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Kinetic Analysis of *Enterococcus faecium* L,D-Transpeptidase Inactivation by Carbapenems

Vincent Dubée,[a,b,c] Michel Arthur,[a,b,c] Hélène Fief,[d,e] Sébastien Triboulet,[a,b,c] Jean-Luc Mainardi,[a,b,c,f] Laurent Gutmann,[a,b,c,f] Matthieu Sollogoub,[d,e] Louis B. Rice,[g] Mélanie Ethève-Quelquejeu,[d,e] and Jean-Emmanuel Hugonnet[a,b,c]

Centre de Recherche des Cordeliers, LRMA, Équipe 12, Université Pierre et Marie Curie, Paris, France[a]; INSERM, U872, Paris, France[b]; Université Paris Descartes, Sorbonne Paris Cité, Paris, France[c]; Institut Parisien de Chimie Moléculaire, Équipe GOBS, Université Pierre et Marie Curie, Paris, France[d]; Centre National de la Recherche Scientifique, Paris, France[e]; AP-HP, Hôpital Européen Georges Pompidou, Paris, France[f]; and Rhode Island Hospital, Brown University, Providence, Rhode Island, USA[g]

Bypass of classical penicillin-binding proteins by the L,D-transpeptidase of *Enterococcus faecium* (Ldt_{fm}) leads to high-level ampicillin resistance in *E. faecium* mutants, whereas carbapenems remain the lone highly active β-lactams. Kinetics of Ldt_{fm} inactivation was determined for four commercial carbapenems and a derivative obtained by introducing a minimal ethyl group at position 2. We show that the bulky side chains of commercial carbapenems have both positive and negative effects in preventing hydrolysis of the acyl enzyme and impairing drug binding.

The last cross-linking step of peptidoglycan synthesis is catalyzed by high-molecular-weight penicillin-binding proteins (PBPs) with D,D-transpeptidase activity, which have historically been considered the lone essential targets of β-lactam antibiotics. However, we recently showed that carbapenems inactivate a second class of cross-linking enzymes (9), the L,D-transpeptidases, which bypass the classical D,D-transpeptidases by forming unusual peptidoglycan cross-links and confer high-level ampicillin resistance in mutants of *Enterococcus faecium* selected *in vitro* (10). Transpeptidases with D,D- and L,D-specificities are structurally unrelated and harbor different catalytic nucleophiles in the form of invariant Ser (14) and Cys (1, 7) residues. L,D-Transpeptidases are sporadically distributed in Gram-positive and Gram-negative bacteria and generally have nonessential roles in peptidoglycan synthesis (6, 11), except in *Mycobacterium tuberculosis* (4), *Mycobacterium abscessus* (5), and *Clostridium difficile* (13). Carbapenems are currently being investigated as therapeutic alternatives in the treatment of extensively drug-resistant tuberculosis (3, 8, 17), and L,D-transpeptidases are the likely targets of the drugs in *M. tuberculosis* (2, 4). Here, we investigated kinetics of inhibition of a model L,D-transpeptidase from *E. faecium* (Ldt_{fm}) by different carbapenems in order to gain insight into the role of the antibiotic side chains in the efficiency of L,D-transpeptidase inactivation.

Four carbapenems—imipenem, meropenem, ertapenem, and doripenem (Fig. 1A)—are used to treat human infections in North America and Europe (12). These drugs differ by the nature of the side chain (R [Fig. 1B]) and the absence (imipenem) or presence (meropenem, doripenem, and ertapenem) of a methyl group (Fig. 1A) that has been introduced into the carbapenem ring to decrease inactivation by human renal dehydropeptidase I (12). To gain insight into the effects of structural variations in the carbapenem side chains on the efficiency of Ldt_{fm} inactivation, we compared the four aforementioned drugs and synthesized an additional carbapenem, designated β-32, which contains a minimal ethyl side chain (Fig. 1C). Ldt_{fm} was purified from recombinant *Escherichia coli* by affinity and size exclusion chromatographies (16). Kinetic constants of Ldt_{fm} were determined by spectrofluorimetry and spectrophotometry (16) for the three steps of the reaction, which comprises noncovalent binding of the drug, acylation of the catalytic Cys residue, and hydrolysis of the corresponding acyl enzyme (Fig. 1D). Replacement of the meropenem side chain by an ethyl group (meropenem versus carbapenem β-32) led to a 15-fold increase in the second-order rate constant k_1 for formation of the noncovalent complex (Fig. 2A). Thus, the bulky side chain of meropenem (Fig. 1A) impairs access of the drug to the active site. In contrast, the rate constant of the chemical step of the reaction (k_{inact}) was similar for meropenem and β-32 (1.3 and 1.9 min^{-1}, respectively). This observation indicates that the side chain of meropenem is nonessential for efficient acylation.

In order to compare the hydrolysis rates (k_{hydrol}) of the acyl enzymes, meropenem and β-32 were incubated with increasing concentrations of Ldt_{fm}, and rupture of the β-lactam ring was monitored by spectrophotometry over 1,000 min (Fig. 2B). Under the conditions used, Ldt_{fm} was totally converted to acyl enzyme in less than 3 min, and the subsequent decrease in absorbance revealed slow enzyme turnover (Fig. 2B). The hydrolysis rate (k_{hydrol}) was determined by subtracting the rate of spontaneous hydrolysis observed in the absence of enzyme. Replacing the meropenem side chain by an ethyl side chain led to a 3-fold increase in k_{hydrol} indicating that the meropenem side chain slows acyl enzyme hydrolysis. Together, these results show that the bulky side chain of meropenem has a negative effect on drug binding but a positive effect on acyl enzyme stability.

Since kinetic analyses of Ldt_{fm} inactivation were previously performed only with imipenem (16), we determined kinetic constants for Ldt_{fm} inactivation of commercially available carbapenems (Fig. 3). Imipenem was the most efficient Ldt_{fm} inhibitor, with respect to both drug binding ($k_1 = 0.065\ \mu M^{-1}\ min^{-1}$) and

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Address correspondence to Jean-Emmanuel Hugonnet, jean-emmanuel.hugonnet@crc.jussieu.fr, or Mélanie Ethève-Quelquejeu, melanie.etheve@upmc.fr.

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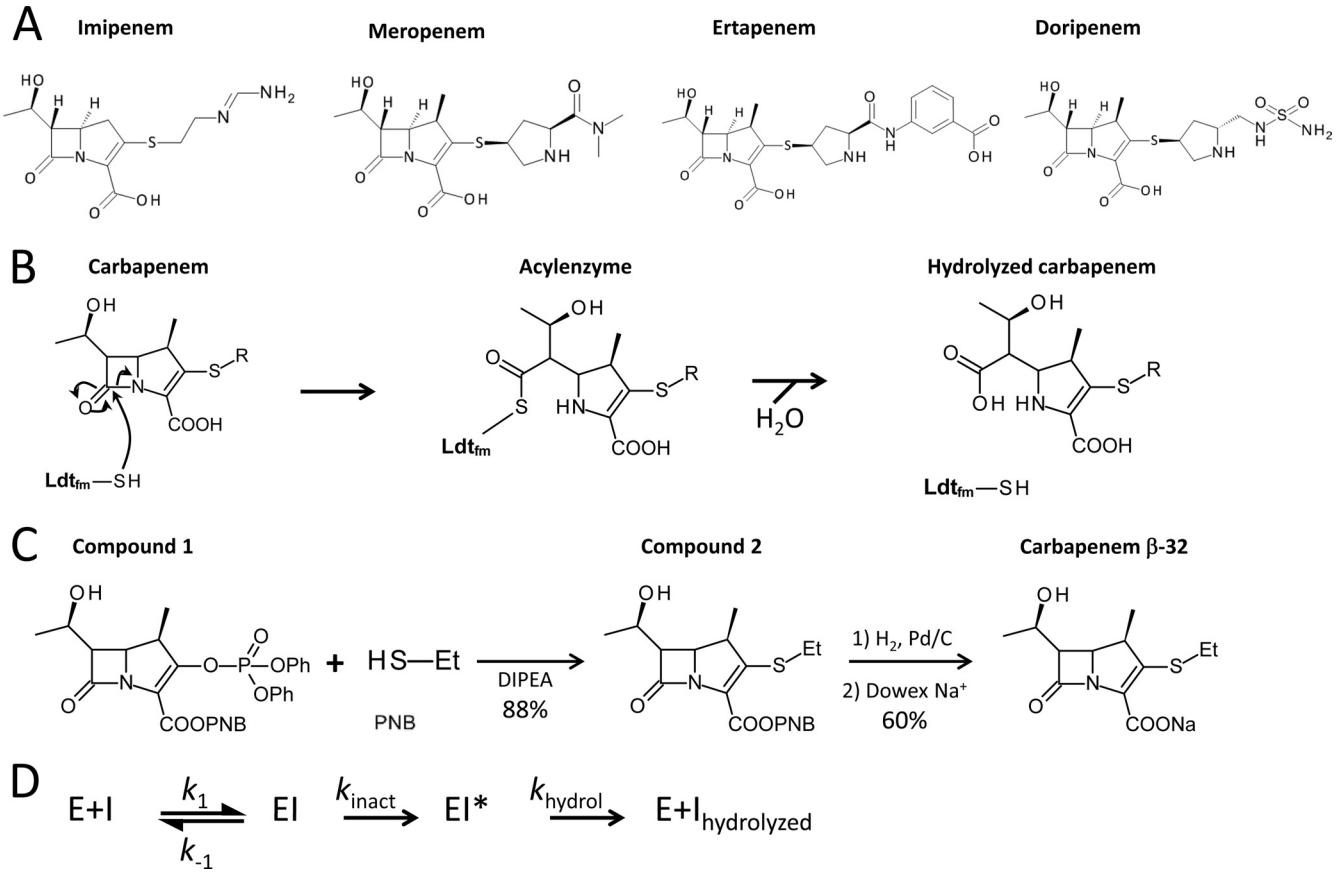

FIG 1 (A) Structures of carbapenems. (B) Mechanism of inactivation of *E. faecium* L,D-transpeptidase (Ldt_{fm}) by carbapenems. The sulfhydryl (SH) of the active-site cysteine of Ldt_{fm} attacks the carbonyl of the β-lactam ring to form a covalent adduct (acyl enzyme). Hydrolysis of the resulting thioester bond leads to native enzyme and hydrolyzed carbapenem. R, carbapenem side chain. (C) Synthesis of carbapenem β-32. An addition-elimination reaction in *N*,*N*-diisopropylethylamine (DIPEA) afforded compound 2 from commercially available compound 1 with an 88% yield (15). Palladium-catalyzed (Pd/C) hydrogenation followed by ion exchange (Dowex resin) (15) afforded the sodium salt of carbapenem β-32 with a 60% yield. Et, ethyl; PNB, paranitrobenzoate. (D) Reaction scheme used in kinetic analyses. Binding of carbapenem (I) to Ldt_{fm} (E) leads to reversible formation of a noncovalent complex (EI). Formation of the acyl enzyme (EI*) and its hydrolysis are irreversible. k_1 and k_{-1} are second- and first-order constants for formation and dissociation of the noncovalent complex. k_{inact} and k_{hydrol} are the rate constants for the acylation and hydrolysis reactions.

acylation (k_{inact} = 4.5 min^{-1}). The other carbapenems, meropenem, ertapenem, and doripenem, bound to Ldt_{fm} less rapidly (k_1 = 0.011 to 0.018 μM^{-1} min^{-1}). The rates of acylation were also lower (k_{inact} = 1.3 to 1.8 min^{-1}). Thus, kinetics of Ldt_{fm} inactivation by meropenem, ertapenem, and doripenem were very similar. This observation confirms that the carbapenem side chains do not contribute to efficient drug binding or acylation, in agreement with the aforementioned comparison of meropenem and β-32. The absence of a methyl group on the carbapenem ring of imipenem may account for the ca. 3-fold-higher Ldt_{fm} acylation rate observed for this drug (k_{inact} = 4.5 min^{-1}) in comparison to rates for other carbapenems (k_{inact} = 1.3 to 1.9 min^{-1}). The rate constant for binding of imipenem (k_1 = 0.065 μM^{-1} min^{-1}) was intermediate between that of β-32 (k_1 = 0.17 μM^{-1} min^{-1}) and those of meropenem, ertapenem, and doripenem (k_1 = 0.011 to 0.018 μM^{-1} min^{-1}). The rate constant k_1 therefore appears to be inversely correlated with the size of the side chain (Fig. 1A).

Comparison of acyl enzyme stability (Fig. 3) indicated that the rate constant for hydrolysis was 2- to 3-fold higher for carbapenem β-32 (1.87×10^{-3} min^{-1}) than for ertapenem, doripenem, and meropenem (0.60 to 0.91 $\times 10^{-3}$ min^{-1}). These results confirmed that the bulky side chain of carbapenems stabilizes the acyl enzyme. Hydrolysis of imipenem by Ldt_{fm} was undetectable ($<0.37 \times 10^{-3}$ min^{-1}).

In order to compare the antibacterial activities of carbapenems, MICs were determined for *E. faecium* M512, which is resistant to ampicillin due to activation of the L,D-transpeptidation pathway. M512, formerly designated D344M512 (10), was obtained by serial selection on media containing increasing concentrations of ampicillin and derives from hypersusceptible *E. faecium* D344S, which does not harbor the gene encoding low-affinity PBP5. MICs of carbapenems were determined in the presence of ampicillin (200 μg/ml), a drug concentration which was chosen to fully inactivate the D,D-transpeptidation pathway (9). Under such conditions, M512 relies exclusively on Ldt_{fm} for peptidoglycan cross-linking, thereby providing an estimate of *in vivo* enzyme inhibition (9). The lowest MIC (0.5 μg/ml) was observed for imipenem that combines relatively high k_1 and k_{inact}. For β-32, the large increase in k_1 was not associated with the expected decrease of the MIC, indicating, as expected, that acyl enzyme stability and efficient drug binding are both important for antibacterial activity of carbapenems. Together, these data show that the bulky side

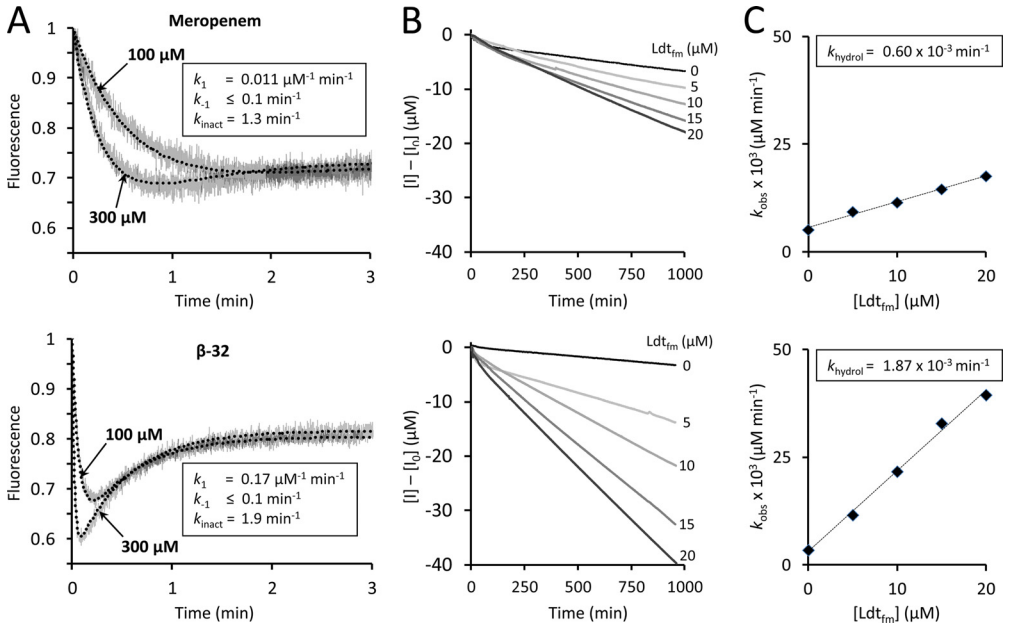

FIG 2 Determination of kinetic constants of Ldt_{fm}. (A) The kinetic constants k_1, k_{-1}, and k_{inact} were determined by spectrofluorimetry, as previously described (16). Ldt_{fm} (10 μM) was incubated with meropenem or carbapenem β-32 (100 and 300 μM) in sodium phosphate buffer (100 mM; pH 6.0) and fluorescence was recorded at 10°C on a Cary Eclipse spectrofluorimeter (Varian SA) equipped with a stopped-flow apparatus (RX-2000; Applied Photophysics). Trp residues were excited at 225 nm with a slit of 5 nm and an optical path length of 2 mm. Fluorescence emission was determined at 335 nm with a slit of 5 nm and an optical path length of 10 mm (gray lines). The detector voltage was set to 600 V. Fluorescence was normalized to the fluorescence intensity of free Ldt_{fm}, which was the lone form of the enzyme at the onset of the reaction. Kinetic constants were determined by fitting simulations (dotted lines) to experimental data (gray lines), as previously described (16). Fitting was simultaneously performed with four drug concentrations (30, 100, 300, and 1,000 μM; results for only two concentrations are shown for clarity). (B) The kinetic constant k_{hydrol} was determined spectrophotometrically. Carbapenems (100 μM) were incubated with Ldt_{fm} (0, 5, 10, 15, and 20 μM) in sodium phosphate buffer (100 mM; pH 6.0), and absorbance at 299 nm was recorded at 20°C on a Cary 100-Bio spectrophotometer (Varian SA). Alkaline hydrolysis of the β-lactam ring of meropenem and of β-32 led to similar decreases in the molar extinction coefficient ($\Delta\varepsilon = -7{,}200$ and $-7{,}400$ M^{-1} cm^{-1}, respectively). The velocity of the reaction (k_{obs}) increased with Ldt_{fm} concentration, indicating that the enzyme catalyzed carbapenem hydrolysis in addition to spontaneous drug hydrolysis. (C) Hydrolysis velocity (k_{obs}) was plotted as a function of Ldt_{fm} concentration, and enzyme turnover (k_{hydrol}) was deduced from the slope.

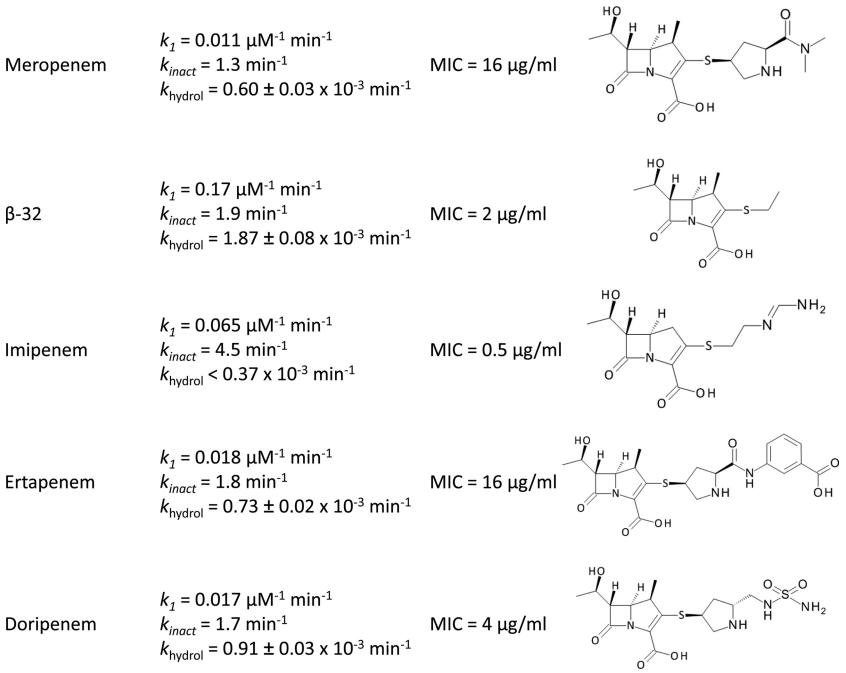

FIG 3 Inactivation of Ldt_{fm} by various carbapenems. Kinetic constants were determined by spectrofluorimetry (k_1 and k_{inact}) and spectrophotometry (k_{hydrol}). MICs of carbapenems for *E. faecium* M512 were determined at 37°C in brain heart infusion broth (Difco) containing 200 μg/ml of ampicillin to inactivate the D,D-transpeptidases (9).

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