase (24, 25). Here, the same Gly170Ser substitution was identified in GES-6, possibly leading to a significant carbapenemase activity, explaining the positivity of the Rapid Carba NP test. Apart from this critical Ser residue at position 170, the Lys at position 104 was previously shown to expand the hydrolytic activity of GES enzymes toward several β-lactams, including oxyimino-cephalosporins (26).

In addition, the intrinsic bla_{ampC} gene and its upstream-located sequences (containing the promoter sequences) were amplified by PCR and sequenced. It revealed a perfect identity with those of the wild-type strain PAO1, therefore suggesting that this bla_{ampC} gene was expressed at low level. This result correlates with the identical MIC value of aztreonam (6 μg/ml) observed for *P. aeruginosa* BOR when testing on Mueller-Hinton agar plates supplemented or not with cloxacillin (200 μg/ml).

Phylogenetic analysis. Multilocus sequence typing analysis performed as described previously (27) showed that isolate BOR belonged to sequence type 235 (ST235). This clone has been previously identified in different parts of the world and is considered "high-risk" in terms of dissemination (9). Of note, a GES-6-producing ST235 isolate was very recently identified in Portugal, but the susceptibility of that isolate to C/T was not evaluated (9). Since the patient from which isolate BOR was recovered had been transferred from Portugal, it is likely that our isolate corresponded to the same clone.

Relative properties of GES-6 and GES-1. Expression of the bla_{GES-1} and bla_{GES-6} genes in *E. coli* TOP10 conferred resistance or reduced susceptibility to all β-lactams except aztreonam (Table 1). However, MICs of imipenem were higher for *Escherichia coli*-producing GES-6 than GES-1, confirming that the Ser170 residue was involved in this higher carbapenemase activity of GES-6. Interestingly, MIC of ceftolozane was 4-fold higher for the GES-6- than for the GES-1-producing *E. coli* recombinant strain (128 versus 32 μg/ml). Furthermore, the addition of tazobactam (4 μg/ml) to ceftolozane only marginally decreased the MIC of ceftolozane (128 to 64 μg/ml) for the GES-6 *E. coli* recombinant strain, while it decreased to 8 μg/ml for the GES-1 producer.

Besides the activity of C/T combination, we measured the activity of the recently launched association CZA. Very interestingly, we observed that this combination remained very effective against both the GES-6 and GES-1 producers, while the ceftazidime-clavulanate or C/T combinations did not show significant efficacies against the GES-6 producer but were excellent against the GES-1 producer (Table 1). This observation suggested that avibactam is an excellent inhibitor of GES-6 activity, in contrast to other β-lactamase inhibitors.

In order to evaluate the impact of bla_{GES-6} expression in *P. aeruginosa* compared to bla_{GES-1}, recombinant plasmids were transformed in wild-type *P. aeruginosa* PAO1. MIC of ceftolozane was 2-fold higher in GES-6 than in the GES-1 producer (128 versus 32 μg/ml), and the addition of clavulanic acid or tazobactam did not significantly lower the MIC values (Table 1). Of note, the MICs of CZA remained in the susceptibility range for both transformants, further highlighting that avibactam very effectively inhibits these two GES enzymes.

TABLE 2 Kinetic parameters of purified β-lactamase GES-6[a]

	GES-6			GES-1		
β-Lactam	**k_{cat} (s^{-1})**	**K_m (μM)**	**k_{cat}/K_m (mM^{-1} s^{-1})**	**k_{cat} (s^{-1})**	**K_m (μM)**	**k_{cat}/K_m (mM^{-1} s^{-1})**
Benzylpenicillin	90	450	200	3	40	75
Amoxicillin	40	300	130	10	200	50
Ticarcillin	3	110	30	0.3	400	0.7
Piperacillin	20	300	660	8	900	9
Cephalothin	110	170	650	180	3,400	50
Cefoxitin	12	1,600	7	1	30	30
Cefotaxime	11	2,200	5	70	4,600	15
Ceftazidime	13	3,300	4	380	2,000	190
Cefepime	2	1,400	1.5	3	1,800	1.6
Ceftolozane	10	1,800	5.5	5	2,200	2.3
Aztreonam	<0.1	ND	ND	<0.1	ND	ND
Imipenem	0.25	8	30	0.003	45	0.06

[a]That is, in comparison to previously reported values of GES-1 (5). Standard deviations were below 15%. ND, not determined due to a low initial rate of hydrolysis. k_{cat}, catalytic efficiency; K_m, Michaelis constant; k_{cat}/K_m, specificity constant.

β-Lactamase GES-6 hydrolyzed all tested β-lactams, including ceftazidime, cefotaxime, cefoxitin, and carbapenems, but spared aztreonam, presenting a substrate profile mirroring that of metallo-β-lactamases. Kinetic data showed that GES-6 significantly hydrolyzed imipenem, with a catalytic efficiency above that of the broad-spectrum cephalosporins (Table 2). In contrast, the catalytic activity of GES-6 for ceftazidime was much less than that of GES-1 (Table 2). In addition, GES-6 hydrolyzed ceftolozane significantly but only twice more than GES-1 (Table 2). The reduced activity of C/T against GES-6 producers might thus be explained much more by a reduced sensitivity to tazobactam rather than an increased hydrolysis activity toward ceftolozane.

Since data obtained through this study suggested that GES-6 likely exhibited reduced sensitivity toward tazobactam, the determination of 50% inhibitory concentrations (IC_{50}) but also on-rate (k_2/K) and off-rate (k_{off}) were performed. Using nitrocefin as the substrate, the comparative inhibitory activities of clavulanic acid, tazobactam, and avibactam were evaluated for GES-6 and GES-1, respectively. Significant differences were observed for clavulanic acid and tazobactam (with sharped reduction of sensitivity toward GES-6), while the activity of avibactam remained almost identical (Table 3). This shows that the Ser residue at position 170, while significantly impacting the sensitivity of GES-6 respect to the "classical" β-lactamase inhibitors clavulanic acid and tazobactam, does not have any significant impact on the efficacy of the recently marketed avibactam molecule (which is not a β-lactam molecule by contrast to clavulanic acid or tazobactam).

Genetic support of bla_{GES-6}. Plasmid DNA of *P. aeruginosa* BOR was extracted by using the Kieser method (28) and analyzed by agarose gel electrophoresis. No plasmid could be visualized, and electro-transformation experiments performed using electrocompetent *P. aeruginosa* PAO1 recipient strain remained unsuccessful. Then, PCR mapping was performed and confirmed that the bla_{GES-6} gene was present in the same genetic context than that of the bla_{GES-6}-positive ST235 clone from Portugal, therefore

TABLE 3 IC_{50} and steady-state kinetic inhibition parameters of different β-lactamase inhibitors against GES-6 and GES-1

	IC_{50} (μM)[a]		K_i (μM)[b]		K_I (μM)[c]		k_{inact} (s^{-1})[d]		k_{inact}/K_I (μM^{-1} s^{-1})	
Inhibitor	**GES-6**	**GES-1**	**GES-6**	**GES-1**	**GES-6**	**GES-1**	**GES-6**	**GES-1**	**GES-6**	**GES-1**
Clavulanic acid	146	5.5	68	1.96	21	3.75	0.048	0.080	0.002	0.021
Tazobactam	16	3.6	7.7	0.9	5.15	0.8	0.11	0.046	0.021	0.06
Avibactam	3.7	3	2	1.5	1.1	0.83	0.09	0.063	0.08	0.076

[a]IC_{50} represents the concentration of a drug that is required for 50% inhibition of the enzymatic activity.
[b]K_i corresponds to the relative k_{off}/k_{on} ratio for the inhibitor for the enzyme.
[c]K_I corresponds to the concentration of inhibitor required to reach $1/2k_{inact}$.
[d]k_{inact} is the number of molecules of enzyme that are inactivated per second.

assessing its chromosomal location (29). Noteworthy, the *bla*GES-6 gene was located into a class 1 integron (In1076) being itself located into a chromosomal Integrative Conjugative Element (ICE) named ICEPae690 (29).

Conclusion. We reported here a multidrug-resistant *P. aeruginosa* isolate producing the GES-6 enzyme which is not only an ESBL but whose substrate profile also encompasses carbapenems and whose sensitivity to β-lactamase inhibitors is actually diminished compared to classical ESBLs. In a way, the substrate profile of GES-6 mirrors that of metallo-β-lactamases, with a broad-spectrum hydrolysis activity, including carbapenems, and some resistance to inhibitors, with the exception of avibactam. We also showed that β-lactamase GES-6 was responsible for acquired resistance to C/T, which usually is an effective therapeutic option for treatment of infections caused by multidrug-resistant *P. aeruginosa*. It is worth noting that GES-6 showed increased activity toward ceftolozane combined with reduced affinity toward tazobactam, both factors leading to the resistance phenotype observed. Increased activity toward ceftolozane may well be related to the presence of both Lys140 and Ser170, while reduced sensitivity to tazobactam is likely related primarily to Ser170.

GES-6 was produced by an ST235 strain, which is now increasingly reported. Recently, a fatal case of infection by an ST235 isolate was reported in the United Kingdom in a patient with cystic fibrosis who experienced a failure in C/T treatment (15). However, whether this strain produced GES-6 remains unknown. Very interestingly, isolate BOR remained susceptible to the CZA combination, and our data indicate that GES-6 is significantly inhibited by avibactam. This recent combination might offer an effective therapeutic option for treating infections caused by GES-6-producing *P. aeruginosa*.

MATERIALS AND METHODS

Bacterial strains and plasmids. *P. aeruginosa* R1190 producing GES-1 and *P. aeruginosa* BOR producing GES-6 were clinical isolates used in the present study, *P. aeruginosa* PAO1 and *Escherichia coli* TOP10 were used as recipient strains. Plasmid pUCp24 (30) was used as shuttle vector for cloning and expression in *E. coli* and *P. aeruginosa* recipient strains.

Susceptibility testing. Susceptibility testing was performed by disk diffusion assay (Sanofi-Diagnostic Pasteur, Marnes-la-Coquette, France) as previously described (31). Results were interpreted according to the CLSI guidelines (32). MICs were determined by Etest (AB bioMérieux; Solna, Sweden) on Mueller-Hinton agar plates at 37°C. Production of MBL was tested using Etest MBL (AB bioMérieux). The antimicrobial agents were obtained from standard laboratory powders and were used immediately after their solubilization. The agents and their sources were as follows: amoxicillin, piperacillin, cefepime, cephalothin, ceftazidime, and clavulanic acid (Sigma, Saint-Quentin Falavier, France); ticarcillin and cefoxitin (ROTH, Arlesheim, Switzerland), benzylpenicillin and tazobactam (Abcam, Cambridge, UK); cefotaxime, gentamicin, and aztreonam (Acros Organic, Geel, Belgium); imipenem (Carbosynth, Berkshire, UK); avibactam (MedChem Express, Luzern, Switzerland); and ceftolozane (ACS DOBFAR, Tribiano, Italy). MICs were determined by an agar dilution technique on Mueller-Hinton agar (Bio-Rad, Marnes-la-Coquette, France) with an inoculum of 10^4 CFU according to CLSI guidelines (32). All plates were incubated at 37°C for 18 h. The MICs of β-lactams were determined alone or in combination with a fixed concentration of clavulanic acid (2 μg/ml), tazobactam (4 μg/ml), or avibactam (4 μg/ml). The MIC of plazomicin was evaluated by the Etest method (Liofilchem, Italy).

In order to rapidly identify by a biochemical-based method whether *P. aeruginosa* BOR produced an ESBL or a carbapenemase, both the Rapid ESBL NP and the Rapid Carba NP tests were performed (21, 22).

Molecular characterization and cloning experiments. Whole-cell DNA of *P. aeruginosa* isolate BOR was extracted using QiaAmp minikit according to the manufacturer's recommendations (Qiagen, Courtaboeuf, France), and DNA was used as a template for the detection of different β-lactamases genes using specific primers (31).

In order to evaluate and compare the spectrum of hydrolysis of GES-6 to that of GES-1, cloning of the bla_{GES-6} and bla_{GES-1} genes was performed using the broad-host-range pUCp24 vector (30), followed by expression in the same *E. coli* TOP10 background (31). Selection was based on plates containing 100 μg of ampicillin per ml plus 30 μg of gentamicin per ml. The PCR amplicon encompassing the entire sequence of the bla_{GES} genes used for cloning was obtained with primers GES-fw (5′-GATGATGAGCTC TTCCATCTCAAGGGATCACC-3′) and GES-Rv (5′-GATGATGGATCCAGACGGGCGTCAACTATTTG-3′) (31). Cloning in pUCp24 was performed by BamHI and SacI restriction enzymes. The pUCp24-GES-1 and pUCp24-GES-6 were transformed in *E. coli* TOP10 and *P. aeruginosa* PAO1. Transformants were selected onto gentamicin containing Luria-Bertani agar plates (15 μg/ml).

Genetic context of bla_{GES-6}. In order to assess whether the bla_{GES-6} gene was indeed located on the chromosome of *P. aeruginosa* BOR as reported for the GES-6-producing ST235 isolate FFUP_PS_690 (29), PCR mapping was performed with a series of primers designed inside and outside the ICE reported in that genome (see Table S1 in the supplemental material).

β-Lactamase purification. Cultures of *E. coli* TOP10 harboring plasmid pUCp24-GES-1 and pUCp24-GES-6 were grown overnight at 37°C in 1 liter of brain heart infusion with amoxicillin (100 μg/ml). The bacterial suspension was pelleted, resuspended in 10 ml of 100 mM phosphate buffer (pH 7), disrupted by sonification (20 min for 30 s of sonication and 50 s of rest at 20 kHz with a Vibra Cell 75186), and centrifuged for 1 h at 11,000 × *g* and 4°C. This suspension was dialyzed overnight against 20 mM Bis-Tris (pH 6.5) at 4°C (31). The enzyme extract was loaded onto a pre-equilibrated HiTrap Q HP column (GE Healthcare) with the same buffer. The resulting enzyme extract was recovered in the flowthrough and dialyzed against 20 mM Tris-HCl (pH 7.5) overnight at 4°C. This extract was then loaded onto a pre-equilibrated (20 mM Tris-HCl [pH 7.5]) HiTrap Q HP column. Proteins were then eluted with a linear NaCl gradient (0 to 1 M). The fractions showing the highest β-lactamase activity were pooled and dialyzed against 100 mM phosphate buffer (pH 7.0), prior to a 10-fold concentration with a Vivaspin 20 (GE Healthcare). The purified β-lactamase extract was immediately used for enzymatic determinations.

Determination of the β-lactamase relative molecular mass. The relative purity of β-lactamase GES-6 was estimated by sodium dodecyl sulfate (SDS)–12% polyacrylamide gel electrophoresis analysis. Enzyme extracts and marker proteins were boiled for 10 min in a 1% SDS–3% β-mercaptoethanol solution and then subjected to electrophoresis (25 mA for 4 h) at room temperature.

Kinetic measurements. Purified β-lactamase was used for kinetic measurements performed at 30°C in 100 mM sodium phosphate (pH 7.0). The initial rates of hydrolysis were determined with a Genesys 10S UV-Vis spectrophotometer (Thermo Scientific). The following wavelengths and absorption coefficients were used: benzylpenicillin (232 nm/$\Lambda\varepsilon$ = −1,100 M^{-1} cm^{-1}), amoxicillin (240 nm/$\Lambda\varepsilon$ = −1,100 M^{-1} cm^{-1}), ticarcillin (235 nm/$\Lambda\varepsilon$ = −1,050 M^{-1} cm^{-1}), piperacillin (235 nm/$\Lambda\varepsilon$ = −1,070 M^{-1} cm^{-1}), cephalothin (262 nm/$\Lambda\varepsilon$ = −7,960 M^{-1} cm^{-1}), cefoxitin (265 nm/$\Lambda\varepsilon$ = −7,380 M^{-1} cm^{-1}), ceftazidime (260 nm/$\Lambda\varepsilon$ = −8,660 M^{-1} cm^{-1}), cefepime (264 nm/$\Lambda\varepsilon$ = −8,240 M^{-1} cm^{-1}), cefotaxime (265 nm/$\Lambda\varepsilon$ = −6,260 M^{-1} cm^{-1}), ceftolozane (254 nm/$\Lambda\varepsilon$ = −6,810 M^{-1} cm^{-1}), imipenem (297 nm/$\Lambda\varepsilon$ = −9,210 M^{-1} cm^{-1}), and aztreonam (318 nm/$\Lambda\varepsilon$ = −640 M^{-1} cm^{-1}).

The 50% inhibitory concentrations (IC_{50}s) were determined for clavulanic acid, tazobactam, and avibactam. Various concentrations of these inhibitors were preincubated with the purified enzyme for 5 min at 30°C to determine the concentrations that reduced the hydrolysis rate of 200 μM nitrocefin by 50%. The results are expressed in micromolar units. The total protein content was measured by Bradford assay.

The K_i value was determined by direct competition assays using 200 μM nitrocefin. Inverse initial steady-state velocities ($1/V_0$) were plotted against the inhibitor concentration ([I]) to obtain a straight line. The plots were linear and provided y-intercept and slope values used for K_i determination. K_i was determined by dividing the value for the *y*-intercept by the slope of the line and then corrected by taking into account the nitrocefin affinity by the following equation: K_i (corrected) = K_i (observed)/(1 + [S]/K_m) (33). Here, "[S]" is the concentration of nitrocefin (200 μM) used in the assay and K_m is the Michaelis constant determined for nitrocefin (108 μM for GES-6 and 115 μM for GES-1). Progress curves with a fixed concentration of enzyme and nitrocefin, and increasing inhibitor concentrations were used to determine the k_{inact} and K_I values, as previously described. The k_{obs} was plotted versus the inhibitor concentrations of the experiment and fit to determine k_{inact} according to the following equation: $k_{obs} = k_{inact}[I]/(K_I + [I])$ (34). The K_I value obtained was corrected using the same equation as that used to calculate the K_i. To calculate k_{inact}, the k_{obs} versus $k_{obs}/[I]$ value was plotted, considering the following: $V_0 = V_{max} - K_m \times (V_0/[S]) \rightarrow K_{obs} = k_{inact} - K_I \times (K_{obs}/[I])$.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.01809-18.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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