# Supplemental material

## Stability of novel siderophore cephalosporin S-649266 to clinically relevant carbapenemases.

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## Materials and Methods

### Antibacterial agent

S-649266 synthesized at Shionogi & Co., Ltd. (Osaka, Japan) and commercially available antibacterial agents were used; meropenem, ceftazidime, and cefepime were from United States Pharmacopeial Convention (Rockville, MD), and nitrocefin was from Oxoid (Hampshire, UK).

### Antibiotic susceptibility testing

Minimum inhibitory concentrations (MICs) were determined using cation-adjusted Mueller-Hinton broth (CAMHB; BBL, Franklin Lakes, NJ) according to the Clinical and Laboratory Standards Institute (CLSI) recommendations (1) except that CAMHB for the MIC determination of S-649266 was suppelemented with 20 μM of human apo-transferrin (apo-T; BBI Solutions, Cardiff, UK) to create ferric iron-deficient condition that mimic human fluids such as blood (2-4). It has been reported that the antibacterial activity of siderophore β-lactams including S-649266 are affected by the surrounding free-iron concentrations (5), and *in vivo* efficacy of S-649266 is best correlated with the MIC values determined in ferric iron-deficient condition (6).

Bacterial strains used in this study were kindly provided by Dr. Patrice Nordmann (University of Fribourg, Switzerland), Dr. Yoshichika Arakawa (Nagoya, Japan), Dr. Tse Hsien Koh (Singapore General Hospital, Singapore), and JMI laboratories (North Liberty, IA).

**Purification of β-lactamases**

The β-lactamases IMP-1, VIM-2, NDM-1, KPC-3, and OXA-23 were purified as a native form without any affinity tags from *E. coli* BL21 derived strains with each expression vector of pET9a or pET28a system (Table S2). The detailed procedures for culture and purification of each β-lactamase are described in the supplemental materials. L1 was purified from clinical isolate *Stenotrophomonas maltophilia* ULA511 according to the previous report (7). All the purified enzymes yielded a single band in sodium dodecyl sulfate polyacrylamide gel electrophoresis and estimated protein purity was >90% by Coomassie Brilliant Blue staining. The detailed purification procedures are as follows.

To obtain IMP-1, *E. coli* TUM3919 was cultured in Luria-Bertani (LB) broth (Becton, Dickinson and Company) containing 50 μg/mL kanamycin at 35°C. When the absorbance value was 0.5 to 0.7 at 600 nm, 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added and incubated for 2 more hours. The cells were harvested by centrifugation and re-suspended in 50 mM HEPES containing 50 μM $ZnSO_4$ (pH 7.5) (buffer A). The cells were disrupted by sonication on ice, followed by ultracentrifugation and purification using HiTrap SP HP column (GE Healthcare, Buckinghamshire, UK) equilibrated with buffer A. The enzyme was eluted with a linear salt gradient (0 to 1 M of NaCl). The fractions hydrolysing nitrocefin were collected and concentrated. The sample was dialyzed overnight at 4°C with buffer A

and concentrated, and then loaded onto a MonoS HR 5/5 (GE Healthcare, Buckinghamshire, UK) equilibrated with buffer A. The enzyme was eluted with a linear salt gradient (0 to 1 M of NaCl). The fractions hydrolysing nitrocefin were collected and concentrated.

To obtain VIM-2, *E. coli* TUM4664 was cultured in LB broth containing 50 µg/mL kanamycin at 35°C. The supernatant was obtained by centrifugation and 50% solid ammonium sulphate was added. After gentle stirring for 1 hour at 4°C, the supernatant was obtained by centrifugation and 80% solid ammonium sulphate was added. The precipitate was collected by centrifugation and solubilized in 20 mM triethanolamine (pH 7.2) (buffer B). After overnight dialysis with buffer B, the sample was loaded onto a Source 15Q (GE Healthcare, Buckinghamshire, UK) equilibrated with buffer B and the crude enzyme was eluted with a linear salt gradient (0 to 1 M of NaCl). The fractions hydrolysing nitrocefin were collected and concentrated. The concentrated sample was injected onto a Source 15Q (GE Healthcare, Buckinghamshire, UK) equilibrated with buffer A and the crude enzyme was eluted with a linear salt gradient (0 to 1 M of NaCl) again. The fractions hydrolysing nitrocefin were collected and concentrated.

To obtain NDM-1, *E. coli* SR08592 was cultured for 3 hours in Buffered Super Broth (yeast extract 20 g/L, tryptone 35 g/L, NaCl 5 g/L, buffered with 50 mM sodium phosphate buffer pH7.0) (8) containing 30 µg/mL kanamycin at 35°C. When the absorbance value was 1 at 600 nm, 0.2% arabinose was added and incubated for 5 hours at 25°C. The cells were collected and disrupted by sonication on ice. The crude extract was loaded onto a Q Sepharose FF (GE Healthcare, Buckinghamshire, UK) equilibrated with 20 mM Bis-Tris containing 5 µM $ZnSO_4$ (pH 6.0) and the crude enzyme was

eluted with 60 mM NaCl. The fractions hydrolysing nitrocefin were collected and purified with Superdex 200 pg 26/60 (GE Healthcare, Buckinghamshire, UK) equilibrated with 20 mM HEPES containing 50 μM $ZnSO_4$ (pH 7.0).

To obtain KPC-3, *E. coli* TUM3923 was cultured in LB broth containing 50 μg/mL kanamycin at 35°C. When the absorbance value was 0.5 to 0.7 at 600 nm, 1 mM IPTG was added and incubated for 4 more hours. The cells were harvested by centrifugation and re-suspended in 30 mM Tris-HCl containing 30% sucrose (pH 8.0). Purification was conducted as described previously (9).

To obtain OXA-23, *E. coli* TUM3920 was cultured in LB containing 50 μg/mL kanamycin (Wako Pure Chemical Industries, Osaka, Japan) at 20°C. When the absorbance value was 0.7 at 600 nm, 1 mM IPTG was added and incubated for 18 more hours. The cells were collected and disrupted on ice by sonication. The crude extract was loaded onto a SOURCE 15Q (GE Healthcare, Buckinghamshire, UK) equilibrated with 10 mM Bis-Tris-propane (pH 9.4) (buffer C) and the crude enzyme was eluted with a linear salt gradient (0 to 1 M of NaCl). The fractions hydrolysing nitrocefin were collected and purified with HiLoad 16/600 Superdex 200 pg (GE Healthcare, Buckinghamshire, UK) equilibrated with buffer C.

**Determination of kinetic parameters and relative hydrolysis velocity**

Hydrolysis of β-lactams was detected by monitoring the changes in the absorbance of β-lactam solution at 30°C in 20 mM HEPES buffer (pH 7.5) containing 50 μM $ZnCl_2$ for IMP-1, VIM-2, L1, and NDM-1, 50 mM phosphate buffer (pH 7.0) for KPC-3, and 100 mM Tris-$H_2SO_4$, 300 mM $K_2SO_4$ (pH 7.0) containing 10 mM $NaHCO_3$ for OXA-23. The absorbance of β-lactam solution was monitored by UV-2550 spectrophotometer (Shimadzu, Japan) or U-3010 (Hitachi, Japan). The wavelengths (nm) and molar

extinction coefficients (Δε) for S-649266 was determined by measuring the change in the absorbance divided by the molarity for complete hydrolysis of S-649266 by addition of NaOH, and those for each β-lactam and reporter substrate were referred from the previous report (7): S-649266 ($\Delta\varepsilon_{259\ nm}$ =−9430 $M^{-1}$ $cm^{-1}$), imipenem ($\Delta\varepsilon_{278\ nm}$ =−5660 $M^{-1}$ $cm^{-1}$), meropenem ($\Delta\varepsilon_{298\ nm}$ =−9530 $M^{-1}$ $cm^{-1}$), ceftazidime ($\Delta\varepsilon_{265\ nm}$ =−10300 $M^{-1}$ $cm^{-1}$), cefepime ($\Delta\varepsilon_{267\ nm}$ =−9120 $M^{-1}$ $cm^{-1}$), nitrocefin ($\Delta\varepsilon_{482\ nm}$ =10000 $M^{-1}$ $cm^{-1}$). Bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO) was added to diluted solutions of β-lactamase to prevent denaturation (final concentration of 20 μg/mL). The steady-state kinetic parameters ($k_{cat}$ and $K_m$) were determined by using the Hanes linearization of the Michaelis-Menten equation (10). For poor hydrolysing substrates, the competitive inhibition constant ($K_i$) instead of $K_m$ was determined in the presence of 100 μM of reporter substrate. Nitrocefin was used as a reporter substrate for IMP-1, VIM-2, KPC-3, and OXA-23, and imipenem was used as a reporter substrate for L1. The $k_{cat}$ was determined at more than 5-fold higher concentration of the $K_i$ value. When hydrolysis of tested β-lactam was not observed, the $k_{cat}$ was described as no hydrolysis detected (NH); change in absorbance was too small to calculate initial hydrolysis velocity, i.e. ≤0.001 after measurement of 90 seconds with 100 μM substrate. The $k_{cat}$ value was shown as not determined (ND) in the following cases; 1) the $K_i$ value was too high and 5-fold higher concentration of the $K_i$ value was exceeded the detection limit of spectrophotometer, 2) hydrolysis was too weak to calculate the $k_{cat}$. The $k_{cat}$, $K_m$, and $K_i$ were determined as average values for three independent experiments. The relative hydrolysis velocity was determined for NDM-1. The initial velocity of each antibacterial agent was determined at 100 μM for 60 seconds and 200 μM for 100 seconds, and relative velocity was calculated as average values for three independent

experiments, assuming those of meropenem to be 100.

## References


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**Table S1.** MICs of S-649266 and other antibacterial agents against clinical strains with various β-lactamases.

| Species | Strains | β-lactamases | MIC (μg/mL)[a] | | | |
|---|---|---|---|---|---|---|
| | | | S-649266 | Ceftazidime | Cefepime | Meropenem |
| *P. aeruginosa* | SR27001 | IMP-1 | 0.5 | >256 | >256 | >256 |
| *P. aeruginosa* | NUBL-1099 | IMP-1 | 0.016 | >256 | 256 | >256 |
| *P. aeruginosa* | NUBL-1101 | IMP-1 | 0.5 | >256 | 256 | 128 |
| *P. aeruginosa* | NTU | VIM-2 | 1 | 256 | 64 | 128 |
| *P. aeruginosa* | NUBL-7807 | VIM-2 | 0.5 | 128 | 128 | 256 |
| *P. aeruginosa* | NUBL-7809 | VIM-2 | 0.25 | 128 | 128 | 32 |
| *K. pneumoniae* | NCTC13443 | NDM-1 | 2 | >256 | >256 | 128 |
| *K. pneumoniae* | KOW5 | NDM-1, SHV-11 | 0.5 | >256 | 32 | 64 |
| *K. pneumoniae* | KI2 | NDM-1, TEM-1, SHV-28, CTX-M-15, CMY-6, OXA-1, OXA-9 | 1 | >256 | >256 | 256 |
| *K. pneumoniae* | ATCC BAA-2146 | NDM-1 | 0.5 | >256 | 256 | 128 |
| *E. coli* | GUE | NDM-1, TEM-1, OXA-1 | 0.5 | >256 | 64 | 16 |
| *E. coli* | ALL | NDM-1, TEM-1, CTX-M-15, OXA-1, OXA-2 | 1 | >256 | >256 | 32 |
| *S. maltophilia* | SR21970 | L1 | 0.125 | 128 | 64 | 256 |
| *S. maltophilia* | SR44358 | L1 | 0.125 | 128 | 64 | 256 |
| *S. maltophilia* | SR44362 | L1 | 0.5 | 256 | 128 | 128 |
| *K. pneumoniae* | 286 | KPC-3 | 0.06 | 256 | 64 | 16 |
| *K. pneumoniae* | 6598 | KPC-3 | 0.06 | >256 | >256 | 256 |
| *K. pneumoniae* | 10886 | KPC-2 | 0.5 | 256 | 64 | 128 |
| *K. pneumonaie* | 13717 | KPC-2 | 0.25 | >256 | >256 | 256 |
| *A. baumannii* | NCTC13301 | OXA-23 | 0.5 | >256 | 32 | 32 |
| *A. baumannii* | DR25547 | OXA-23, OXA-64 (OXA-51group) | 0.03 | 256 | 16 | 16 |
| *A. baumannii* | DU32628 | OXA-23, OXA-66 (OXA-51 group) | 0.06 | 128 | 16 | 16 |
| *A. baumannii* | NUBL-7727 | OXA-23 | 0.5 | 128 | 128 | 32 |
| *A. baumannii* | NUBL-7728 | OXA-23 | 0.25 | 128 | 32 | 32 |

[a] Cation-adjusted-Mueller Hinton broth was used as a medium, but supplemented with 20 μM human apo-transferrin for S-649266 to obtain iron-deficient condition.

**Table S2.** Strains and plasmids used for preparation of β-lactamases.

| β-Lactamase | Molecular class[a] | Stain name | Expression vector[b] | Host strain[c] |
|---|---|---|---|---|
| IMP-1 | B | *Escherichia coli* TUM3919 | pET28a | *Escherichia coli* BL21 (DE3) |
| VIM-2 | B | *Escherichia coli* TUM4664 | pET28a | *Escherichia coli* BL21 (DE3)/pLysS |
| NDM-1 | B | *Escherichia coli* SR08592 | pET9a | *Escherichia coli* BL21-AI |
| KPC-3 | A | *Escherichia coli* TUM3923 | pET28a | *Escherichia coli* BL21 (DE3)/pLysS |
| OXA-23 | D | *Escherichia coli* TUM3920 | pET28a | *Escherichia coli* BL21 (DE3) |
| L1 | B | *Stenotrophomonas maltophilia* ULA511 | - | - |

[a] Classification as described in Reference 11.
[b] The coding region of the β-lactamase was amplified from clinical isolate by PCR and cloned into the expression vector.
[c] Both series of pET28a and pET9a vectors and *E. coli* BL21 strains were obtained from Novagen and Invitrogen, respectively.