strands of the (D)-pr-, (L)-pl-, and (D)-px series with complementary strands of the (D)-r series. For (L)-pr sequences, see (5). β-pr = ribo-, α-pl = lyxo-, β-px = xylo-, and α-pa = arabinopyranosyl; D = 2,6-diaminopurine.
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Reduced Immunotoxicity and Preservation of Antibacterial Activity in a Releasable Side-Chain Carbapenem Antibiotic

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A carbapenem antibiotic, L-786,392, was designed so that the side chain that provides high-affinity binding to the penicillin-binding proteins responsible for bacterial resistance was also the structural basis for ameliorating immunopathology. Expulsion of the side chain upon opening of the beta-lactam ring retained antibacterial activity while safely expelling the immunodominant epitope. L-786,392 was well tolerated in animal safety studies and had significant in vitro and in vivo activities against methicillin- and vancomycin-resistant Staphylococci and vancomycin-resistant Enterococci.

The increasing prevalence of resistance in Staphylococci and Enterococci to currently available antimicrobials has resulted in the significant diminution of therapeutic options available. Patients infected with multi–drug resistant organisms are once again succumbing to sepsis (*1*). One approach to counter this is to directly target the molecular mechanism of resistance in an existing class of antimicrobials, restoring their effectiveness. Methicillin resistance in Staphylococci is generally dependent on the production of a unique penicillin-binding protein (PBP), PBP2a, which like other high molecular weight PBPs, catalyzes the transpeptidation of peptidoglycan. PBP2a has a relatively low affinity to all the common β-lactam antibiotics and, in the presence of antibiotic, is able to take on the critical tasks of cell wall remodeling normally performed by the more antibiotic-susceptible PBPs (*2*).

Carbapenems acylate a broad spectrum of PBPs with high affinity. They are rapidly bactericidal, with potent activity against methicillin-sensitive staphylococci. These intrinsic properties, together with multiple PBP targets, result in low frequency of resistance selection. In addition, the carbapenem nucleus is resistant to most serine β-lactamases, whether of staphylococcal, enterococcal, or Gram-negative origin (*3*). We previously described a series of carbapenems with improved affinities for PBP2a, and concomitant activity against methicillin-resistant *Staphylococcus aureus* (MRSA) (*4*). Improved affinity for PBP2a was related to the presence of a large lipophilic substituent attached to the carbapenem nucleus. Unfortunately, carbapenems optimized for their PBP2a affinity provoked immune responses in rhesus monkeys (*5*), including high-frequency immune-mediated hemolytic anemia with Coombs positivity, a peripheral blood lymphocytosis, and diffuse lymphoid hyperplasia of spleen and lymph nodes at subhemolytic doses (15 mg/kg body weight/day of L-742,728). These nonanaphylactic autoimmune syndromes are occasionally observed with lower frequency and at higher doses in response to β-lactams in clinical use (*6*).

Sera from rhesus macaques exposed to the carbapenems L-742,728, L-741,462, and L-695,256 (*7*) all showed high titer, drug-specific antibodies that could be affinity purified on drug-sepharose columns and agglu-

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Table 1. Comparative in vitro antibacterial activity of L-786,392, imipenem, and vancomycin on Staphylococci and Enterococci.

Organism (number of strains)	MIC_{90} (μg/ml)		
	L-786,392	Vancomycin	Imipenem
Methicillin-susceptible *S. aureus* (15)	0.06	2	0.03
Methicillin-resistant *S. aureus* (42)	4	2	256
Methicillin-susceptible coagulase-negative *Staphylococcus* spp. (23)	0.016	2	0.03
Methicillin-resistant coagulase-negative *Staphylococcus* spp. (25)	4	4	256
Vancomycin-susceptible *E. faecalis* (17)	1	2	1
Vancomycin-resistant *E. faecalis* (15)	4	1024	2
Vancomycin-susceptible *E. faecium* (12)	2	2	64
Vancomycin-resistant *E. faecium* (31)	8	2048	>128
Enterococcus gallinarum (14)	1	8	16

tinated rhesus red blood cells (RBCs) haptenated with 5 mg/ml of these carbapenems (*8, 9*). Antibodies eluted from the surface of affected erythrocytes possessed the same specificity (*10*). Serum antibodies showed distinct patterns of cross-reactivity by free-drug competition in hemagglutination assays using in vitro haptenated erythrocytes with titers in the range of 1:512 to 1:2048. (Fig. 1A). These carbapenems (aminolysed structures in Fig. 1) all share structural elements of the MRSA pharmacophore and cationic substituents (such as diazonia-bicyclo-octyl or imidazolium groups). Other carbapenems, such as meropenem, share only the same carbapenem nucleus. It was therefore possible to measure the relative contributions of ring-opened carbapenem nucleus and side chain to the immunogenic epitopes generated in rhesus monkeys. Antisera from rhesus monkeys exposed to L-742,728 did not cross-react with meropenem-haptenated rhesus RBCs. We concluded that antisera specificity was determined solely by one or more immunodominant structural components of the side chain in the 2-position, that is, the essential MRS-pharmacophore necessary for high affinity to PBP2a, and not to the opened carbapenem nucleus. Drug-specific T cell proliferation (*11*) was also dependent on the presence of the MRS-active side chain (Fig. 1B). We further concluded that both humoral and cellular responses to L-742,728 were dependent on an immunodominant side-chain epitope and were distinct from the carbapenem nucleus.

To limit immunotoxicity in a situation where the immunodominant epitope was also a key contributor to PBP2a binding, we coupled the β-lactam ring opening with a chemical fragmentation resulting in the quantitative expulsion of the carbapenem side chain upon opening of the β-lactam ring (Fig. 2). In this way, the pharmacophore was present to support the initial interaction of the carbapenem with the hydrophobic pocket of PBP2a, but the immunodominant epitope would be expelled once either acylation of the specific target had taken place or nucleophilic attack by bystander host proteins had occurred. The remaining ring-opened nucleus had to be similar both in size and structure to that of well-tolerated carbapenems such as imipenem, and although capable of eliciting an antibody response analogous to that seen for the benzylpenicilloyl epitope, it should not result in high-frequency immunopathology. A reduction of this model to experimental practice resulted in the identification of the 2-(naphthosultamyl)methyl-carbapenem L-786,392 (Fig. 2), which did not cross-react with specific L-742,728 antisera or stimulate the proliferation of L-742,728–dependent lymphocytes (Fig. 1).

L-786,392 was effective against MRSA and methicillin-resistant coagulase-negative staphylococci [minimal inhibitory concentration $(MIC)_{90} \leq 4$ μg/ml], and resistant *Enterococcus faecium* ($MIC_{90} \leq 8$ μg/ml) and *E. faecalis* ($MIC_{90} \leq 4$ μg/ml) (Table 1) (*12*). Good activity (MIC ≤ 2 μg/ml) was demonstrated against vancomycin-intermediate *S. aureus* (VISA) strains (*13*). L-786,392 showed bactericidal activity against both MRSA (5 log reduction at the MIC in 24 hours) and on vancomycin-resistant Enterococci (3 log reduction in 8 hours at the MIC). These activities correlated with the improved binding affinity that L-786,392 showed for key PBPs (*14*), specifically the PBP2a (IC_{50} = 1.7 μg/ml, compared to 128 μg/ml for

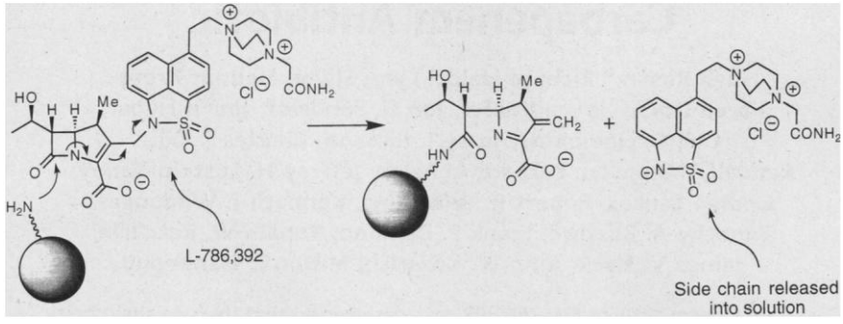

Fig. 2. The releasable hapten hypothesis. Coupled β-lactam ring opening with a chemical fragmentation results in the release of the immunogenic side chain. The figure illustrates primary aminolysis, but also is applicable to spontaneous or enzymatic hydrolysis and to PBP active-site serinolysis.

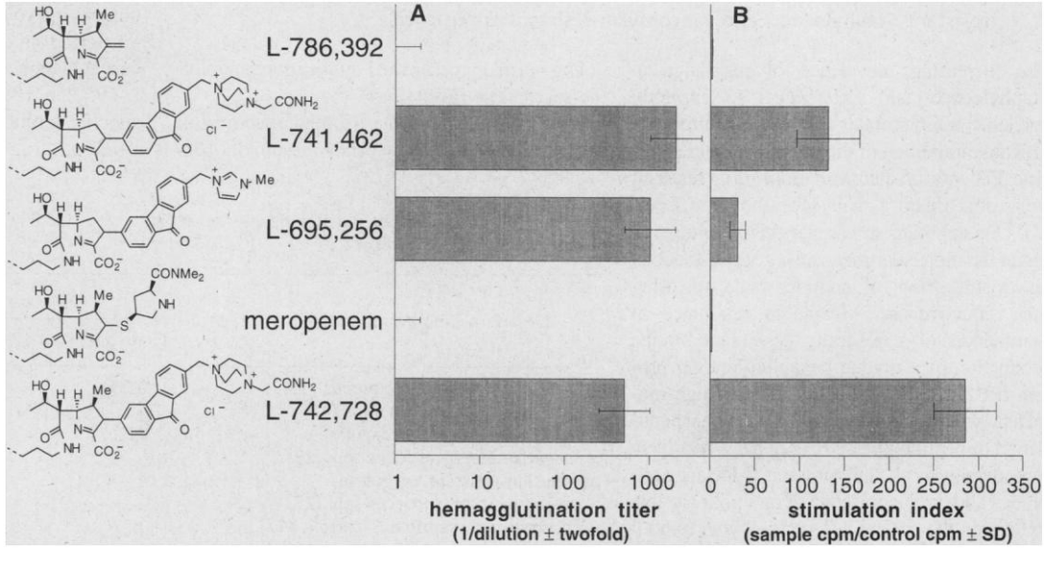

Fig. 1. (**A**) Cross-reactivity of L-742,728 antiserum in the IAT (*9*). Plasma sample was obtained from a rhesus monkey on day 23 after the start of nine doses (50 mg/kg/day) of L-742,728. Test cells: washed erythrocytes from a β-lactam–naïve rhesus were incubated for 2 hours at 37°C with 5 mg/ml of compounds in neutral buffered saline. The expected surface residue structures formed from the named carbapenems are shown to the left. Antibody specificity was associated with the aryl-cationic side chain. There was no recognition of the nuclear portion despite its presence on the immunizing hapten. (**B**) Cross-reactivity of L-742,728 T cell stimulation in the LTT (*11*). Peripheral blood mononuclear cells were obtained from the same rhesus blood sample described in (A). Continuous exposure of test compounds at 1.2 mg/ml was maintained for 72 hours prior to harvest. Cellular specificity was qualitatively similar to humoral response in (A). The common nuclear portion was not recognized in the response despite its presence on the immunizing hapten.

imipenem) of MRSA (*15*) and PBP5 (IC_{90} = 2.4 μg/ml, compared to 19 μg/ml for imipenem) of *E. hirae* (*16*).

L-786,392 was effective in complement C5-deficient DBA/2N mice against the homotypically resistant and β-lactamase positive MRSA "76 Virginia" clinical strain at a high inoculum [1.71×10^7 colony-forming units (CFU) per mouse] (*17*). L-786,392 [median effective dose (ED_{50}) = 3.9 mg/kg per dose] was more active than vancomycin (ED_{50} = 9.2 mg/kg per dose). Imipenem, with its low PBP2a affinity, was significantly inferior in protecting the animals (ED_{50} = 31.5 mg/kg). L-786,392 was as active as imipenem (ED_{50} = 0.024 and 0.035 mg/kg, respectively) against the methicillin-sensitive *S. aureus* "SMITH" strain.

To determine whether the side chain was being expelled upon opening of the β-lactam ring, we analysed the products formed after dehydropeptidase (DHP, renal dipeptidase E.C. 3.4.13.19) degradation. DHP readily hydrolyzes L-786,392 by opening the β-lactam ring and is therefore expected to release the side chain. DHP treatment of L-786,392 generated a pair of product peaks upon high-performance liquid chromatography (HPLC) analysis (*18*). The kinetics of disappearance of L-786,392 matched the appearance of ring-opened β-lactam and the liberated side chain (Fig. 3B) within experimental error, supporting the immediate and complete expulsion reaction. The expected identities of the resulting fragments shown in Fig. 3A were confirmed by liquid chromatography–mass spectrometry (LC-MS) (*19*) and nuclear magnetic resonance spectroscopy.

To demonstrate side-chain expulsion upon aminolysis at physiological pH, reaction of L-786,392 with Tris was used as a model. The only products formed were confirmed by LC-MS to be the side chain and the aminoacyltris-conjugated nucleus (Fig. 3A). The rate of aminolysis of L-786,392 was the same as imipenem (k = 0.0127 min^{-1} for both compounds) and similar to L-742,728. The designed fragmentation mechanism should offer little opportunity for immune presentation of the side chain.

The immunopathology observed in animal safety studies with MRS-active carbapenems was dose-dependent. Effective comparison of agents requires similar levels of drug exposure, best measured as the plasma concentration area under the curve (AUC) of the pharmacokinetic curve in the target species. The intravenous pharmacokinetics of L-786,392 were determined at 10 mg/kg (*20*). L-786,392 has a plasma half-life [$T_{1/2}$ (β) = 1.34 hours] and an AUC of 119 μg·hour/ml (*21*). Although the plasma half-life of L-742,728 was 1.35 hours, the AUC was 56.44 μg·hour/ml. The level of drug exposure in the 4-week study was therefore twofold greater for L-786,392 compared to the equivalent dose of L-742,728.

Support for the amelioration of immunotoxicity by expelling the MRS-pharmacophore was evident from the absence of any pathological changes in rhesus monkeys after administration of L-786,392 at doses up to 100 mg/kg/day for 4 weeks (n = 8). There was no anemia, lymphocytosis, nor any evidence of increases in lymphoid tissue weights nor histological evidence for lymphoid hyperplasia (Table 2). Only low titers of drug-specific antibodies were observed in rhesus monkeys (30- to 60-fold less than seen with L-742,728). These antibodies were specific for the ring-opened β-lactam moiety (like penicillin) and not the immunodominant 2-aryl side chain as seen with previous compounds. Thus, hemagglutination was inhibited by the ring-opened nucleus (<0.1 μg/ml) and not by a fully elaborated side chain at up to 250 μg/ml, a measured selectivity of >2500:1. In this respect, L-786,392 at dos-

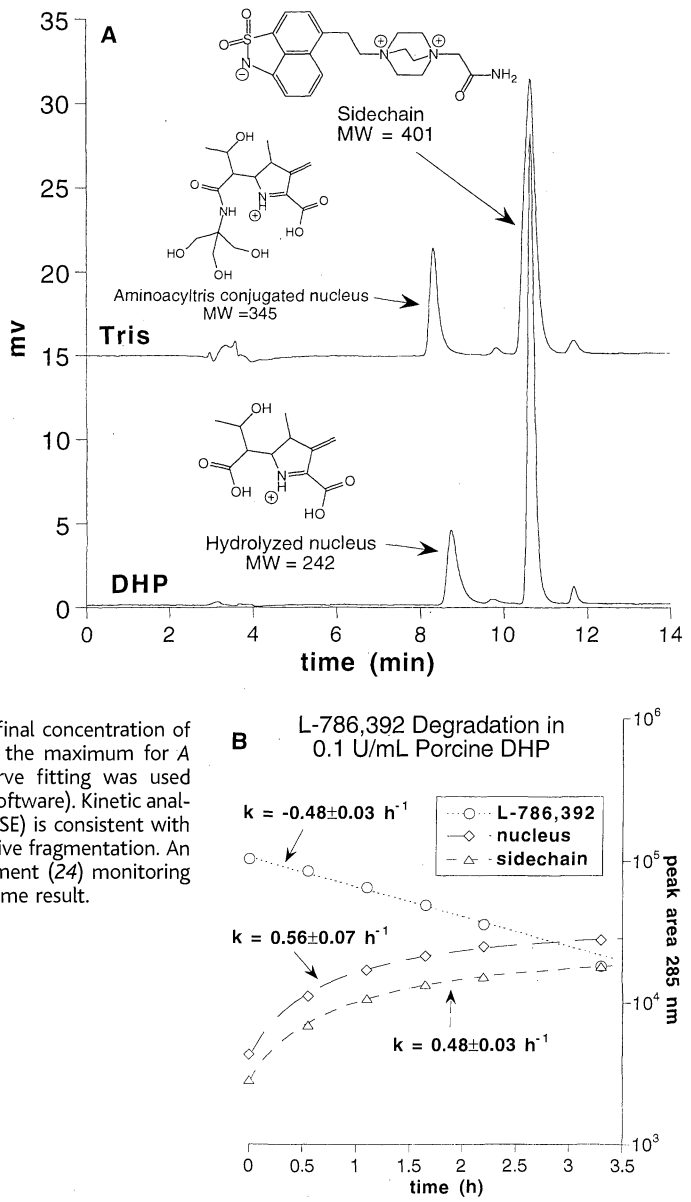

Fig. 3. (**A**) Monitoring fragmentation chemistry by HPLC (*18*). Molecular weights of products of hydrolysis and aminolysis were verified by LC-MS (*19*). The proposed structures of the monocationic species detected are illustrated along with their molecular weights. mv, millivolt. (**B**) First-order disappearance of L-786,392 by enzyme hydrolysis is well matched to the appearance rates of the product fragments. Equations: $A = A_o e^{-kt}$, $A = B(1 - e^{-kt}) + B_o$, where k is the first-order rate constant; t is time; A = measured peak area; A_o and B_o are the t = 0 areas of L-786,392 and the fragment, respectively; and B is a scalar which embodies the detector response factor and the final concentration of product, thereby setting the maximum for A above B_o. Nonlinear curve fitting was used (KaleidaGraph, Synergy Software). Kinetic analysis (equivalence of $k \pm$ SE) is consistent with immediate and quantitative fragmentation. An analogous kinetic experiment (*24*) monitoring aminolysis yielded the same result.

Table 2. Comparison of drug week-4 hematology and spleen weights in rhesus monkeys treated iv with either L-742,728 or L-786,392. Values indicate average percent change from control. HCT, hematocrit; HgB, hemoglobin.

Parameter	L-742,728 (45 mg/kg)	L-786,392 (50 mg/kg)	L-786,392 (100 mg/kg)
RBCs	−8.7	0	1.8
HCT	−9.8	2.7	4.2
HgB	−8.0	3.2	3.2
Spleen weight	73	−9.0	8.9

es of 50 to 100 mg/kg/day for 28 days behaved similarly to high-dose penicillin, in which measurable serum titers to the ring-opened β-lactam nucleus epitope are found with high frequency and do not impede acceptable tolerability in humans. In contrast to L-742,728, which induced T cell proliferation in naïve rhesus and human lymphocytes in vitro to at least sixfold above background, L-786,392 lacked any lymphoproliferative capacity on both rhesus and human lymphocytes in vitro (*22*).

Erythrocyte-bound immunoglobulin G (IgG), induced by treatment of rhesus macaques with L-786,392 (50 mg/kg/day) for 28 days, was determined by ^{125}I–protein A binding (*23*). There were 210 ± 36 IgG molecules per erythrocyte versus 53 ± 10 for control, which when acid-eluted from these cells, showed no drug or side-chain specificity despite being above the limit of detection in the DAT assay. These IgGs remained bound in the presence of sufficient free L-786,392 that displaced the drug-specific serum antibody from the surface of in vitro haptenized RBCs. We conclude that the observed small increase in erythrocyte surface immunoglobulin was unrelated to the serum antibodies to ring-opened nucleus. In vitro exposure of RBCs to 10 mg/ml 2-aryl-carbapenems resulted in the formation of more than 20-fold more drug-specific IgG binding sites on the RBC surface compared to L-786,392, suggesting that this contributed quantitatively to the potential for anemia.

The "releasable hapten" strategy used in the design of L-786,392 was successful in reducing the immunogenicity of this molecule, both quantitatively and qualitatively, after 4-week high-dose exposure in rhesus macaques.

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7. Blood was obtained at necropsy from rhesus monkeys treated with the following compounds: L-741,462, L-742,728, meropenem, and L-786,392. Separated plasma and whole blood (diluted with an equal volume of Alsever's solution) were stored at 4°C until use. Control blood was obtained from healthy, adult rhesus monkeys naïve to all β-lactam antibiotics. All animal studies were performed under veterinary supervision and approved by the Institutional Animal Care and Use Committee.
8. The DAT (direct antiglobulin test) was performed as described [L. D. Petz, in *Manual of Clinical Laboratory Immunology*, N. R. Rose, H. Friedman, J. L. Fahey, Eds. (American Society for Microbiology, Washington, DC, ed. 3, 1986), pp. 613–629].
9. The IAT (indirect antiglobulin test, a measure of drug-specific antibodies) was performed as described in (*8*) with test RBCs (prepared by incubation with 4 to 16 mg of drug per milliliter for 2 hours at 37°C) exposed to a dilution series of the serum in question and washed. Sensitized cells for all agents except L-786,392 were stable for at least 1 week stored at 10% hematocrit in Alsever's solution at 4°C. L-786,392–sensitized cells had to be used immediately after drug exposure.
10. Antibody eluates from DAT-positive cells were tested in the IAT substituting the eluates for plasma. Eluates were obtained by brief treatment of RBCs with 0.1 M glycine HCl (pH 2.5 with NaOH), centrifugation, and neutralization of the supernatant with tris.
11. Lymphocyte transformation test (LTT) was performed according to C. Brander *et al.* [*J. Immunol.* **155**, 2670 (1995)]. Results were expressed as a stimulation index (SI) calculated as the mean sample counts per minute of ^{3}H-thymidine divided by mean counts per minute of the medium only controls. Student's *t* test (Microsoft Excel) was evaluated for all comparisons. However, analysis of variance (F-statistic, StatView version 4.1, Abacus Concepts, Berkeley, CA) indicated that in most tests where there appears to have been a response (≥0.6 mg/ml), variances were not well matched; therefore, the nonparametric Mann-Whitney U test was performed for all tests as well. SI was judged significant at the $P < 0.05$ level.
12. The MIC against each of the strains was determined by microdilution in Mueller-Hinton broth according to the National Committee for Clinical Laboratory Standards guidelines after incubation for 24 hours. Enterococci were tested in cation-supplemented Mueller-Hinton broth at 3×10^5 to 7×10^5 CFU/ml. MIC is defined as the lowest concentration of antibiotic inhibiting visible growth.
13. VISA strains Mu3 and Mu50 were kindly provided by K. Hiramatsu, Juntendo University, Tokyo, Japan [K. Hiramatsu *et al.*, *Lancet* **350**, 1670 (1997); K. Hiramatsu, *Am. J. Infect. Control* **25**, 405 (1997)].
14. Binding affinity for PBPs was determined in a competition assay with [^{3}H]-benzylpenicillin ([^{3}H]-benzylpenicillin *N*-ethylpiperidinium salt prepared at Merck Research Laboratories) using the procedure described by B. G. Spratt [*Eur. J. Biochem.* **72**, 341 (1977)]. IC_{50} and IC_{90} are calculated as the concentration of carbapenem that reduced the binding of [^{3}H]-benzylpenicillin by 50% and 90%, respectively.
15. MRSA membranes were prepared from the COL strain growing exponentially at 37°C in trypticase soy broth. Cells were cooled, collected by centrifugation, resuspended in 50 mM sodium phosphate buffer (pH 7), and disrupted by lysostaphin treatment (20 U/ml at 37°C) followed by sonication. Unbroken cells were removed at 8000*g*; membranes were collected at 40,000*g* and resuspended in 50 mM sodium phosphate buffer (pH 7). Protein concentration was determined by the Bio-Rad microassay method, and membranes were stored at −80°C.
16. *E. hirae* ATCC 9790 membranes were prepared by the procedure described [J. Coyette, J. Ghuysen, H. R. Perkins, *Eur. J. Biochem.* **75**, 225 (1977)].
17. Single-dose subcutaneous antibiotic protection from septicemic infections was measured in five mice per dose per test as described [C. J. Gill *et al.*, *Antimicrob. Agents Chemother.* **42**, 1996 (1998)]. L-786,392 was prepared in MOPS (morpholino propane sulfonic acid, Sigma) containing 40 mg/kg cilastatin. Survival was monitored for 7 days. ED_{50}s and LD_{50}s were determined by the method of L. F. Knudsen and J. M. Curtis [*J. Am. Stat. Assoc.* **42**, 282 (1947)]. All animal studies were performed under veterinary supervision and approved by the Institutional Animal Care and Use Committee.
18. C_{18} reversed-phase HPLC was performed using a 0.1% trifluoroacetic acid gradient solvent system with methanol to measure the parent, L-786,392, and its fragmented products of lactam hydrolysis. Urine samples were diluted with MOPS [33 or 50 mM (pH 7.4), 1 to 19 volumes]. L-786,392 was quantified using fluorescence (excitation = 345 nm, emission = 480 nm) or ultraviolet (340 nm) detection. Assay sensitivity for the side chain was similar to that of L-786,392 (that is, ~1 μg/ml in plasma).
19. The identities of the chromatographic peaks were confirmed in mass-spectral studies using a SCIEX API III^{+} in positive-ion mode employing pneumatically assisted electrospray ionization, *m/e* (mass-to-charge) scan range = 100 to 700 daltons. Reported ratio equals the molecular mass when the ion carries a single positive charge. The side-chain peak from both reactions produced a signal at 401 daltons, corresponding to a deprotonated structure.
20. Pharmacokinetics of carbapenems in rhesus monkeys were measured according to J. Sundelof *et al.* [*Antimicrob. Agents Chemother.* **41**, 1743 (1997)].
21. L-786,392 was measured by bioassay and HPLC from biological samples as described in (*20*). Zones of inhibition which fell in the linear (correlation coefficient, $r^2 \geq 0.992$, 0.078 to 10 μg/ml) region of the millimeter zone versus log μg/ml standard curve. The sensitivity of the assays was ~0.05 μg/ml for L-786,392 and imipenem. The coefficients of plate-to-plate variation were approximately 1.8 to 3% at 0.625 μg/ml for L-786,392.
22. R. Hajdu, unpublished observation.
23. Quantitative IgG determinations using protein A–RBCs (2 to 10 μl packed cell volume) were carried out with incubation at room temperature for 1.5 hours in 250 μl of ^{125}I–protein A (New England Nuclear; specific radioactivity 9.30 μCi/μg, 1:50 in diluent), followed by five washes in diluent. Molecules per cell were calculated from the γ-counts per minute measured with a counting efficiency of 82% applied after blank subtraction, with 2×10^6 cells per microliter packed cell volume.
24. R. Hajdu and R. Thompson, data not shown.

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