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Glutathione-*S*-transferase FosA6 of *Klebsiella pneumoniae* origin conferring fosfomycin resistance in ESBL-producing *Escherichia coli*

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Objectives: The objectives of this study were to elucidate the genetic context of a novel plasmid-mediated *fosA* variant, *fosA6*, conferring fosfomycin resistance and to characterize the kinetic properties of FosA6.

Methods: The genome of fosfomycin-resistant *Escherichia coli* strain YD786 was sequenced. Homologues of FosA6 were identified through BLAST searches. FosA6 and FosA^{ST258} were purified and characterized using a steady-state kinetic approach. Inhibition of FosA activity was examined with sodium phosphonoformate.

Results: Plasmid-encoded glutathione-*S*-transferase (GST) FosA6 conferring high-level fosfomycin resistance was identified in a CTX-M-2-producing *E. coli* clinical strain at a US hospital. *fosA6* was carried on a self-conjugative, 69 kb IncFII plasmid. The Δ*lysR-fosA6-*Δ*yjiR_1* fragment, located between IS*10R* and ΔIS*26*, was nearly identical to those on the chromosomes of some *Klebsiella pneumoniae* strains (MGH78578, PMK1 and KPPR1). FosA6 shared >99% identity with chromosomally encoded FosA^{PMK1} in *K. pneumoniae* of various STs and 98% identity with FosA^{ST258}, which is commonly found in *K. pneumoniae* clonal complex (CC) 258 including ST258. FosA6 and FosA^{ST258} demonstrated robust GST activities that were comparable to each other. Sodium phosphonoformate, a GST inhibitor, reduced the fosfomycin MICs by 6- to 24-fold for *K. pneumoniae* and *E. coli* strains carrying *fosA* genes on the chromosomes and plasmids, respectively.

Conclusions: *fosA6*, probably captured from the chromosome of *K. pneumoniae*, conferred high-level fosfomycin resistance in *E. coli*. FosA6 functioned as a GST and inactivated fosfomycin efficiently. *K. pneumoniae* may serve as a reservoir of fosfomycin resistance for *E. coli*.

Introduction

Escherichia coli accounts for the majority of urinary tract infections. Recent surveillance studies indicate very low rates of fosfomycin resistance in this species.[1,2] As such, fosfomycin was included as one of the first-line treatment options for uncomplicated urinary tract infections in the most recent treatment guidelines published by the IDSA and ESCMID.[3] Fosfomycin belongs to an antimicrobial class of its own and functions by inactivating the cytosolic *N*-acetylglucosamine enolpyruvyl transferase (MurA), which prevents the formation of *N*-acetylmuramic acid from *N*-acetylglucosamine and phosphoenolpyruvate, the initial step in peptidoglycan chain formation of the bacterial wall.[4] However, *E. coli* can acquire resistance to fosfomycin through several mechanisms, including impaired transport, target modification or overexpression, and inactivation of fosfomycin itself.[5] Fosfomycin-modifying enzymes can confer fosfomycin resistance by breaking its epoxide ring and inactivating the agent.[6] Of the three major classes of fosfomycin resistance enzymes (FosA, FosB and FosX), FosA is the group of enzymes most frequently reported among Gram-negative pathogens including *E. coli*.[7–11] FosA enzymes can catalyse the nucleophilic addition of glutathione to carbon-1 of fosfomycin.[6] An increasing number of studies report identification of ESBL-producing *E. coli* isolates that are resistant to fosfomycin due to plasmid-mediated production of FosA3 from both animal and human sources in East Asian countries.[7–11] We recently reported a case of FosA3-producing *E. coli* identified in a hospital in Pennsylvania.[12] In addition, plasmid-mediated production of FosA5, also termed FosKP96, has been reported in *E. coli* and *Klebsiella pneumoniae* from China and Hong Kong.[11,13,14] Here, we report the identification of a novel plasmid-mediated FosA variant, FosA6, in an ESBL-producing

E. coli strain and characterize its kinetic properties as well as genetic context.

Materials and methods

Strains

Fosfomycin-resistant *E. coli* strain YD786 was identified from the urine of a female inpatient who had recurrent urinary tract infections, but did not have a documented history of prior fosfomycin therapy. *K. pneumoniae* clinical strains NDM01,[15] CRKpE6 and CRKpC1, available in our research laboratory, were used as strains producing FosA^{PMK1}, FosA^{ST37} and FosA^{ST258}, respectively. FosA^{PMK1}, FosA^{ST37} and FosA^{ST258} are some of the most commonly observed chromosomally encoded FosA in *K. pneumoniae* (GenBank accession numbers WP_004146118, WP_004182826 and WP_002887377) and are closely related to FosA6 described in this study.

Susceptibility testing

MICs of fosfomycin and other commonly used agents were determined by Etest (bioMérieux, Durham, NC, USA) and commercially available broth microdilution testing plates (Sensititre GNX2F), respectively, and interpreted according to CLSI guidelines.[16] *E. coli* ATCC 25922 (susceptible to fosfomycin) was used as the quality control strain. Inhibition of the glutathione-*S*-transferase activity of FosA was examined with sodium phosphonoformate as reported previously[17] with the following modification, where fosfomycin Etest was placed on Mueller–Hinton agar plates with or without 500 mg/L sodium phosphonoformate. *E. coli* 55B8 was used as the fosfomycin-resistant, *fosA*-negative control strain. This clinical strain does not possess any *fosA* gene, but rather lacks the hexose phosphate transporter gene *uhpT* as the fosfomycin resistance mechanism, as evidenced by PCR and RT–PCR.

PCR and cloning

PCR for *fosA3* was conducted as previously described.[12] The chromosome of YD786 was extracted, digested with restriction enzyme Sau3AI and ligated with cloning vector pUC19 (Thermo Scientific, Waltham, MA, USA) which was digested with BamHI. *E. coli* TOP10 (Thermo Scientific) was transformed with this ligated product and transformants were identified by growth on LB agar plates containing 50 mg/L ampicillin, 50 mg/L fosfomycin and 25 mg/L glucose-6-phosphate. Nucleotide and protein BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) were used to find homologues of *fosA6* and FosA6.

fosA6, *fosA*^{PMK1}, *fosA*^{ST37} and *fosA*^{ST258} were cloned into pBCSK− (Agilent Technologies, Santa Clara, CA, USA) using the following primers: FosA-XbaI-F, 5′-TGCTCTAGATGCTGAGTGGACTGAATCAC-3′; FosA-HindIII-R, 5′-TCCAAGCTTCACTGATCAAAAAACACCATCC-3′; and FosA258-HindIII-R, 5′-TCCAAGCTTCACTGTTCAAAAAACACCATCC-3′.

Transferability of plasmids

Transformation and conjugation were performed as described previously,[18] using *E. coli* TOP10 and azide-resistant *E. coli* J53 as recipients, respectively. Transformants and transconjugants were selected on LB agar plates containing fosfomycin and glucose-6-phosphate as above, whereas 100 mg/L sodium azide was also added for selection of the transconjugants.

WGS

The YD786 genome was sequenced by HiSeq 2500 (Illumina, San Diego, CA, USA) and PacBio RS II (Pacific Biosciences, Menlo Park, CA, USA) as previously described,[19] resulting in full assembly of the chromosome and two plasmids (pYD786-1 and pYD786-2) and partial assembly of pYD786-3 and pYD786-4. Gaps in pYD786-3 and pYD786-4 were filled with HiSeq reads and verified by PCR and sequencing (data not shown). The chromosomal and plasmid sequences were submitted under accession numbers CP013112.1 and KU254578–81, respectively.

Purification of FosA6 and FosA^{ST258} and steady-state kinetic assays

fosA6 and *fosA*^{ST258} were synthesized by GenScript (Piscataway, NJ, USA) and cloned into the pE-SUMOstar prokaryotic expression vector (LifeSensors, Malvern, PA, USA) according to the manufacturer's instructions. Fosfomycin-dependent glutathione conjugation was detected spectrophotometrically using monochlorobimane (Sigma–Aldrich). A standard curve was prepared using 0–750 μM glutathione. Data were fitted to Michaelis–Menten equations using SigmaPlot (Systat Software, San Jose, CA, USA). Details of the purification and kinetic assays are available as Supplementary data at *JAC* Online.

Results and discussion

Antimicrobial susceptibility of E. coli YD786

E. coli YD786 was resistant to cephalosporins, aztreonam, fluoroquinolones and doxycycline and intermediate to minocycline (Table S1). Notably, it showed high-level resistance to fosfomycin with an MIC of 512 mg/L (susceptibility breakpoint, 64 mg/L).[16] The MIC was reduced by 16-fold to 32 mg/L in the presence of 500 mg/L sodium phosphonoformate, which suggested the presence of FosA-group glutathione-*S*-transferase activity. However, PCR was negative for *fosA3*, which is the most commonly reported *fosA* gene in *E. coli* worldwide. Sequences of *murA*, *glpT* and *uhpT*, the three genes commonly implicated in fosfomycin resistance,[5] were identical to those of fosfomycin-susceptible reference strain ATCC 25922. In comparison, fosfomycin resistance could not be reversed by sodium phosphonoformate in the *fosA*-negative, fosfomycin-resistant control strain 55B8 (Table 1).

Cloning and sequencing of fosA6

Genomic cloning of the fosfomycin resistance determinant from strain YD786 yielded *E. coli* TOP10 harbouring recombinant plasmid pYD786S14, which was highly resistant to fosfomycin with an MIC of >1024 mg/L. Sequencing of pYD786S14 revealed an 815 bp insert, which shared 99% nucleotide identity with multiple chromosomal sequences of *K. pneumoniae*, including those of the epidemic carbapenem-resistant ST258 strains and *K. pneumoniae* PMK1 (ST15, CP008929.1), which is an NDM-producing strain that caused an outbreak of neonatal infections in a Nepali hospital.[20] The insert contained a single 420 bp ORF encoding FosA, hereafter referred to as FosA6 since it was located on a plasmid and shared 96% and 79% identity with FosA5 and FosA3 at the amino acid level, respectively. BLAST searches identified FosA6 homologues to be widely encoded on the chromosomes of *K. pneumoniae* (~700 *K. pneumoniae* out of 800 Enterobacteriaceae sequences found in GenBank; data not shown). The amino acid sequence of FosA6 shared >99% identity with FosA^{PMK1} with one amino acid substitution (Pro130Gln) and 98% identity with FosA^{ST258} differing by only three amino acids (Val91Ile, Pro130Gln and Glu138Asp; Table 1). FosA^{PMK1} is distributed in 84 *K. pneumoniae*

Table 1. MICs for *E. coli* clones carrying various *fosA* genes and *K. pneumoniae* clinical isolates with chromosomal *fosA* genes

Strain	MIC of fosfomycin (mg/L)	MIC of fosfomycin in the presence of PPF (mg/L)	Amino acid alteration compared with FosA^{PMK1}		
			Ile91	Pro130	Asp138
E. coli TOP10 (pBCSK−)	0.38	0.50			
E. coli TOP10 (pFosA^{PMK1})	16	0.75			
E. coli TOP10 (pFosA6)	12	0.75		Gln	
E. coli TOP10 (pFosA^{ST37})	16	1	Val		
E. coli TOP10 (pFosA^{ST258})	12	0.75	Val		Glu
K. pneumoniae NDM01(FosA^{PMK1})	24	4			
K. pneumoniae CRKpE6 (FosA^{ST37})	16	2	Val		
K. pneumoniae CRKpC1(FosA^{ST258})	24	1	Val		Glu
E. coli 55B8 (*fosA* negative)	>1024	>1024			

MICs were determined by Etest. Sodium phosphonoformate (PPF) was added to Mueller–Hinton agar at 500 mg/L. The *E. coli* clones harbour *fosA* genes carried on vector pBCSK−. *E. coli* 55B8 was included as a fosfomycin-resistant, *fosA*-negative control strain, which has a defective *uhpT* gene.

strains, represented by 33 STs in different clonal complexes (Table S2 and Figure S1), including reference strains MGH78578 (ST38, CP000647.1), NTUH-K2044 (ST23, AP006725.1) and an NDM-producing strain reported from our hospital previously (*K. pneumoniae* NDM01; ST14, CP006798.1).[15] FosA^{ST258} is identified in 427 *K. pneumoniae* strains (data not shown), of which 119 were assigned STs based on the MLST scheme (https://cge.cbs.dtu.dk/services/MLST/), including 111 ST258 and 5 ST11 strains (Table S3 and Figure S1). FosA^{ST258} appears to be common in the epidemic CC258 strains, including ST258, ST11 and ST512 among others, suggesting a wide distribution of closely related homologues of FosA6 in this species (Table S3 and Figure S1).

Genome analysis of E. coli YD786 and transferability of fosA6

E. coli YD786 had a genome of 4.9 Mb in length and belonged to ST410. It contained typical quinolone resistance-determining region substitutions Ser83Leu and Asp87Asn in GyrA and Ser80Ile in ParC. The plasmids were assembled as 227 kb $bla_{CTX\text{-}M\text{-}2}$-carrying IncHI2 plasmid pYD786-1, 69 kb *fosA6*-carrying IncFII plasmid pYD786-2, 45 kb IncX1 plasmid pYD786-3 and 26 kb IncX2 plasmid pYD786-4.

pYD786-2 carried *fosA6* as well as an IncFII replicon region, conjugative transfer operon (*tra-trb*), toxin/antitoxin addiction system (*hok-mok*) and plasmid stability and partition system (*parB*, *parM*). This overall structure was similar to $bla_{NDM\text{-}1}$-carrying plasmids pGUE-NDM (JQ364967.1) and pMC-NDM (HG003695.1), identified in *E. coli* from India and Poland, respectively,[21,22] except that pYD786-2 contained only *fosA6* and *floR* (encoding chloramphenicol efflux protein) as resistance genes (Figure S2).

pYD786-2, the native *fosA6*-carrying plasmid in YD786, was transferable by transformation and broth mating. *E. coli* TOP10 (pYD786-2) had a fosfomycin MIC of 128 mg/L, which could be explained by the presence of *fosA6*. *E. coli* TOP10 (pYD786-2) was also resistant to chloramphenicol, but otherwise remained susceptible to other classes of antimicrobial agents (Table S1). The transconjugant had an identical resistance phenotype and plasmid profile to the transformant (data not shown).

Genetic environments of fosA6

A transcriptional regulator gene *lysR* truncated by IS*10R* was located upstream of *fosA6* and an aminotransferase truncated by IS*26* was located downstream of *fosA6* (Figure 1). This 1183 bp Δ*lysR-fosA6-*Δ*yjiR_1* region was nearly identical to those in *K. pneumoniae* MGH78578, PMK1 and KPPR1 (ST493, CP009208.1) with only three or four nucleotide differences, suggesting its mobilization from the chromosome of this species. *K. pneumoniae* MGH78578 is also known as ATCC 700721 and was isolated from the sputum of a 66-year-old ICU patient in 1994. KPPR1 is a rifampicin-resistant derivative of ATCC 43816 commonly used in animal studies.

This genetic context was very similar to that of *fosA5*, which was discovered in plasmids pHKU1 (KC960485.1),[11] pKP96 (EU195449.1)[14] and pHS33 (KP143090.1).[13] However, the Δ*lysR* located upstream of *fosA5* in these plasmids was shorter (143 versus 354 bp) than that upstream of *fosA6* in pYD786-2 as a result of more substantial truncation by IS*10R*. In addition, downstream of *fosA5* there was a gene of unknown function truncated by IS*10R* in pHKU1 and pKP96 or by IS*1R* in pHS33. The IS*10R*-flanked transposition unit bounded by direct repeats (DRs) in pHKU1 was identical to that in pKP96 except that in pKP96 subsequent transposition of Tn*1721* and IS*Ecp1B* resulted in the acquisition of $bla_{CTX\text{-}M\text{-}24}$ (Figure 1). In contrast to these *fosA5*-containing plasmids, in pYD786-2, IS*10R*-Δ*lysR-fosA6-*Δ*yjiR_1* interrupted IS*26*. The 5′ end of IS*26* truncated *resA* while the 3′ end truncated an ORF of unknown function, a structure that, together with adjacent *stbAB*, is observed in some non-*fosA*-carrying IncFII plasmids (pEQ011, KF582523.1; pEC_B24, GU371926.1; p12-4374_62, CP012928.1).

Overall, it appeared likely that *fosA6* was mobilized from the chromosome of *K. pneumoniae* closely related to PMK1 by IS*10*, but in a separate mobilization event compared with *fosA5*, given its distinct insertion site and mobilization onto an IncFII plasmid, as opposed to IncN or IncA/C plasmid for *fosA5*, and also the geographical separation where *fosA5* was found in China and Hong Kong whereas *fosA6* was identified in the USA.

Also of note is the fact that *fosA6* is located on a plasmid that only carries one other resistance gene, *floR*, encoding a

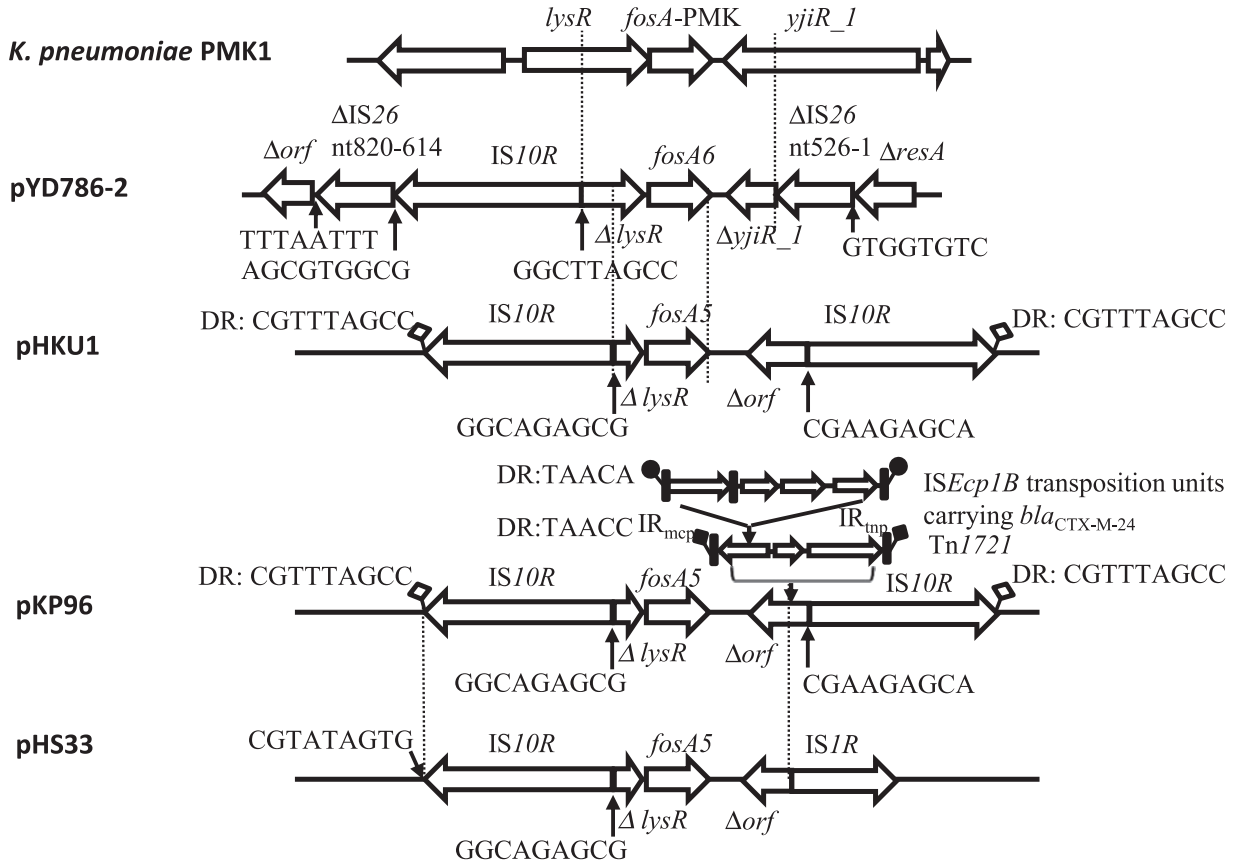

Figure 1. Genetic environment of *fosA6*, *fosA5* and the corresponding region on the chromosome of *K. pneumoniae* PMK1. Fragments between the dotted lines share >99% identity. The predicted ORFs and ISs are indicated by bold arrows and annotated above or below, with arrowheads indicating the direction of transcription. Putative DR sequences are given to indicate their boundaries. Paired filled/unfilled squares or circles represent DRs of transposition units of transposons or ISs. *K. pneumoniae* PMK1, CP008929.1; pHKU1, KC960485.1; pKP96, EU195449.1; and pHS33, KP143090.1.

Table 2. Steady-state kinetic parameters determined for FosA6 and FosA^{ST258}

	Fosfomycin[a]				Glutathione[b]			
Enzyme	K_M (mM)	V_{max} (mM. min^{-1})	k_{cat} (min^{-1})	k_{cat}/K_M (mM^{-1}. min^{-1})	K_M (mM)	V_{max} (mM. min^{-1})	k_{cat} (min^{-1})	k_{cat}/K_M (mM^{-1}. min^{-1})
FosA6	2.5±1.5	0.1±0.0	500.3±70.4	200.1	5.4±4.5	0.2±0.1	937.9±350.7	173.6
FosA^{ST258}	2.1±1.1	0.1±0.0	438.9±49.5	209.0	21.4±13.1	0.5±0.2	2018.2±948.2	94.3

[a]Measured at 20 mM glutathione.
[b]Measured at 20 mM fosfomycin.

chloramphenicol efflux pump. In contrast, *fosA3* has so far been identified exclusively on plasmids carrying a broad-spectrum β-lactamase gene (CTX-M-group ESBL,[8,10,23,24] CMY-2-group plasmid-mediated AmpC β-lactamase[23,25] or KPC and NDM carbapenemases).[24,25] However, the fact that *fosA6* is located on a non-MDR plasmid suggests that these emerging fosfomycin resistance genes may have been overlooked. It is also possible that the acquisition of *fosA6* was a relatively recent event and *fosA6* may find other resistance genes as partners on the same plasmids in the future. Nevertheless, *K. pneumoniae* serving as a ubiquitous reservoir of *fosA* for *E. coli* is a concerning phenomenon given the selective pressure exerted by increasing use of fosfomycin.

Functionality of FosA6 and FosA^{ST258}

The steady-state kinetic parameters for fosfomycin (Table 2) were largely comparable for FosA6 and FosA^{ST258}, suggesting that the three amino acid differences between these genes do not impact

fosfomycin binding. In contrast, the catalytic efficiency for glutathione for FosA6 was ~2-fold higher than the value determined for $FosA^{ST258}$, which was predominantly driven by a change in K_M.

These kinetic data showed that $FosA^{ST258}$, the chromosomally encoded FosA produced by the epidemic KPC-producing ST258 strains, is able to inactivate fosfomycin as robustly as FosA6. Median fosfomycin MICs for KPC-producing *K. pneumoniae* strains are 16–64 mg/L,[26,27] which are substantially higher than those for *E. coli*, which are typically in the 1–2 mg/L range.[1,26] While further studies are needed, these findings suggest that *fosA* probably contributes to the higher baseline fosfomycin MICs for *K. pneumoniae* compared with *E. coli* that lacks chromosomal *fosA* as a species.[28,29] The functionality of various FosA enzymes was also supported by reduction of fosfomycin MICs by 6- to 24-fold in the presence of sodium phosphonoformate for *fosA*-positive *K. pneumoniae* strains as well as *E. coli* clones carrying these *fosA* genes of *K. pneumoniae* origin (Table 1).

Sodium phosphonoformate behaves as a competitive inhibitor of fosfomycin by binding to FosA in the active site including conserved Thr9 and Mn(II).[30] It has been successfully used as a diagnostic tool to detect production of FosA3, FosA4 and FosC2[17] and, in our study, FosA6 and chromosomal FosA of *K. pneumoniae*. It is worth noting that sodium phosphonoformate is approved for clinical use in the treatment of herpes virus infections as the antiviral compound foscarnet.

Conclusions

We report a novel glutathione-*S*-transferase FosA6 that confers high-level fosfomycin resistance in an *E. coli* clinical strain identified in Pennsylvania, USA. The gene was probably mobilized from *K. pneumoniae* chromosome to *E. coli* plasmid through an IS*10*-mediated mobilization event. *K. pneumoniae* may serve as a significant reservoir of fosfomycin resistance in *E. coli* as the use of this agent increases.

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Transparency declarations

Y. D. has served on advisor boards for Shionogi, Meiji Seika Pharma, Tetraphase Pharmaceuticals, Achaogen and Merck, has consulted for Melinta Therapeutics and has received research funding from Merck and The Medicines Company for studies unrelated to this work. All other authors: none to declare.

Supplementary data

Details of purification and kinetic assays, Tables S1 to S3 and Figures S1 and S2 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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