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Imitation of β-lactam binding enables broad-spectrum metallo-β-lactamase inhibitors

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Carbapenems are vital antibiotics, but their efficacy is increasingly compromised by metallo-β-lactamases (MBLs). Here we report the discovery and optimization of potent broad-spectrum MBL inhibitors. A high-throughput screen for NDM-1 inhibitors identified indole-2-carboxylates (InCs) as potential β-lactamase stable β-lactam mimics. Subsequent structure–activity relationship studies revealed InCs as a new class of potent MBL inhibitor, active against all MBL classes of major clinical relevance. Crystallographic studies revealed a binding mode of the InCs to MBLs that, in some regards, mimics that predicted for intact carbapenems, which includes with respect to maintenance of the Zn(II)-bound hydroxyl, and in other regards mimics binding observed in MBL–carbapenem product complexes. InCs restore carbapenem activity against multiple drug-resistant Gram-negative bacteria and have a low frequency of resistance. InCs also have a good in vivo safety profile, and when combined with meropenem show a strong in vivo efficacy in peritonitis and thigh mouse infection models.

The increase in antibiotic resistance raises concerns that, at least in some regions, we are returning to a pre-antibiotic era, in particular for Gram-negative infections. The increased prevalence of extended-spectrum serine-β-lactamases (SBLs) and metallo-β-lactamases (MBLs) means β-lactams are increasingly ineffective in treating Gram-negative infections[1,2]. The advent of mobilized colistin resistance-1 in 2015[3] and the novel transferable tigecycline resistance genes (tetX3–tetX5) in 2019[4], which mediate resistance to colistin and tigecycline, respectively, means all clinically vital antibiotics for serious Gram-negative infections are com-

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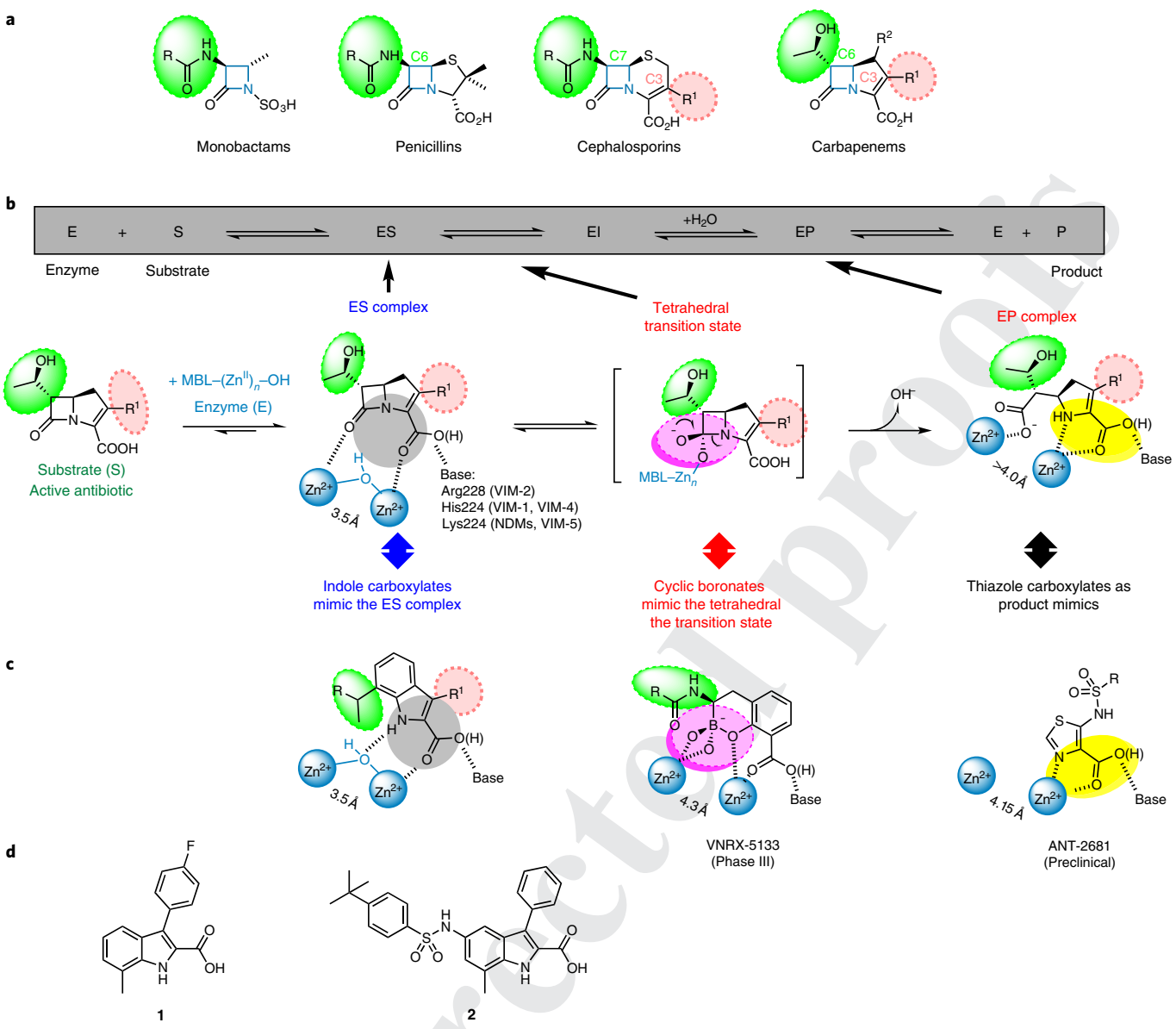

Fig. 1 | InCs binding to MBLs mimics that of intact β-lactam substrates and/or products. a, Clinically used β-lactam classes. **b**, Outline of B1 MBL mechanism exemplified with a carbapenem (note that the protonated form of the enamine tautomer of the product is shown in the enzyme–product (EP) complex[15,30,46]). **c**, InC binding mimics that of carbapenem or carbapenem-derived products to B1 MBLs, with maintenance of the di-Zn(II) complex hydroxide and conservation of the Zn–Zn distance observed in unligated MBLs (Zn:Zn distance 3.5 Å, Protein Data Bank (PDB) ID 4BZ3[43]). Most MBLs displace Zn–hydroxyl, as observed for bi(cyclic) boronates (Zn:Zn distance 4.3 Å, PDB ID 5FQC[38], which mimics the tetrahedral transition state), and triazole-carboxylates (Zn:Zn distance 4.15 Å, PDB ID 6ZGM[17], the binding of which is related to that of hydrolysed penemsas and penicillins). **d**, Examples of InC hits identified by the ELF. ES, enzyme–substrate.

promised. There are few novel anti Gram-negative drugs entering clinical trials, and therefore overcoming resistance to restore the activity of existing drugs with an excellent safety record, for example β-lactams, is important (Fig. 1a)[5].

Carbapenems, often 'drugs of last resort', manifest stability to extended-spectrum SBLs, although they are hydrolysed by SBL carbapenemases and all MBLs[6–8]. Avibactam, relebactam and vaborbactam are recently introduced SBL carbapenemase inhibitors[9–11], but apart from vaborbactam, which has a relatively limited activity spectrum[12,13], these and classical SBL inhibitors (SBLIs) (for example, clavulanate) are increasingly susceptible to β-lactamases, which include MBLs that degrade all β-lactam classes[6,14]. The development of MBL inhibitors (MBLIs), in particular to protect carbapenems, is thus an unmet clinical need, especially in the developing world, where MBL-producing bacteria are widely disseminated.

MBL inhibition is challenging because of the structural diversity in MBL active sites (Fig. 1b)[15,16]. By contrast with the SBLs, no clinically useful MBLI is available. Most reported MBLIs (Fig. 1b and Supplementary Fig. 1)[17–19] lack the breadth of potency against relevant MBL variants that is required for clinical use[7,20]. Most MBLIs inhibit by tight Zn(II) chelation at the active site or in solution, the latter a property that may make it difficult to achieve selectivity over human metalloenzymes[6]. Aspergillomarasmine A[18], a Zn(II) chelator and the preclinical candidate ANT-2681[17], a site Zn(II) binder active in a mouse model, both have limited MBL coverage, as does

the SBL-inhibiting bicyclic boronate taniborbactam (which is in phase 3 trials[19]).

Given that imitation of the initial substrate binding mode has been successfully employed for SBL inhibition (for example, by β-lactam-mediated SBL inhibition by clavulanate) and that β-lactam antibiotics are mimics of the substrates of their transpeptidase targets[9,21–24], we envisaged that an analogous 'substrate-focused' approach may enable the identification of broad-spectrum MBLis. Here we report on efforts by two public–private partnerships, the European Lead Factory (ELF)[25] and the European Gram-negative Antibacterial Engine (ENABLE (http://nd4bb-enable.eu/)) Oxford MBLI project that led to the identification and optimization of indole carboxylates (InCs) (Fig. 1c,d) as broad-spectrum MBLis. The InCs have an unprecedented MBL binding mode, which is different with regards to those of both carbapenem substrates and products. InCs protect carbapenems from MBL activity in multidrug-resistant (MDR) and extensively drug-resistant (XDR) Gram-negative pathogens, as shown by in vitro and in vivo mouse infection models.

Results

Hit finding. To identify new broad-spectrum MBLis, we carried out a fluorescence-based[26] high-throughput screen that employed NDM-1 and the ELF compound collection. This led to the identification of several new, but typical, MBLis, that is, heteroaromatic Zn(II) chelators[27,28]. Several InCs (**1** and **2**) (Fig. 1d)[29] were identified as novel reversibly binding, non-covalent, competitive NDM-1 inhibitors (pIC_{50} (negative log of the half maximal inhibitory concentration) values of 7.7) that are not influenced by the added of excess of zinc(II) ions; these attracted our attention because of their structural similarity to carbapenems and carbapenem-derived products[30–33], because they can be efficiently prepared and because they are not obvious potent Zn(II) chelators. The potential of InCs as a broad-spectrum B1 subfamily of MBLis was demonstrated by studies with VIM-1, VIM-2 and IMP-1. At this stage, we used crystallography to investigate the InC binding mode. As described below, these structures revealed a novel binding mode, which in part mimicked that of β-lactams prior to their hydrolysis (Fig. 1).

Structure–activity relationship studies. We initiated structure–activity relationship (SAR) studies that employed NDM-1, VIM-1, VIM-2 and IMP-1, in which positions amenable to derivatization (N1 and C2 to C7) were modified. The results revealed the roles of the indole NH and the C2 carboxylate in potent inhibition; *N*-methylation or replacement of the C2 carboxylate reduced activity by ~1,000 fold (**3–7**; Supplementary Tables 1 and 2). C3 modification was found to be useful, as shown by studies with C3 aryl InCs (**8–26**; Supplementary Table 3). In general, the C4, C5 and C6 positions were less amenable to diverse derivatization (**27–37**; Supplementary Tables 4 and 5). A C7 substituent was found to be important in inhibition, with an isopropyl group being preferred in the initial studies, although the initial SAR importantly revealed that larger groups could be accommodated at C7 (**1** and **38–46**; Supplementary Table 6).

InCs with *para*-substituted phenyl groups at C3 and a C7 methyl displayed good activity, with pIC_{50} values of 6.0–6.5 for NDM-1, VIM-1, VIM-2 and IMP-1 (**16**, **18** and **20**; Supplementary Table 3). Further C3 SARs, which included with disubstituted phenyls, led to pIC_{50} values > 8 against NDM-1, VIM-1, VIM-2 and IMP-1 (**47–50**, **52** and **55**; Supplementary Table 7); in some cases, pIC_{50} values >9 were achieved for NDM-1, which indicates the potential for a highly potent inhibition (**5**, **47**, **48** and **52**; Supplementary Table 7). Binding of selected compounds, that is, with a C7 methyl (**1**, **8**, **9**, **11–19**, **24–26** and **38**), OMe (**39**), F (**40**), CF_3 (**43**), ^{t}Bu (**46**) or *i*Pr (**32**), to NDM-1, VIM-1 and IMP-1 was analysed by surface plasmon resonance[34]. A good correlation was observed between the pIC_{50} and surface plasmon resonance results (Supplementary Fig. 2), with surface plasmon resonance derived on rates of 10^5–10^6 M^{-1} s^{-1} and off rates of up to 10^{-2} s^{-1} (Supplementary Tables 8–10); **16** has a K_D of 7 nM for NDM-1. Overall, the early SAR study achieved substantial improvements (up to 100-fold versus that of **1**) in potency and revealed the amenability of the C3 and C7 InC positions to modification.

Antimicrobiological activity, permeation and efflux studies. We evaluated the in vitro activity of InCs in combination with carbapenems against MDR and XDR bacteria that possessed various MBLs. Given the global rise in carbapenem resistance in Enterobacterales, we targeted these pathogens. Early studies revealed a reduced activity for **5** and **50–55** with *Klebsiella pneumoniae* clinical isolates compared with that for *Escherichia coli* (carrying NDM, IMP or VIM MBLs; Supplementary Table 11), which revealed that factors other than potency against isolated MBLs are important for inhibition in cells.

To optimize the InC activity against clinically relevant MDR and XDR pathogens, which included with altered permeation and/or elevated efflux properties, we engineered strains of *K. pneumoniae* NCTC 5055 that carried pBAD (*ramA*), Ecl8 and Ecl8Δ*ramR*[35] with plasmids that expressed NDM-1, VIM-1 or IMP-1 MBLs[35]. (Supplementary Tables 12 and 13 and Supplementary Fig. 3). The results demonstrate the ability of InCs to restore meropenem activity in MDR and/or XDR Enterobacterales to produce NDM-1, VIM-1, VIM-4, IMP-1 and IMP-4 (Supplementary Tables 11–13). Note, despite the excellent InC activity versus isolated VIM-2 and NDMs, the InC combinations show limited activity against VIM and NDM that produce *Pseudomonas aeruginosa* and *Acinetobacter* spp, probably because of cell permeability constraints (Supplementary Table 14).

Physicochemical, ADME, receptor testing, in vivo tolerability and efficacy for the InC hits. InC **49** was selected to assess the suitability of InCs for use in vivo—**49** does not interact with 30 human receptors and has acceptable physicochemical and ADME (absorption, distribution, metabolism and excretion) properties (Supplementary Tables 15 and 16). **49**, however, exhibits a high plasma protein binding and minimum inhibitory concentration (MIC) studies in the presence of serum reduced its activity (Supplementary Table 17). No adverse effects were observed in mice with single doses of 10 or 100 mg kg^{-1} of **49** (Supplementary Table 18). A proof-of-concept study revealed synergy between meropenem and **49** for the treatment of mice infected with *E. coli* 91N[36] (**49** at a 100 mg kg^{-1} dose, data not shown). The two weaknesses of the initial InCs as exemplified by **48**, **49** and **53**, that is, a relatively low metabolic stability and high plasma protein, binding (Supplementary Table 18) were then addressed by SAR studies guided by crystallography.

Structural studies. We obtained >50 high-resolution structures (<1.5 Å) with clinically relevant B1 and/or B3 subfamily MBLs, exemplified by structures of InCs with both B1 MBLs (VIM-1:**49**, VIM-2:**11** and NDM-7:**48**) and the B3 MBL L1:**49** (Fig. 2a–d; Supplementary Figs. 4 and 5 and Supplementary Tables 20 and 21). These reveal a highly conserved and unprecedented MBL binding mode for the InCs (Fig. 2a–d).

The InCs inhibit via a binding mode that contrasts with those of most active site binding MBLis, which displace the bridging water. In all the InC structures, both Zn(II) ions and, importantly, the bridging water or hydroxide are present in high occupancy, with one of their C7 isopropyl methyl groups apparently locking the hydroxide in its Zn–Zn bridging location. The Zn–Zn and Zn–water or –hydroxide ion distances in the MBL–InC complexes correspond to those observed for MBLs without inhibitors (~3.5 Å; Fig. 1 and Supplementary Fig. 4[15,28,37]); these distances increase in the product or intermediate–intermediate mimic MBL complexes[15,38] (Fig. 1 and

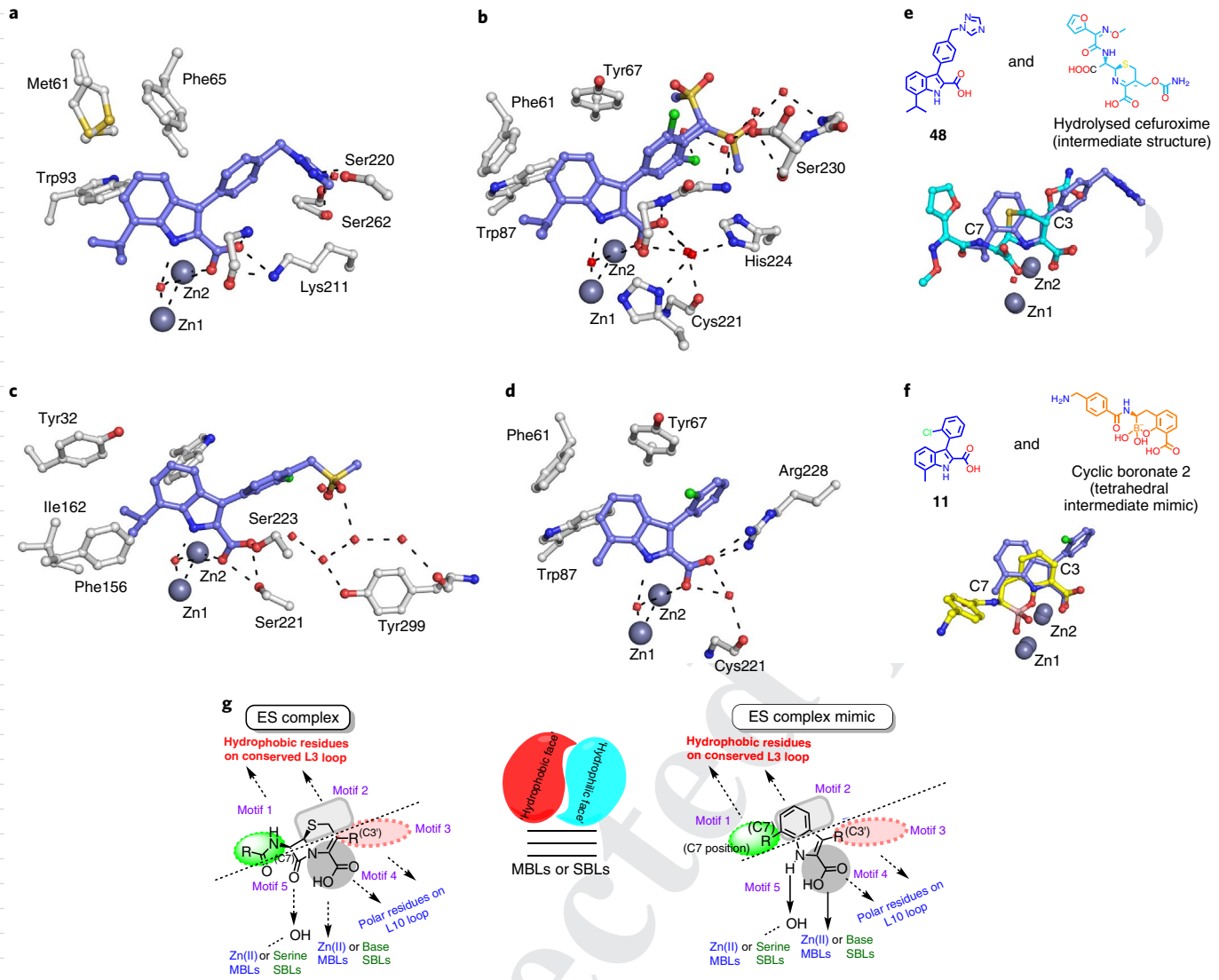

Fig. 2 | InCs inhibit B1 and B3 MBLs via an unprecedented MBL binding mode. a–d, Views of **48** with NDM-7 (PDB ID 7AEZ, 1.1 Å) (**a**), **49** with VIM-1 (PDB ID 7AEX, 1.1 Å) (**b**), **11** with VIM-2 (PDB ID 7AFY, 1.6 Å) (**c**) and **49** with L1 (PDB ID 7AFZ, 1.5 Å) (**d**). Note the presence of a di-Zn(II) ion-bridging hydroxide, the position of the C7 alkyl group relative to that of the bridging water, and that the C2 carboxylate is positioned to interact with the different types of binding motifs employed in MBL substrate carboxylate binding. **e**,**f**, Overlays comparing modes of InCs, hydrolysed β-lactams and analogues of tetrahedral transition state, with two MBLs—hydrolysed cefuroxime with NDM-1 (PDB ID 4RL0) (**e**) and cyclic boronate 2 with VIM-2 (PDB ID 5FQC) (**f**). **g**, Active site interactions made by InCs reveal their ability to mimic the proposed binding mode for bicyclic β-lactams and/or subsequently formed intermediates.

Supplementary Fig. 1). Thus, at least with respect to retention of the hydrolytic hydroxide and the Zn–Zn distance, the InC binding mode mimics that anticipated for substrates. However, note that the binding mode of the pyrrole ring of the InCs is similar to that observed for the pyrroline ring in active-site bound hydrolysed carbapenems, especially when in its enamine form (as shown in Fig. 1b and Supplementary Fig. 1), which is proposed to be the major nascent product for MBL-catalysed carbapenem hydrolysis[30]. Note that most MBL-hydrolysed carbapenem structures show the complexed product in the imine tautomeric form, although this is not necessarily relevant in efficient catalysis (Supplementary Fig. 1). Thus, the binding mode of the InCs resembles those of both substrates and products, albeit in different regards.

The C2 InC carboxylate ligates to Zn2 in a manner reminiscent of the carboxylate of β-lactam substrates (Fig. 3). Importantly, this binding mode enables InCs to engage with the different motifs employed in MBL substrate carboxylate binding, for example VIM-1 (B1) uses a histidine-, VIM-2 (B1) an arginine-, NDM-7 (B1) a lysine- and L1 (B3) a serine residue in substrate carboxylate binding (Fig. 2a–d).

The InC C3 substituent interacts with residues at the active site surface in a manner analogous to that of cephalosporin C3′ and/or carbapenem C2 sidechains (Fig. 2g). At least one of the methyl groups of the C7 isopropyl points towards the surface of the active site at which the C6 or C7 groups of the penicillins or cephalosporins, respectively, bind (Fig. 2a–d). Thus, the binding of C7, C3 alkyl or aryl and C2 carboxylate substituted InCs mimics those of β-lactams and the structures rationalize the SAR observations that both the InC C3 and C7 positions are amenable to substitution with relatively large groups (Fig. 2e,f).

β-Lactamase inhibition profile. Comparison of the potency of six InCs (**1**, **11**, **16**, **48**, **49** and **53**) with that of the cyclic boronate taniborbactam (a dual MBLI and SBLI) and avibactam (an SBLI)

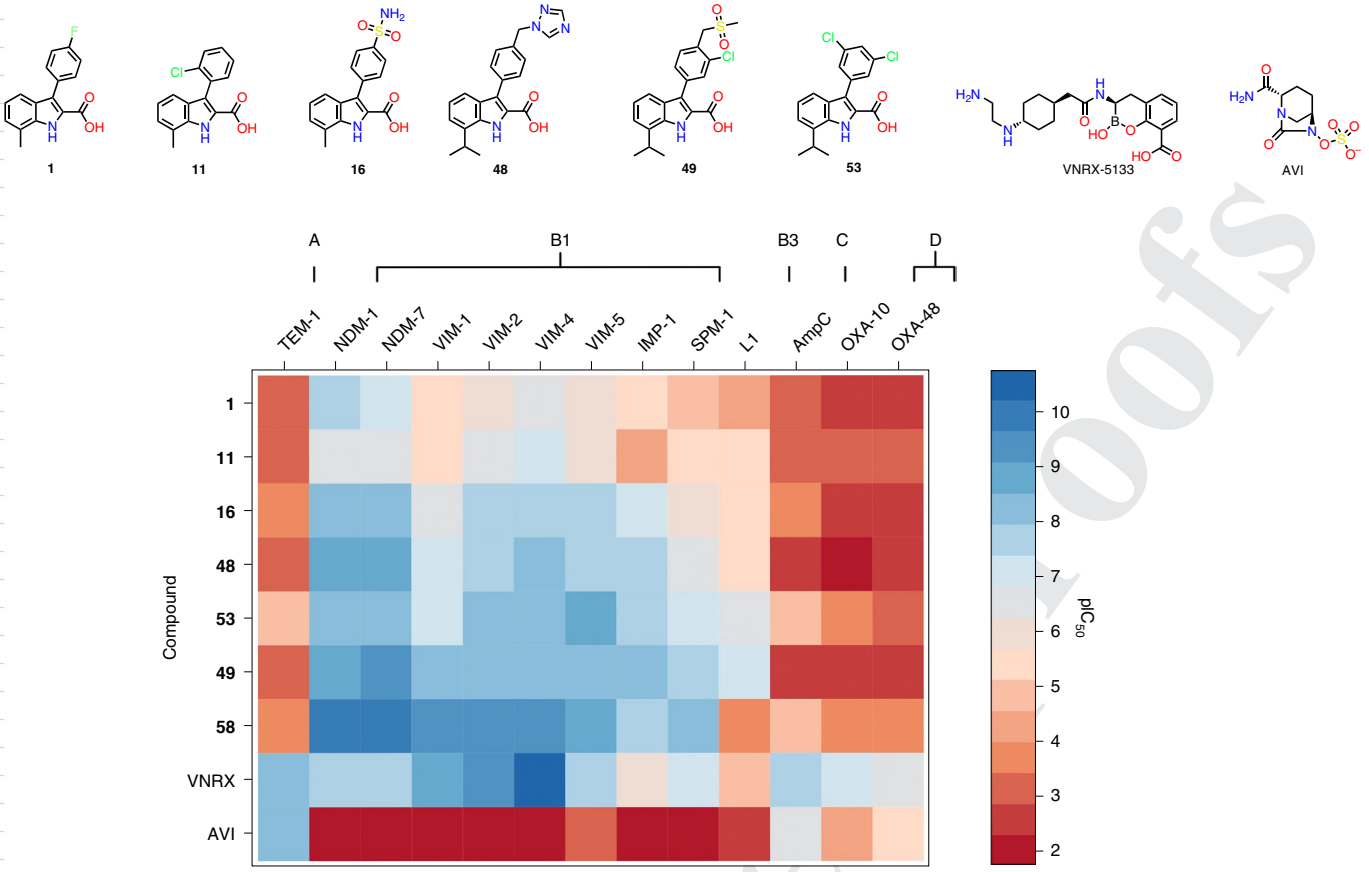

Fig. 3 | Heat-map analysis comparing the potency of InCs for selected clinically important SBLs and MBLs with β-lactamase inhibitors. InCs are potent MBLis and less potent SBLIs. VNRX, VNRX-5133 (taniborbactam); AVI, avibactam. SBLs and MBLs tested: TEM-1, a class A SBL; NDM-1, NDM-7, VIM-1, VIM-4, VIM-5, IMP-1 and SPM-1, all class B1 MBLs; L1, a class B3 MBL; AmpC from *P aeruginosa*, a class C SBL, and OXA-10 and OXA-48, class D SBLs. Q15 Note that the variations in potency versus the VIM-1, VIM-2, VIM-4 and VIM-5 variants may reflect substitutions at residues 224 and/or 228 on the L10 loop, which is involved in substrate–InC carboxylate and C3 binding.

against representative SBLs and/or MBLs reveals the superiority of the InCs for MBL inhibition. The InCs potently inhibit all the tested clinically relevant B1 and B3 MBLs; for several MBLs (SPM-1, L1, IMP-1, NDM-7 and VIM-1) a 100–1,000-fold improved activity compared with that of VNRX-5133 was observed (Supplementary Table 22). Given that both SBLs and MBLs recognize the same substrates (Fig. 2g), it is interesting that InCs are also SBLIs (pIC_{50} values of 2.4–5.1), although at a much lower level than that for MBLs (Fig. 3 and Supplementary Table 22)[38].

Lead optimization. We then carried out SAR studies at the InC C3 and C7 positions employing structural information to improve the metabolic stability and plasma protein binding of **49**, while maintaining and/or improving the activity versus that of isolated MBLs (Supplementary Table 23). The general synthetic routes used to prepare the InCs are shown in Fig. 4. Modification at C3 was achieved via Pd-catalysed arylation of bromo or iodo precursors, prepared from commercial indoles or from anilines. Racemic C3 and C7 amide derivatives (for example, **56**) were prepared by reductive amination of a C7 methyl ketone, followed by amide formation. Chiral C7 amides (for example, **57** and **58**) were prepared from the same starting materials via imine formation with (*R*)-*tert*-butylsulfinamide, then an organoborane (L-selectride)-mediated reduction and hydrolysis to give a chiral C7 amine, which was coupled to give **57** and **58**.

The SAR studies improved NDM-1 activity, as exemplified by (±)-**56**, which manifests more than tenfold improvement over that of **49**. However, these studies did not translate into improved VIM-1 activity (Fig. 3). Docking studies indicated the (*S*)-enantiomer of racemic **56** would preferentially bind to NDM-1. Indeed, **57** showed an improved activity versus NDM-1 ($pIC_{50} > 10.2$ against NDM-1, the limit of detection in our standard assay).

To improve the VIM-1 activity, we installed a novel amide isostere, that is a spirocyclic oxazolidinone at C7 together with a primary amine, the latter to improve the accumulation in Gram-negative bacteria[39]. Separation of the diastereomers of the spirocyclic oxazolidinone gave **58** and **59** (Fig. 4), which showed a similar 2 log-fold activity improvement versus that of VIM-1 (compared with **57**); **59** shows an improved NDM-1, VIM-2 and IMP-1 activity compared with that of **58** (0.6–0.8-fold; Supplementary Table 23). Thermal shift assays measured using differential scanning fluorimetry reveal that binding of **58** stabilizes NDM-1 (the melting temperature of NDM-1 increase by 14 °C in the presence of **58**; Supplementary Fig. 6a) and its resistance to proteolytic degradation also increases, as revealed by mass spectroscopy studies (Supplementary Fig. 6b–d).

Microbiological studies reveal similarly good activities for **58** Q18 and **59** (Supplementary Table 24), which manifest an excellent metabolic stability ($t_{1/2} > 450$ minutes in mouse, rat and human hepatocytes), although the fraction of unbound compound to human and mouse plasma was higher for **58** (mouse/human f_u (%) 6/38 versus 1.8/28) than for **59**. In the case of **58**, this represents a >500-fold improvement compared with that of **49** (Supplementary Table 25).

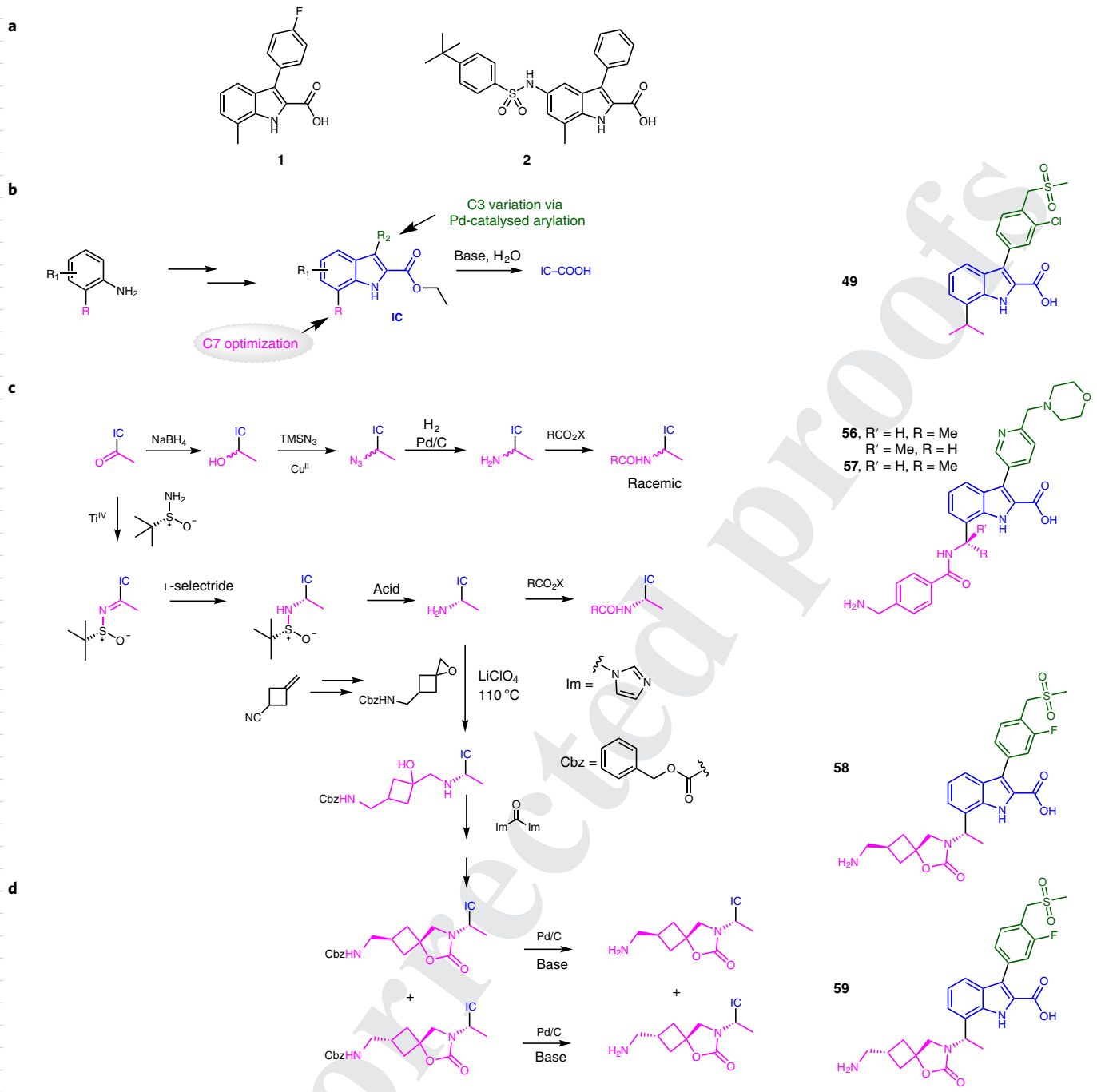

Fig. 4 | Strategies for InC synthesis. a, HTS hits. C3 groups were introduced by the Pd-catalysed arylation of C3 bromo or iodo precursors, prepared from commercial indoles or aniline precursors. In general, the C3 acid was protected as an alkyl ester, which was cleaved by base-mediated hydrolysis. **b**, Synthesis of simple derivatives in early hit finding and SAR studies (for example, **49**) involved Pd coupling. **c**, Preparation of C7 derivatives during the lead optimization required development. Racemic C3 and C7 amide derivatives (for example, **56**) were prepared via a two-step reductive amination of the requisite C7 methyl ketone via an alcohol intermediate, followed by amide formation. Subsequent C3 iodination enabled Pd-catalysed C3 arylation. **d**, Optimization at C7 for improved VIM activity. Chiral C7 amides (for example, **57**) were prepared via an initial $Ti(OEt)_4$-catalysed imine formation with (*R*)-*tert*-butylsulfinamide, then L-selectride-mediated reduction and acid hydrolysis to give a chiral C7 amine, which was used to prepare diastereomerically pure **58** and **59**. TMS, tetramethylsilyl.

Restoration of carbapenem activity against Enterobacterales pathogens and determining frequency of resistance. We investigated the level to which **58** (8 mg l^{-1}) restores carbapenem activity against 280 (Fig. 5 and Supplementary Table 27) genome-sequenced MDR and XDR global Gram-negative clinical isolates (Supplementary Table 26). The MIC_{90} values for meropenem, imipenem and doripenem alone against the panel were all >64 mg l^{-1} (Supplementary Table 24). Combinations of **58** and a carbapenem resulted in potent activity and broad coverage against Enterobacterales (MIC_{90} 1–2 mg l^{-1}). MIC_{90} values were, in several cases, up to fourfold below the carbapenem breakpoint. Excellent activity was obtained against *Citrobacter* spp, *Serratia marcescens*

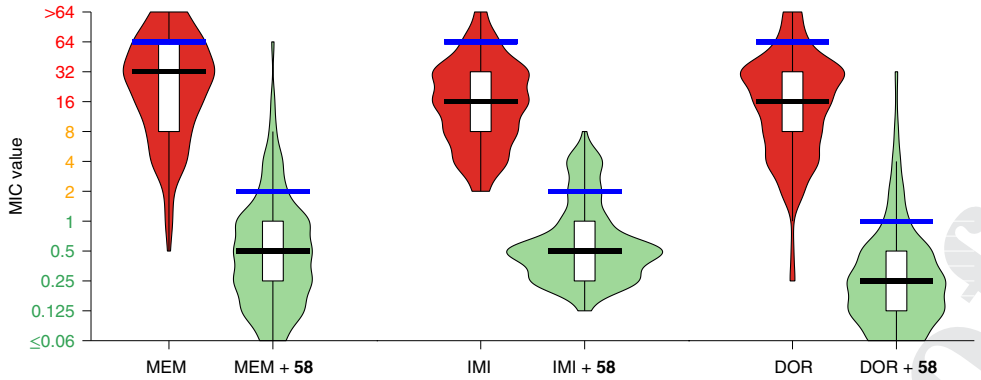

Fig. 5 | InC 58 potentiates meropenem activity in vitro against clinically relevant strains. Coverage of clinically relevant strains for **58** carbapenem combinations. Agar dilution series MIC susceptibility testing showing the combined effect of **58** and meropenem (MEM), imipenem (IMI) or doripenem (DOR) against a globally acquired representative MBL-producing Enterobacterales collection (total $n=280$, *E. coli* $n=97$, *Klebsiella* spp $n=47$, *E. cloacae* $n=51$, other *Enterobacter* $n=20$, *P. mirabilis* $n=6$, *S. marcescens* $n=12$, *Citrobacter* spp $n=28$ and *Providencia* spp $n=20$). The Enterobacterales panel reflects pathogens commonly isolated from complicated urinary tract infections (Supplementary Table 23)[47]. Sequencing revealed the presence of up to seven β-lactamase genes, which represent all Ambler classes (Supplementary Table 26), including VIM (VIM-1, VIM-2, VIM-12, VIM-20 and VIM-40) and NDM variants (NDM-1, NDM-5, NDM-7 and NDM-16; Supplementary Table 26c). Violin plots show the kernel probability density of the data, which range from the minimum to the maximum observed value. The lower and upper hinges of each Tukey's box plot correspond to the first and third quartiles of the data distribution, respectively, with whiskers that extend 1.5 times the interquartile range from each hinge. Outliers are not represented. Black lines show MIC_{50} values (which correspond to each median) and blue lines show MIC_{90} values. The results were obtained from a single biological sample of each bacterial isolate.

and *Proteus mirabilis* (meropenem MIC_{90} 0.125–0.5 μg ml^{-1}). The MIC_{90} values against other tested strains (*E. coli*, *K. pneumoniae*, *Providencia* spp and *Enterobacter* spp.) were 2–4 mg l^{-1} and 0.5–1 mg l^{-1} for **58** with meropenem and doripenem, respectively. Compared with the combination of taniborbactam with cefepime or meropenem, the **58**–meropenem combination showed an up to sixfold higher activity (Supplementary Table 28).

The FoR (frequency of spontaneous mutational resistance) to combinations (imipenem and meropenem) with **58** was low against three strains used for the in vivo studies. The corresponding figure for the hypermutable (100× mutation rate) strain *E. coli* GB20 (*ΔampC mutS::Tn10*) strain[40] the FoR was $<10^{-10}$ (below the level of detection at 4×MIC) (Supplementary Table 29). Similarly, a seven-day serial passage experiment revealed that **58** reduced the development of resistance to meropenem or imipenem when compared with meropenem or imipenem alone (Supplementary Fig. 7).

Safety and in vivo activity. InC **58** showed no evidence for major interactions with 69 human receptors (Supplementary Table 26) and was well tolerated in mice (300 mg kg^{-1}, subcutaneous seven-day repeat dose) with only mild side effects (Supplementary Table 31). Macroscopic organ changes were not observed and plasma kidney and liver markers (blood urea nitrogen, kidney injury molecule-1, aspartate aminotransferase and alanine aminotransferase) were not elevated.

We examined the efficacy of the **58**–meropenem combination in a total of eight murine infection models using four different strains (Fig. 6a–c). To identify suitable clinically derived isolates, we screened >30 strains in pilot murine models. Overall, we did not find correlation between meropenem MIC and in vivo virulence or EC_{50} (half-maximum effective concentration). For **58**, single intravenous doses (10 or 30 mg kg^{-1}) were used, which, based on single-dose pharmacokinetic studies, resulted in peak **58** concentrations of 75 and 224 μg ml^{-1}, respectively. At this dose, the $t_{1/2}$ of **58** was ~0.69 hours, considerably longer than that of meropenem (0.17 hours) (Supplementary Fig. 8).

With the peritonitis/septicaemia models (Fig. 6b and Supplementary Figs. 9–12) using three *E. coli* strains and one *K. pneumoniae strain*, the **58**–meropenem combination significantly reduced the mean colony-forming units (c.f.u.) compared with meropenem alone, by as much as ~5 log-fold c.f.u. With the thigh infection models (Fig. 6c and Supplementary Figs. 13–16) using the same strains at a dose of **58** (10 mg kg^{-1}), the effect was reduced, being a <1 log-fold c.f.u. reduction for two of the *E. coli* strains and an ~1 log-fold c.f.u. reduction for the *E. coli* IHMA and the *K. pneumoniae* B-68–1 strains. However, at a higher **58** dose (30 mg kg^{-1}) the c.f.u. count was reduced up to ~2 log-fold c.f.u.

Discussion

The clinical need for new treatments against MDR and XDR Gram-negative infections, in particular for Enterobacterales. is critical[41]. Owing primarily to the excellent safety profiles and efficacy of β-lactams in the absence of β-lactamases, one successful strategy is the development of SBLIs[5]. However, there are no MBLis in clinical use and only taniborbactam, which has a narrow spectrum of MBL activity, is in clinical trials.[19] The lack of MBLis is, in part, due to the challenge of achieving the breadth of potency against different subclasses of clinically relevant MBLs (Figs. 1 and 2)[42]. Our collaboration involving academic and pharmaceutical partners that operate across multiple sites, has enabled the development of a novel type of MBLi, **58**, that potentiates the in vivo activity of carbapenems.

We used a high-throughput screen to search for novel NDM-1 inhibitors with a binding efficiency that mimics that of β-lactam substrates, based on the premise that most β-lactamases have evolved to bind their substrates with remarkable efficiency (Figs. 1 and 2). We envisaged that inhibitors that mimic the elements of bicyclic β-lactam substrate, which are involved in MBL binding, might enable the breadth of MBL inhibition required for clinical application (Fig. 2g). By contrast with our substrate-focused approach, a (single) protein-based structure-based design approach might not favour the identification of such broad-spectrum inhibitors.

After crystallographic studies on several new MBLI classes identified from the NDM-1 inhibition high-throughput screening, we focused on the InCs. We found that C3 and C7 functionalized InCs are broad-spectrum NDM, VIM and IMP B1 MBLis, but they also inhibit B3 MBLs, as shown by studies with L1. SAR studies guided

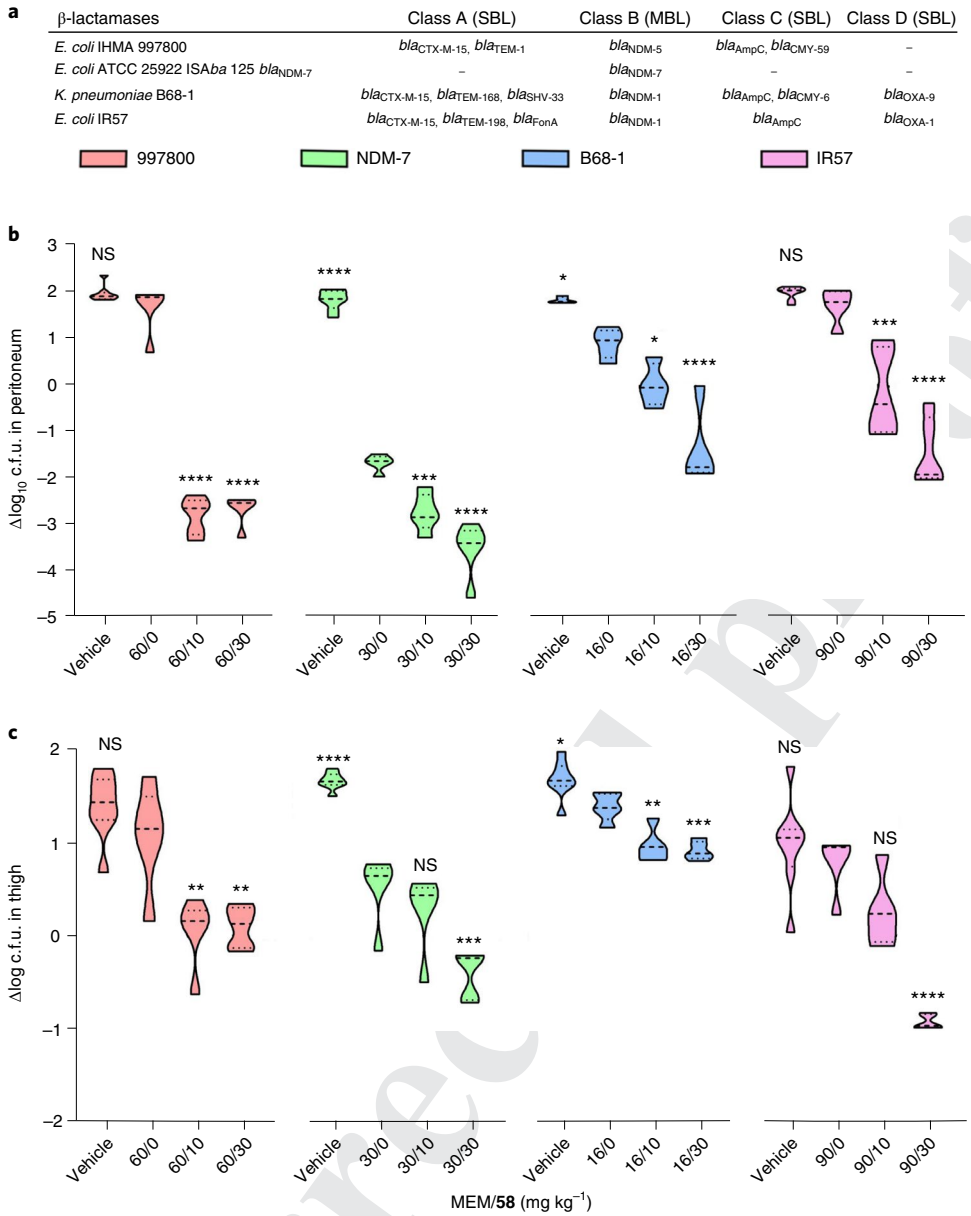

Fig. 6 | InC 58 potentiates meropenem activity in vivo against clinically relevant strains. a, Strains used for the in vivo efficacy studies. **b**,**c**, In vivo peritonitis/septicaemia (**b**) and in vivo thigh mouse model (**c**) results for **58**–meropenem and meropenem alone. In both murine neutropenic and peritonitis/septicaemia models, we used a 4 h infection followed by a single subcutaneous injection of meropenem and a single intravenous dose of **58**. For the murine neutropenic models, colistin was used as a positive control; animals were made neutropenic by cyclophosphamide treatment. For the murine peritonitis/septicaemia model, 5% porcine mucin was used to achieve virulence. For the thigh model, the final inoculum was ~2×10^7 c.f.u. ml^{-1} and for the peritonitis/septicaemia model 5.7×10^5 was adequate. Meropenem doses were based on dose-dependence studies. Statistical comparisons were performed with Prism 8 using one-way analysis of variance and Dunnett's multiple comparisons test, and all the differences between means with $P \leq 0.05$ are indicated. NS, not significant. *P* values for the in vivo peritonitis/septicaemia model are: for 997800, 60/0 versus 60/10 $P \leq 0.0001$, 60/0 versus 60/30 $P \leq 0.0001$; for NDM7, 30/0 versus vehicle $P \leq 0.0001$, 30/0 versus 30/10 $P = 0.0006$, 30/0 versus 30/30 $P \leq 0.0001$; for B68-1, 16/0 versus vehicle $P = 0.0282$, 16/0 versus 16/10 $P = 0.0450$, 16/0 versus 16/30 $P \leq 0.0001$; for IR57 90/0 versus 90/10 $P = 0.0002$, 90/0 versus 90/30 $P \leq 0.0001$). *P* values for the in vivo thigh mouse model are: for 997800, 60/0 versus 60/10 $P = 0.0019$, 60/0 versus 60/30 $P = 0.0030$; for NDM7 30/0 versus vehicle $P \leq 0.0001$, 30/0 versus 30/30 $P = 0.0003$; for B68-1, 16/0 versus vehicle $P = 0.0181$, 16/0 versus 16/10 $P = 0.0023$, 16/0 versus 16/30 $P = 0.0009$; for IR57, 90/0 versus 90/30 $P \leq 0.0001$.

by a structure-based design and analyses in solution revealed the importance of the InC indole NH, C2 carboxylate and C3 and C7 alkyl and/or aryl groups for potent MBL inhibition (Fig. 2a–d). The InC core can be readily synthesized and is amenable to modification at C3 and C7 via established and new procedures (Fig. 5).

In some regards, the biophysical analysis supports the proposal that the InC binding mode mimics that of intact bicyclic β-lactams, in particular carbapenems (Fig. 2g). Most strikingly, the indole NH and C7 alkyl groups cooperate to form a stable complex in which the di-Zn(II) ion-bridging hydroxide is retained and the Zn–Zn

distance is the same as that in the unligated MBLs. This binding mode contrasts with those of nucleophilic MBLis (for example, thiols, such as captopril[43]) and transition state analogues as bicyclic boronates[38], both of which displace the Zn-complexed hydroxide. The C7 alkyl group appears to enclose the bridging water/hydroxide and the indole NH is positioned to hydrogen bound to the bridging water, that is, it may mimic protonation of the β-lactam nitrogen that must occur during hydrolysis (Fig. 1).

However, the binding mode of the InCs also resembles those of carbapenem-derived products bound to the MBL active site, in particular in their enamine tautomeric form, which has been proposed to represent the major nascent product of MBL-catalysed carbapenem hydrolysis[30] (Supplementary Fig. 1). Thus, binding of the InCs appears to take advantage of elements included in both the substrate and product binding, a property that may contribute to their high potency.

Further, the InC C2 carboxylate binding mode is similar to that of the carboxylate of β-lactam substrates (and β-lactam derived intermediates), including with respect to interaction with the different active site elements used in substrate carboxylate binding by all of NDM-1, VIM-1, VIM-2 and L1, which probably contributes to their breadth of InC potency (Fig. 2).

The InC binding mode contrasts with those of other bidentate MBLis in different stages of development, for example Aspergillomarasmine A[18], thioenolates (derived from the corresponding rhodanines)[44], bicyclic boronates and pyridine and thiazole derivatives. Some of these are strong metal ion chelators and therefore could inhibit multiple human metalloenzymes, which include human MBLs[45], and potentially lead to toxicity. Whether or not these differences will be reflected in in vivo efficacy, selectivity and/or safety profiles is of interest with respect to future studies.

Overall, the InCs represent a new type of metalloenzyme inhibitor that works via an unprecedented mechanism of action that locks the zinc-complexed hydroxide, rather than displacing it. Importantly, compared with most reported MBLis, the InCs are relatively weak metal ion chelators. The metal-hydroxyl trapping mechanism of action manifested by the InCs for MBL inhibition may be of utility in the inhibition of other classes of MBL fold hydrolases that are medicinal chemistry targets[45] and more generally for the inhibition of metallohydrolases.

The carbapenem-type binding mode of the InCs enabled us to fine-tune MBL activity. Optimization of the C3 and C7 side chains enabled the identification of **58**, which potently inhibits all three targeted B1 MBL types, with pIC_{50} values >9 for NDMs and VIM, and >7 for IMP-1 obtained (Supplementary Table 23). We also optimized in vitro microbiology and ADME properties, the latter with regards to plasma protein binding and metabolic stability. It is notable that some InCs inhibit SBLs, although less potently than MBLs (Fig. 3), a difference that may reflect the different interactions employed by MBLs and SBLs in binding and/or reacting with β-lactams, that is, nucleophilic catalysis by SBLs versus metal-ion-enabled catalysis by MBLs. Also note that all the potent small-molecule SBLIs inhibit via a covalent reaction[16].

To assist SAR studies on optimizing the cell penetration and potential efflux properties, we used engineered strains with an efflux pump and porin deficiencies, as well as clinical isolates, which mainly compromised *E. coli* and *K. pneumoniae* strains that produce VIM, NDM and IMP variants (Supplementary Table 26). **58**–carbapenem combinations reduced MICs up to fourfold below the carbapenem breakpoints against an MDR and XDR Enterobacterales panel (Fig. 5). The activity of the **58**–meropenem combination compared with those for taniborbactam plus meropenem or taniborbactam plus cefepime reveals the impressive efficacy of **58** in repressing B1 MBL-mediated resistance (Supplementary Table 28). The results for **58** combined with meropenem or imipenem demonstrate a lower FoR when compared with those of the carbapenems alone (Supplementary Table 29).

InC **58** is tolerated well by mice and when combined with meropenem it shows significant in vivo efficacy in multiple murine peritonitis/sepsis and thigh models with infection by carbapenem-resistant XDR strains. A single dose of **58** (10 mg kg^{-1}) plus meropenem (16–90 mg kg^{-1}) reduced the bacterial load in murine infection models by up to 7 log-fold. These results reveal that InCs have a significant potential for clinical development with β-lactam antibiotics. We are actively progressing InCs towards clinical trials in humans, with a particular focus on low-to-middle income countries in which NDM-mediated resistance is widespread.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41557-021-00831-x.

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Methods

Methods, full experimental section and all data supporting the findings of this study are available within the paper and its supplementary information files. The atomic coordinates and structure factors are deposited in the Protein Data Bank, accession codes: PDB ID 7AEZ (**48** with NDM-7), PDB ID 7AEX (**49** with VIM-1), PDB ID 7AFY (**11** with VIM-2) and PDB ID 7AFZ (**49** with L1). The raw data for the InC **58** and meropenem microbial activity dataset can be accessed in Zenodo at https://doi.org/10.5281/zenodo.4438867.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

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Author contributions

J.B., T.P., J.H., P.D., A.F., K.S., G.G.C., P.K., S.S., D.K., R.L., J.S., D.L., A.G.B., J.R., L.R., A.R., S.D.S.P., A.D.P., M.M., M.P., A.K.B., P.B., J.Y.-K., E.B., M.G.P.P., F.B., P.S.J., E.S., A.M. and C.J.S. conducted the medicinal chemistry analysis and/or chemical synthesis. J.U.H., E.L., E.N., J.K. and S.G. led the safety and/or *in vivo* efficacy experiments. A.E., M.B. and P.B. led or conducted the physicochemical and ADME testing. L.E., M.C.T., A.F.A., J.C.J.-C., E.W. and J.M.T. conducted the microbiological experiments under the guidance of M.B.A., M.G., R.C., F.B. and T.M.R. K.C., M.E.K., G.W.L., M.S., A.R., I.H.N., P.A.L., S.P.M. and J.B. conducted the biochemical or biophysical testing. P.H., M.M., T.L., J.S. and J.B. conducted the X-ray crystallography work and analysis. J.B. oversaw all the studies. J.B. and C.J.S. wrote the first draft of the manuscript with input from all the authors.

Competing interests

A patent has been filed concerning the indole carboxylates as MBL inhibitors (WO2017093727A1)[29]. The inventors may benefit financially from the work. The work described in the manuscript was carried out prior to these employments, which are not relevant to the work. G.W.L. is an employee of Charles River Laboratories, M.S., J.R., L.R., S.P.M., P.S.J. and A.M. are employees of BioAscent Discovery Ltd, A.R. and P.B. are employees of AstraZeneca and E.B. is an employee of Evotec.

Additional information

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Methods, full experimental section and all data supporting the findings of this study are available within the paper and its supplementary information files. The atomic coordinates and structure factors are deposited in the Protein Data Bank. Accession codes: PDB ID: 7AEZ (48 with NDM-7), PDB ID: 7AEX (49 with VIM-1), PDB ID: 7AFY (11 with VIM-2) and PDB ID: 7AFZ (49 with L1). The raw data for the InC 58 and meropenem microbial activity dataset can be accessed in Zendo: https://doi.org/10.5281/zenodo.4438867 for full data.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	n=5 for mouse efficacy studies. The sample size for PK experiments was determined by pilot experiments. A sample size of 5 animals per group was used as is advised by the relevant EMA and FDA guidelines. IC50 calculations. Points were plotted as the mean average of 4 intra-plate replicates. Dose-response curves was then fitted using the "log(inhibitor) vs normalized response - variable slope" model of GraphPad Prism 12.
Data exclusions	No data were excluded for the efficacy studies and the in vivo PK and Safety experiments. IC50 experiments: Each concentration point for routine screening was conducted with 4 intra-plate replicates. If one point from the 4 repeats was an outlier then it was excluded and the three remaining points used in the analysis; if more than two replicates were suitable for exclusion, all replicates were excluded. If more than two concentration points were excluded, or if a maximum/minimum plateau were not achieved, then the compound was retested separately; adjusting the concentration range for testing.
Replication	Efficacy studies: For the bacterial isolate E. coli IR57, the efficacy of EBL-2915 was evaluated using four independent murine thigh infection experiments and three murine septicemia experiments. Efficacy studies versus the other bacterial isolates were not repeated. For PK studies, the standard 2 replicates were used for LC-MS-MS measurements. IC50 experiments: A control compound was included in each screening run. If the pIC50 of the control compound deviated by more than 0.2 from the reference value, then then plate was considered to have failed.
Randomization	Efficacy studies: Mice were randomly distributed into groups on arrival from the vendor. Mice were randomly assigned to PK and safety experiments. IC50 experiments: randomization not applicable to screening data.
Blinding	Efficacy studies: Experiments were not blinded; however, technicians performing the CFU quantification did not consult the study protocol. In safety experiments researchers were blinded to study groups. In the PK experiments effects in the groups treatment were not compared to a control; blinding was therefore not relevant. IC50 experiments: Blinding is not applicable to biochemical screening data. Compounds were treated identically to control inhibitors of known potency.

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Materials & experimental systems

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☐	☒ Animals and other organisms
☒	☐ Human research participants
☒	☐ Clinical data
☒	☐ Dual use research of concern

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
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☒	☐ MRI-based neuroimaging

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals	Efficacy studies: mouse female NMRI from ENVIGO or TACONIC, PK, or tox studies: We used only 8-12 weeks old NMRI female mice from ENVIGO.
Wild animals	No wild animals were used

Field-collected samples	no field-collected samples
Ethics oversight	Efficacy studies. Standards for housing and care of animals comply with the latest and most comprehensive international guidelines, i.e. Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. All animal experiments were approved by the National Committee of Animal Ethics, Ministry of Environment and food of Denmark. PK and Safety experiments: Ethical permission for animal experiments is stated in the methods section. The experimental procedures were approved by the Latvian Animal Protection Ethical Committee, Food and Veterinary Service, Riga, Latvia.

Note that full information on the approval of the study protocol must also be provided in the manuscript.