RESEARCH ARTICLE

In silico identification and experimental validation of hits active against KPC-2 β-lactamase

Raphael Klein1☯, Pasquale Linciano2☯, Giuseppe Celenza3, Pierangelo Bellio3☯, Sofia Papaioannou2, Jesus Blazquez4, Laura Cendron5, Ruth Brenk6*, Donatella Tondi2*  

1 Institute of Pharmacy and Biochemistry, Johannes Gutenberg University, Mainz, Germany, 2 Dipartimento di Scienze della Vita, Università di Modena e Reggio Emilia, Modena, Italy, 3 Dipartimento di Scienze Cliniche Applicate e Biotecnologie, Università dell’Aquila, L’Aquila, Italy, 4 Department of Microbial Biotechnology, National Center for Biotechnology, Consejo Superior de Investigaciones Científicas (CSIC), Campus de la Universidad Autonoma-Cantoblanco, Madrid, Spain, 5 Dipartimento di Biologia, Università di Padova, Padova, Italy, 6 Department of Biomedicine, University of Bergen, Bergen, Norway  

☯ These authors contributed equally to this work.  
* donatella.tondi@unimore.it (DT); Ruth.Brenk@uib.no (RB)

Abstract

Bacterial resistance has become a worldwide concern, particularly after the emergence of resistant strains overproducing carbapenemases. Among these, the KPC-2 carbapenemase represents a significant clinical challenge, being characterized by a broad substrate spectrum that includes aminothiazoleoxime and cephalosporins such as cefotaxime. Moreover, strains harboring KPC-type β-lactamases are often reported as resistant to available β-lactamase inhibitors (clavulanic acid, tazobactam and sulbactam). Therefore, the identification of novel non-β-lactam KPC-2 inhibitors is strongly necessary to maintain treatment options. This study explored novel, non-covalent inhibitors active against KPC-2, as putative hit candidates. We performed a structure-based in silico screening of commercially available compounds for non-β-lactam KPC-2 inhibitors. Thirty-two commercially available high-scoring, fragment-like hits were selected for in vitro validation and their activity and mechanism of action vs the target was experimentally evaluated using recombinant KPC-2. N-(3-(1H-tetrazol-5-yl)phenyl)-3-fluorobenzamide (11a), in light of its ligand efficiency (LE = 0.28 kcal/mol/non-hydrogen atom) and chemistry, was selected as hit to be directed to chemical optimization to improve potency vs the enzyme and explore structural requirement for inhibition in KPC-2 binding site. Further, the compounds were evaluated against clinical strains over-expressing KPC-2 and the most promising compound reduced the MIC of the β-lactam antibiotic meropenem by four-fold.

Introduction

The emergence of KPC-2 class-A β-Lactamase (BL) carbapenemase, which confers resistance to last resort carbapenem, poses a serious health threat to the public. KPC-2, a class A BL,
uses a catalytic serine to hydrolyze the β-lactam ring. Specifically, the hydrolysis reaction proceeds through a series of steps involving: (i) the formation of a pre-covalent complex, (ii) the conversion to a high-energy tetrahedral acylation intermediate, (iii) followed by a low-energy acyl-enzyme complex, (iv) a high-energy tetrahedral de-acylation intermediate consequent to catalytic water attack, and (v) finally the release of the hydrolyzed β-lactam ring product from the enzyme. [1–6].

Notably to treat infections caused by bacteria that produce class A BLs, mechanism-based inhibitors (i.e., clavulanic acid, sulbactam, and tazobactam) are administered in combination with β-lactam antibiotics. However, strains harboring KPC-type β-lactamases are reported to be resistant to available β-lactamase inhibitors. Moreover, because of KPC-2’s broad spectrum of activity (which includes penicillins, cephalosporins, and carbapenems) treatment options against KPC-2-producing bacteria are scarce, and “last-resort” carbapenems are ineffective as well [7]. Therefore, studies directed to the discovery of novel, non-β-lactam KPC-2 inhibitors have multiplied in the last years. Recently, new drugs able to restore susceptibility to β-lactams i.e. the novel inhibitor avibactam in combination with ceftazidime (CAZ) and RPX7009 (vaborbactam) with meropenem have been approved (Fig 1)[8–10].

As attention on KPC-2 rises, the number of crystal structures of its apo and complexed form disclosed in the PDB has increased, making KPC-2 a druggable target for structure based drug design efforts and for the study of novel, non-β-lactam like inhibitors of this threatening carbapenemase [9–12]

Recently, two crystal structures of the hydrolyzed β-lactam antibiotics cefotaxime and faropenem in complex with KPC-2 were determined (PDB codes 5UJ3, 5UJ4; Fig 2).[13]

Both ligands form hydrogen-bond interactions with their C4-carboxyl group to Ser130, Thr235 and Thr237. The dihydrothiazine moiety of cefotaxime and the dihydrothiazole moiety of faropenem forms π-π-stacking interactions with Trp105. In the apo-enzyme, this side chain adopts two rotamers, upon binding of a ligand just one. Mutagenesis studies have shown the importance of Trp105 in substrate recognition [7]. The faropenem ring nitrogen forms a hydrogen-bond interaction with Ser130, whereas the ring nitrogen of cefotaxime a hydrogen bond with Ser70. The aminothiazole ring of cefotaxime forms van-der-Waals contacts with Leu167, Asn170, Cys238 and Gly239, while the oxyimino group and the hydroxyethyl group of faropenem are solvent exposed (Fig 2).[13]
Based on this and other structural information, we used a hierarchical screening cascade for the discovery of non β-lactam like KPC-2 inhibitors. The selected 32 candidates, most of them fragment-like, were then validated as hits against isolated recombinant KPC-2. Among the tested compounds 9a, a benzothiazole derivative, and 11a, a tetrazole-containing inhibitor, showed the highest activity against KPC-2 and behaved as competitive inhibitors of the targeted carbapenemase (Fig 1). Subsequently, compound 11a, in light of its promising ligand efficiency and chemistry, was selected to undergo chemical optimization for potency improvement and to explore structural requirement for inhibition in KPC-2 binding site. Further, the obtained compounds were evaluated against clinical strains overexpressing KPC-2 and the most promising compound reduced the MIC of the β-lactam antibiotic meropenem by four fold.

**Materials and methods**

**Pharmacophore hypothesis**

A search for similar binding sites of KPC-2 was carried out using the online tool PoSSuM—Search K [14,15]. Based on shared ligand interactions in the retrieved structures (Table 1), a
pharmacophore was defined based on a *K. pneumoniae* KPC-2 protein structure (PDB code 3RXW) [16] and the ligand OJ6 of CTX-M-9 β-lactamase (PDB code 4DE1) [17]. The derived pharmacophore contained a hydrogen-bond acceptor feature for interaction with Thr237, Thr235 and Ser130, a hydrophobic feature for π-stacking with Trp105 and a hydrogen bond acceptor feature for interactions with Asn132 (Fig 3).

**Virtual screening**

Our in-house MySQL-database of commercially available compounds was used as basis for virtual screening. This database contains catalogues from the following suppliers: Apollo

Table 1. Result of PoSSuM Search K for similar binding sites. Structures with binding sites similar with structure 3RXW in complex with a non-covalent ligand were reported.

| PDB-code | Protein name              | Resolution | Ref. |
|----------|---------------------------|------------|------|
| 1        | 4BD0 *E. coli* β-lactamase TOHO-1 | 1.21 Å     | [18] |
| 2        | 3G30 *E. coli* β-lactamase CTX-M-9a | 1.80 Å     | [19] |
| 3        | 4DE1 *E. coli* β-lactamase CTX-M-9a | 1.26 Å     | [17] |
| 4        | 4DDY *E. coli* β-lactamase CTX-M-9a | 1.36 Å     | [17] |
| 5        | 4DE3 *E. coli* β-lactamase CTX-M-9a | 1.44 Å     | [17] |
| 6        | 4DDS *E. coli* β-lactamase CTX-M-9a | 1.36 Å     | [17] |
| 7        | 4DE0 *E. coli* β-lactamase CTX-M-9a | 1.12 Å     | [17] |
| 8        | 4EUZ *S. fonticola* β-lactamase SFC-1 | 1.08 Å     | [20] |
| 9        | 4DE2 *E. coli* β-lactamase CTX-M-9a | 1.40 Å     | [17] |
| 10       | 3G35 *E. coli* β-lactamase CTX-M-9a | 1.41 Å     | [19] |
| 11       | 3G32 *E. coli* β-lactamase CTX-M-9a | 1.31 Å     | [19] |
| 12       | 3G2Y *E. coli* β-lactamase CTX-M-9a | 1.31 Å     | [19] |
| 13       | 3G31 *E. coli* β-lactamase CTX-M-9a | 1.70 Å     | [19] |

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Scientific, Asinex, Chembridge, Chemdiv, Enamine, InterBioScreen, Key Organics, Life Chemicals, Maybridge, MicroCombiChem, Otava, Peakdale, Sigma-Aldrich, Specs, Timtec, Vitas-M laboratory, and ZyLexa Pharma. The compounds were standardized and filtered for compounds fulfilling the following lead-like criteria: between 10 and 25 heavy atoms, between one and six hydrogen-bond acceptors, between one and three hydrogen-bond donors and a clog P between -3 and 3. In addition, the complexity was limited by only including compounds with less than 7 rotatable bonds and between 1 and 3 ring systems. Compounds containing unwanted reactive or toxic functional groups were excluded as well [22].

In-house python scripts based on OpenEye’s OEChem toolkit (OEChem, version 2016.6.1, OpenEye Scientific Software, Inc., Santa Fe, NM, USA) were used to charge, tautomerize and stereoisomerize the selected compounds (scripts are available at https://github.com/ruthbrenk/compound_preparation). Conformers were generated using OpenEye’s OMEGA toolkit [23]. The pharmacophore filtering was carried out using Molecular Operating Environment (MOE, Chemical Computing Group). Compounds that passed the pharmacophore filter were transformed into a format suitable for docking as described previously [24].

The crystal structure of *K. pneumoniae* KPC-2 (PDB code 3RXW) [16] was used as receptor for docking. The ‘protonate 3D’ tool of MOE was used to add polar hydrogen atoms to the receptor, energy minimize their positions and to assign partial charges based on the AMBER force field parameters. Water molecules and ligands (CIT and SR3) were deleted and the position of the Ser69 side chain was energy minimized with the same force field parameters. The structure was aligned with the crystal structure of *E. coli* CTX-M-9 (PDB code 4DE1) and the ligand 0J6 was used to define spheres as matching points for docking. Grid-based excluded volume, van-der-Waals potential and electrostatic potential as well as solvent occlusion maps were calculated as described earlier [25,26].

The compounds were docked into the binding site of KPC-2 using DOCK3.6 [26–28]. Parameters for sampling ligand orientations were set as follows: bin size of ligand and receptor were set to 0.4 Å, overlap bins were set to 0.2 Å and the distance tolerance for receptor and ligand matching spheres was set to 1.5 Å. Each docking pose which did not overlap with the receptor was scored for electrostatic and van-der-Waals complementarity and penalized according to its estimated partial desolvation energy. For each compound, only the best-scoring pose out of its tautomers, protonation states or ring alignments was saved in the final docking hit list. The docking hit list was filtered with the pharmacophore described above, keeping the ligand positions rigid. Compounds passing this filter were ranked by their calculated ligand efficiency [29,30] and inspected by eye.

### Expression and purification of recombinant KPC-2

The blaKPC-2 gene was kindly provided by Prof. Sergei Vakulenko (University of Notre Dame du Lac, Indiana, USA) and cloned as already reported [31] and transformed into competent *E. coli* BL21 (DE3) cells for protein expression. 50 mL of Tryptic Soy Broth (TSB) (50 mg/L kanamycin) were inoculated with fresh colonies and grown at 37°C. 4 mL of the overnight culture was used to inoculate 1.3 L of TSB (50 mg/L kanamycin) grown at 37°C with shaking to an optical density of 0.5 measured at 600 nm. Then expression of recombinant bla gene was induced by adding 1.0 mM IPTG (isopropyl-D-thiogalactopyranoside) and the cells were again allowed to grow at 20°C overnight. Bacteria were harvested by centrifugation at 4000 rpm for 20 minutes. The pelleted cells were resuspended in Tris-HCl 50 mM pH 7.4–7.5. Periplasmatic proteins were extracted as reported in the pET System Manual (TB055 10th Edition Rev. B 0403) and subsequently dialyzed in sodium acetate buffer (50 mM, pH 5.0). The protein was conveniently purified in a single step using a Macro-Prep High S resin and eluted using
sodium acetate 50 mM pH 5.0 and a sodium chloride (NaCl) linear gradient from 100 to 500 mM. The purified protein was dialyzed overnight in sodium phosphate buffer 50 mM, pH 7.0 [31,32].

**Inhibition assays**

The hydrolytic activity of KPC-2 was measured using the β-lactam substrates CENTA (100 uM, $K_M$ 70 uM) or nitrocefin (114 uM, $K_M$ 36 μM) in reaction buffer consisting of 50 mM of PB at pH 7.0 at 25˚C with 0.01% v/v Triton X-100 to avoid compound aggregation and promiscuous inhibition.[33] Reactions were monitored using a Beckmann DU640 spectrophotometer at 405nM for CENTA and 480 nM wavelength for nitrocefin [34]. The test compounds were synthesized as described below or purchased from Enamine, TimTec, Vitas-M, ChemBridge, Otava, Life Chemicals or Apollo Scientific and assayed without further purification. Compounds were dissolved in dimethyl sulfoxide (DMSO) to a concentration of 25 mM and stored at -20˚C. The highest concentration at which the compounds were tested was up to 1 mM (depending on their solubility). All experiments were performed in duplicate and the error never exceeded 5%. The reaction was typically initiated by adding KPC-2 to the reaction buffer last. To control for incubation effects, protein was added to the reaction buffer first, and the reaction was initiated by the addition of reporter substrate after 10 minutes of enzyme-compound incubation. The results are reported in Fig 4 and Fig 5.

Competitive inhibition mechanism and the $K_i$ for compound 9a was determined by Line-Weaver–Burk (LB) and Dixon plots. For compound 11a, already reported as competitive inhibitor of the extended spectrum β-lactamase (ESBL) CTX-M15, the $K_i$ was calculated by the Cheng-Prusoff equation ($K_i = IC_{50}/(1+ [S]/K_M)$) assuming competitive inhibition [35].

**Synthetic procedures**

All commercially available chemicals and solvents were reagent grade and were used without further purification unless otherwise specified. Reactions were monitored by thin-layer chromatography on silica gel plates (60F-254, E. Merck) and visualized with UV light, cerium ammonium sulfate or alkaline KMnO$_4$ aqueous solution. The following solvents and reagents have been abbreviated: ethyl ether (Et$_2$O), dimethyl sulfoxide (DMSO), ethyl acetate (EtOAc), dichloromethane (DCM), methanol (MeOH). All reactions were carried out with standard techniques. NMR spectra were recorded on a Bruker 400 spectrometer with $^1$H at 400.134 MHz and $^{13}$C at 100.62 MHz. Proton chemical shifts were referenced to the TMS internal standard. Chemical shifts are reported in parts per million (ppm, δ units). Coupling constants are reported in units of Hertz (Hz). Splitting patterns are designed as s, singlet; d, doublet; t, triplet; q quartet; dd, double doublet; m, multiplet; b, broad. Mass spectra were obtained on a 6310A Ion TrapLC-MS(n).

**General procedure for the synthesis of sulfonamides 1-6b.** To a solution of 3-(1H-tetrazol-5-yl)aniline (1 eq.) in DCM dry (25 mL) at room temperature and under nitrogen atmosphere, pyridine (3 eq.) and the appropriate sulfonyl-chloride (1.2 eq.) were added. The mixture was reacted at room temperature for 2–12 h. The reaction was quenched with aqueous saturated solution of NH$_4$Cl and acidified at pH 4 with aqueous 1N HCl. The aqueous phase was extracted with AcOEt, and the organic phase washed with brine, dried over Na$_2$SO$_4$ and concentrated. The crude was crystalized from MeOH or Et$_2$O to give the desired product.

**N-(3-(1H-tetrazol-5-yl)phenyl)-3-fluorobenzene sulfonamide (1b).** Pale yellow solid (150 mg, yield 47%). $^1$H NMR (400 MHz, DMSO-d6) δ 7.19 (dd, $J = 2.2, 8.1$ Hz, 1H), 7.33–7.45 (m, 2H), 7.47–7.58 (m, 3H), 7.62 (d, $J = 7.7$ Hz, 1H), 7.76 (t, $J = 1.8$ Hz, 1H), 10.61 (s, 1H), the H of tetrazole exchanges. MS m/z [M+H]$^+$ 320.1; [M-1]$^{-}$ 318.0.
| ID  | Structure | $IC_{50}$ mM $^{[a,b]}$ | ID  | Structure | $IC_{50}$ mM $^{[a,b]}$ |
|-----|-----------|-------------------|-----|-----------|-------------------|
| 1a  |          | > 0.33 (30)       | 17a |          | > 1.0 (23)        |
| 2a  |          | > 0.50 (38)       | 18a |          | 1.0 (18)          |
| 3a  |          | > 0.25 (13)       | 19a |          | 0.88              |
| 4a  |          | > 0.25 (12)       | 20a |          | > 0.5 (28) $^{[c]}$ |
| 5a  |          | > 0.50 (13)       | 21a |          | > 1.0 (12)        |
| 6a  |          |                  | 22a |          | NI at 1.0         |
| 7a  |          | > 1.0 (18)        | 23a |          | > 1.0 (26) $^{[c]}$ |
| 8a  |          | > 0.50 (37)       | 24a |          | > 0.1 (41)        |
| 9a  |          | 0.15$^{[d]}$      | 25a |          | > 0.4 (26)        |
| 10a |          | 0.07 $^{[e]}$     | 26a |          | > 0.83 (18)       |
| 11a |          | 0.036 $^{[e]}$    | 27a |          | NI at 0.5         |
| 12a |          | 0.70 $^{[e]}$     | 28a |          | > 0.5 (14)        |
| 13a |          | NI at 0.25        | 29a |          | > 1.0 (15)        |
| 14a |          | > 1.0 (26)        | 30a |          | NI at 1.0         |
| 15a |          | > 0.66 (38)       | 31a |          | > 1.0 (11)        |
| 16a |          | > 1.0 (17)        | 32a |          | 1.24              |
N-(3-(1H-tetrazol-5-yl)phenyl)-3-nitrobenzenesulfonamide (2b). Pink solid (62% yield). $^1$H-NMR (400 MHz, DMSO-d$_6$) $\delta$: 7.31 (ddd, $J = 1.0, 2.3, 8.2$ Hz, 1H), 7.51 (t, $J = 8.0$ Hz, 1H), 7.74 (dt, $J = 1.2, 7.8$ Hz, 1H), 7.82–7.96 (m, 2H), 8.18 (dt, $J = 1.3, 7.9$ Hz, 1H), 8.46 (ddd, $J = 1.0, 2.3, 8.2$ Hz, 1H), 8.54 (t, $J = 2.0$ Hz, 1H), 10.90 (s, 1H); the H of tetrazole exchanges.

$^{13}$C-NMR (DMSO-d$_6$) $\delta$: 117.35, 120.09, 123.04, 127.35, 129.17, 129.22, 129.69, 133.21, 136.59, 137.99, 139.81, 148.82, 154.28. MS m/z [M+H]$^+$ Calcd for C$_{13}$H$_{10}$N$_6$O$_4$S: 346.0 Found: 347.2.

N-(3-(1H-tetrazol-5-yl)phenyl)-5-(dimethylamino)naphthalene-1-sulfonamide (3b). White solid (31% yield). $^1$H NMR (400 MHz, DMSO-d$_6$) $\delta$: 2.78 (s, 6H), 7.20 (dd, $J = 1.5, 7.6$ Hz, 1H), 7.24–7.38 (m, 3H), 7.39–7.65 (m, 3H), 8.27 (dd, $J = 1.6, 7.5$ Hz, 1H), 8.41 (ddd, $J = 1.5, 7.5, 19.3$ Hz, 2H), the H of tetrazole exchanges. MS m/z [M+H]$^+$ Calcd for C$_{19}$H$_{18}$N$_6$O$_2$S: 394.1 Found: 395.1.

N-(4-(N-(3-(1H-tetrazol-5-yl)phenyl)sulfamoyl)phenyl)acetamide (4b). Pink solid (52% yield). $^1$H NMR (400 MHz, DMSO-d$_6$) $\delta$: 2.12 (s, 3H), 7.31 (ddd, $J = 1.0, 2.3, 8.2$ Hz, 1H), 7.44 (t, $J = 7.9$ Hz, 1H), 7.65–7.72 (m, 3H), 7.74–7.79 (m, 2H), 7.86 (t, $J = 1.9$ Hz, 1H), the H of tetrazole exchanges. $^{13}$C NMR (100 MHz, DMSO-d$_6$) $\delta$: 22.58, 118.86, 118.95, 122.60, 123.07, 126.32, 127.96, 128.87, 129.96, 133.69, 139.01, 142.97, 170.57. MS m/z [M+H]$^+$ Calcd for C$_{15}$H$_{14}$N$_6$O$_3$S: 358.1 Found: 359.2.

N-(3-(1H-tetrazol-5-yl)phenyl)quinoline-8-sulfonamide (5b). White solid (88% yield). $^1$H NMR (400 MHz, Acetone-d$_6$) $\delta$: 7.28–7.42 (m, 2H), 7.59–7.90 (m, 4H), 8.03 (dt, $J = 1.1, 1.8$ Hz, 1H), 8.25 (dd, $J = 1.5, 8.2$ Hz, 1H), 8.42 (dd, $J = 1.4, 7.3$ Hz, 1H), 8.52 (dd, $J = 1.8, 8.4$ Hz, 1H), 9.21 (dd, $J = 1.8, 4.3$ Hz, 1H), 9.41 (s, 1H), the H of tetrazole exchanges. $^{13}$C NMR (100 MHz, Acetone-d$_6$) $\delta$: 117.35, 120.09, 123.01, 123.04, 125.55, 128.06, 129.17, 129.69, 129.79, 130.11, 133.27, 139.81, 140.02, 140.84, 149.72, 154.28. MS m/z [M+H]$^+$ Calcd for C$_{16}$H$_{12}$N$_6$O$_2$S: 352.1 Found: 353.2.

N-(3-(1H-tetrazol-5-yl)phenyl)-4-chlorobenzenesulfonamide (6b). Light yellow solid (93% yield). $^1$H NMR (400 MHz, Methanol-d$_4$) $\delta$: 7.57 (t, $J = 8.0$ Hz, 1H), 7.74–7.84 (m, 1H), 7.78–7.84 (m, 1H), 7.99 (d, $J = 8.6$ Hz, 2H), 8.06 (d, $J = 8.6$ Hz, 2H), 8.41 (t, $J = 1.9$ Hz, 1H), the H of tetrazole exchanges. $^{13}$C NMR (100 MHz, Methanol-d$_4$) $\delta$: 117.35, 120.09, 123.01, 123.04, 125.55, 128.06, 129.17, 129.69, 129.79, 130.11, 133.27, 139.81, 140.02, 140.84, 149.72, 154.28. MS m/z [M+H]$^+$ Calcd for C$_{13}$H$_{10}$ClN$_5$O$_2$S: 335.0, 337.0 Found: 336.1, 338.2.

**Results and discussion**

**Virtual screening**

The binding sites in the available KPC-2 crystal structures were analyzed to select a suitable receptor for docking. Alignment and superposition of the binding site residues of the seven available crystal structures of *E. coli* and *K. pneumoniae Kp* revealed a rather rigid binding site with only Trp105 adopting two different rotamers, a closed conformation found 6-times and an open one, found 2-times. In one structure, both rotamers were present (Fig 6). Thus, for virtual screening, the structure with the highest resolution was selected (*K. pneumoniae Kp* in complex with the covalent inhibitor penamsulfone PSR-3-226 (PDB code 4F8E).

Fig 4. Inhibitory activity of compounds selected from virtual screening. $^1$A Assays were performed in duplicate (errors were less than 5%) with CENTA as reporter substrate (100 μM, km 70 μM). Kinetic were monitored at 25˚C following the absorbance variation at $\lambda = 405$ nm. $^2$If no IC$_{50}$ has been measured, percent inhibition at the highest tested concentration is given in parentheses. For example, $> 0.50 (26\%)$ implies that the highest concentration tested was 0.50 mM; at this concentration, the enzyme was inhibited by 26%. Therefore, IC$_{50} > 0.50$ mM. When % Inhibition was below 10% No Inhibition (NI) is reported. $^3$Assays were run after 10' incubation of the inhibitor with KPC-2.

Reaction was started by the addition of CENTA.

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| ID  | STRUCTURE | IC₅₀ mM^{(b)} |
|-----|-----------|--------------|
| 11a | ![Structure](image1.png) | 0.036 |
| 1b  | ![Structure](image2.png) | > 0.2 (25) |
| 2b  | ![Structure](image3.png) | > 0.6 (23) |
| 3b  | ![Structure](image4.png) | Not tested not soluble |
| 4b  | ![Structure](image5.png) | > 0.2 (16) |
| 5b  | ![Structure](image6.png) | > 0.2 (13) |
| 6b  | ![Structure](image7.png) | NI at 0.2 |
| 7b  | ![Structure](image8.png) | > 1.0 (30) |
| 8b  | ![Structure](image9.png) | > 1.0 (37) |
| 9b  | ![Structure](image10.png) | NI at 0.5 |
| 10b | ![Structure](image11.png) | NI at 0.5 |
| 11b | ![Structure](image12.png) | > 1.0 (37) |
| 12b | ![Structure](image13.png) | NI at 0.5 |
| 13b | ![Structure](image14.png) | NI at 0.5 |
| 14b | ![Structure](image15.png) | > 500 (20) |
This structure contained both rotamers of Trp105. For virtual screening, the closed conformation was chosen, as this is the most dominant conformation upon ligand binding.

Only little diversity with respect to bound ligands was found in the KpKPC-2 structures. To obtain a more detailed picture on key interactions and to derive a pharmacophore hypothesis, PoSSuM—Search K was used to search for similar binding sites containing non-covalent ligands. This resulted in thirteen structures (Table 1), all having tetrazoles or carboxylates derivatives bound in the hydrophilic pocket formed by the amino acids corresponding to Thr235, Thr237, Ser130 and Ser70 in KpKPC-2 (Fig 6). Seven of the contained ligands were fragment hits for E. coli CTX-M class A extended spectrum β-lactamase (ESBL), and four were derivatives of the most potent screening hit. Further, a structure of S. fonticola SFC-1 S70A β-lactamase in a non-covalent complex with meropenem and one of E. coli Toho-1 R274N: R276N β-lactamase were retrieved. Superposition of the binding site residues of KpKPC-2 (PDB code 3RXW) and the CTX-M β-lactamase structures gave rmsd values for the Cα atoms between 0.72 and 0.82 Å, for superposition of KpKPC-2 and S. fonticola SFC-1 (PDB code 4EUZ) 0.28 Å and for superposition KpKPC-2 and E. coli Toho-1 (PDB code 4BD0) 0.73 Å (Fig 7).

Based on the retrieved structures, a pharmacophore hypothesis was derived. All of the ligands in these structures as well as the β-lactamase binding protein (PDB code 3E2L, 3E2K) and the covalent ligand of the structure used as receptor, formed a hydrogen-bond with Thr235 or Thr237. Accordingly, a hydrogen-bond acceptor at the corresponding ligand position was considered to be crucial for binding (Fig 3). Further, in most of the structures the ligands formed interactions with Trp105 (Ambler numbering) [21]. Therefore, this interaction was also included in the pharmacophore hypothesis. Hydrogen-bond interactions to Asn130 were found in four structures (PDB codes 3RXW, 3G32, 3G30, 4EUZ) and included as well.

A hierarchical approach was adopted for virtual screening. First, our in-house database of around five million purchasable compounds was filtered for lead-like molecules [22]. In the second step, the obtained hits were screened with the above-described pharmacophore resulting in 44658 compounds. Out of these, 31122 compounds could be docked into the Kp KPC-2 binding site. Filtering these binding poses again with the pharmacophore resulted in 2894 compounds. These were divided into three clusters, depending on the functional group placed in the hydrophilic pocket (tetrazoles, carboxylates, sulfonamides) and inspected by eye. Finally, 32 compounds were selected for hit validation (Fig 4).

Most of the selected chemotypes carried an anionic group, mainly a carboxylic group or its bioisostere, the tetrazole ring. Candidates were predicted to orient the anionic side of their moiety in the carboxylic acid binding site of KPC-2, delimited by Ser130, Thr235 and Thr237 and present in all serine-based β-lactamases. In the above mentioned site, in fact, binds the C(3)4' carboxylate of β-lactams antibiotics as well as the sulfate group of avibactam and the carboxylic group of other known BLs inhibitors [12,31,36–38].

**Hit evaluation**

The large majority of the selected candidates were fragment-like as defined by the “rule of three” [39]. Dealing with fragments, potencies in the high micromolar to millimolar range...
were thus expected as well as others important issues such as reaching the solubility limit when testing the compounds and unpredictable precipitation in the employed assays conditions [40,41]. In our hands, the required high concentrations for ligand testing could not always be
achieved due to solubility which might have resulted in false negatives, but their true incidence is difficult to establish [42].

However, some of the tested molecules inhibited the hydrolytic activity of KPC-2 with low millimolar potency and their favorable solubility allowed us to determine full $IC_{50}$ and/or $K_i$ values. Among those, compounds 9a and 11a were the most promising compounds with micromolar affinities ($IC_{50}$ of 0.15 and 0.036 mM, translating to ligand efficiencies (LE) of 0.38 and 0.28 kcal/mol/non-hydrogen atom, respectively; Fig 4) and were thus further investigated.

Compound 9a was predicted to place its carboxylate group in proximity of the catalytic Ser70, in the carboxylic acid binding site mentioned above, forming hydrogen bond interactions with the side chains corresponding to amino acids Ser130, Thr235 and Thr237 (Fig 8). Thr 237 in KPC-2 is known to be necessary for cephalosporinase and carbapenemase activity and is involved in clavulanic acid, sulbactam and tazobactam recognition. [7] This position in β-lactamases that do not have carbapenemase or extended-spectrum β-lactamase (ESBL) activity generally corresponds to an alanine. The side chain hydroxyl groups of Ser130 and Ser70 were predicted to form interactions with the nitrogen of the benzothiazole ring. The predicted position of the aromatic system is well placed to establish ring-ring interactions with Trp105, a residue involved, in turn, in the stabilization of β-lactams through mainly hydrophobic and van der Waals interactions (centroids distances of 4.4 and 4.5 Å between Trp105 and the thiophene and the benzene rings respectively) The role of Trp105 in substrate and inhibitor interactions in KPC-2 β-lactamase has been deeply investigated being essential for hydrolysis of substrates. [7] The methoxy group of the molecule is oriented towards a rather open and solvent accessible area of the binding site and does not contact any of the surrounding residues. Interestingly, the presence of the sulphur atom of the benzothiazole system seems critical for

![Fig 7. Analysis of PoSSuM hits.](https://doi.org/10.1371/journal.pone.0203241.g007)
affinity as the related compound 19a, the benzimidazole analog, resulted 6-fold less active. Similar, the presence of the carboxylic group appeared to be crucial as compound 32a, without such a functionality, was 8-fold less active.

Fig 8. Predicted binding mode of compound 9a (beige) in the KpKPC-2 receptor (blue). Putative hydrogen bond interactions are indicated as black dots (Ambler numbering)[21].

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For compound 9a binding affinity and mode of inhibition was determined by using gradient concentrations of CENTA. Fitting of the obtained data showed that compound 9a behaves as a competitive inhibitor with a determined $K_i$ of 112.0 $\mu$M (Fig 9). Its binding affinity was also determined towards other class A $\beta$-lactamases ($IC_{50}$ vs CTX-M9 160 $\mu$M). For this compound aggregating behavior was also excluded by dynamic light scattering experiment (data not shown) [43]. Compound 9a with its fragment-like characteristic (MW 208.21, determined $K_i$ 112.0 $\mu$M, LE 0.38 kcal/mol/non-hydrogen atom) exerts an interesting activity vs KPC-2 and represents a very promising molecule to be directed to hit to lead optimization.

Among the 32 selected hits evaluated in vitro for their binding affinity vs KPC-2, compound 11a was the most active inhibitor with a micromolar affinity vs KPC-2 (determined $IC_{50}$ 36 $\mu$M, calculated $K_i$ 14.8 $\mu$M, LE 0.28 kcal/mol/non-hydrogen atom).[35] The tetrazole ring of compound 11a, a well-known bioisostere of the carboxylic group, was predicted to lie in the hydrophilic pocket formed by Thr235, Thr237, Ser130 and Ser70, driving the binding of the inhibitor in KPC-2 active site (Fig 10). The phenyl ring attached to the tetrazole was predicted to be sandwiched between the Trp105 side with a distance compatible with weak hydrophobic interactions and the backbone of Thr237. The amide group of 11a was oriented in the canonical site delimited by Asn132, Asn170 and in a further distance Glu166 where the R1 amide side chain of $\beta$-lactams is known to bind. However, the amine linker and the second phenyl ring in 11a were not predicted to form any specific interactions with the protein, except for the amide nitrogen contacting the backbone of Thr237. The distal fluoro-benzene ring was oriented at the entrance of the active site against two hydrophobic patches, one defined by Leu167, closer, and the other by the backbone of Asn170, a residue critical for carbapenemase activity.

**Hit derivatization and evaluation**

Compound 11a appeared to be the most promising compound for optimization. Despite its small size, it has a potency in the low micromolar range (calculated $K_i$ 14.8 $\mu$M) and a suitable ligand efficiency (0.28 kcal/mol/non-hydrogen atom). Therefore, this compound was selected to undergo chemical optimization for potency improvement and to investigate target binding requirements for optimal inhibitor-enzyme interaction. The phenyl-tetrazole moiety, that was predicted to strongly drive the binding, was retained unaltered, whereas structural modifications on the linker and on the distal aromatic ring were introduced in order to explore and maximize the interactions with the pocket formed by Asn132, Asn170 and Leu167 (Fig 10).
Because the amide linker does not contact efficaciously the protein we chose to replace it with a sulfonamide (Fig 11). We meant to target residues proximal to the opening of the active site while investigating the potentiality for sulfonamide derivatives.
Sulfonamides are more stable towards hydrolysis than carboxyamides, possess an additional hydrogen bonding oxygen atom and their NH is a strong hydrogen bond donor. In addition, the dihedral angle \( \omega \) OSNH measures around 90˚ compared with the 180˚ \( \omega \) OCNH angle of amide. Sulfonamides, in addition, have a non-planar configuration that could orient the distal ring towards Leu167 and Asn170 (Fig 12). Therefore, the introduction of a sp\(^3\) geometry could allow a more efficacious spanning of the active site compared to the planar amide [44]. Moreover, modeling suggested that the sulfonamide group could form an additional hydrogen bond with Asn132 residue actively involved in substrate recognition and hydrolysis.

Further, we explored different substitutions on the sulfonamide linker to probe binding interactions. Substituents with different electronic and steric properties (i.e. halogens, nitro, sulfonamide, carboxylic acid, methyl, acetamide, amino groups) were inserted in the different position of the aromatic ring. In addition, the benzene ring was replaced by heterocyclic or extended benzfused systems such as benzimidazole, quinazolinone, naphthalene, or quinolone ring. Based on the availability of compound or building blocks, 6 compounds (1b-6b) were synthesized and 8 compounds (7b-14b) were purchased to test our hypothesis (Fig 5). The fourteen new compounds were tested in vivo vs clinical strains overproducing KPC-2 to evaluate their ability to restore bacteria susceptibility to carbapenem meropenem (Table 2).

Compounds 1b-6b were synthesized in high yield (75–95% yield) and purity (>95%) through direct reaction of 3-(1H-tetrazol-5-yl) aniline and the appropriate sulfonyl chloride in dichloromethane at room temperature for 3 hours (Fig 13).

The derivatives of compound 11a were tested for KPC-2 affinity (Fig 5). However, their solubility limit in the assay condition was often a limitation in accurate affinity determination. Overall, the compounds exhibited either weaker activities than 11a or were not active at all at tested concentration. This data lets us confidently affirm that the sulfonamide linker is not a suitable group to optimize the affinity of this compound series.

The antimicrobial activity of the best hits 9a and 11a and their derivatives was studied in bacterial cell cultures to investigate their ability to cross the outer membrane reaching the periplasmic space, where KPC-2 is secreted and confined in Gram negative bacteria. Compounds were tested for synergy with the \( \beta \)-lactam antibiotic meropenem against four \( K.\ pneumoniae \) clinical strains, isolated from different patients at the Hospital Universitario Son Espases, Palma de Mallorca, Spain. One of the four clinical strains was not a KPC-2 producer and was susceptible to meropenem (strain Kpn (C-); MIC <0.25 ug/mL). The three additional strains harbored the blaKPC-2 gene and were resistant to meropenem (Table 2). [32] Noteworthy none of the tested compounds had intrinsic antibiotic activity (MIC >256 ug/mL), against the employed strains, included the susceptible one. The results show that in most cases the compounds were not able to reverse antibiotic resistance and did not showed synergism with meropenem. However, against strain Kpn 53A8 the MIC value was lowered by a factor of two when meropenem was combined with compounds 32a, 1b, 2b, 5b and 6b while in combination with compound 11a the MIC value was reduced by 4 fold.

Fig 11. Design strategy for amide derivatives. Virtual Screening hit 11a (left), amine 1 (black) and the optimized part of the molecule (red).

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Conclusions

In this study two novel hits for KPC-2 were identified via an in-silico approach (compound 9 and 11a, Fig 4). Both of them had promising LEs with 0.38 and 0.28 kcal/mol/non-hydrogen atom, resp. 14 tetrazole derivatives originating from 11a were designed, synthesized and evaluated for their ability to inhibit KPC-2. We introduced chemical diversity on the distal part of the inhibitor, choosing to keep unchanged the anchor tetrazole ring while modifying the amide and the distal ring. The results suggest that a sulfonamide linker is neither suitable to improve the potency of 11a nor the solubility of final compounds. Future optimization work should instead rather concentrate on exploring secondary binding sites more distal from the pocket that the screening hits are supposed to address [45]. If the amide functionally found in

Fig 12. Predicted binding mode of a sulfonamide derivative of compound 11a (beige) in the KpKPC-2 receptor (blue). Putative hydrogen bond interactions are indicated as black dots (Ambler numbering)[21].

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11a or alternative linkers are best suited remains to be explored. Nevertheless, two promising hit compounds for KPC-2 were retrieved which can serve as starting points to derive more potent inhibitors.

Although a decrease of potency in *in vitro* tests was registered for the designed and synthesized chemical entities, as none of the compounds was able to trigger stronger interactions with the open region of KPC-2 they were meant to target, this study yielded a better comprehension of the catalytic pocket of this enzyme. Our study provided a better understanding of how challenging the target of additional, superficial, binding pockets is and how it could be critical in designing inhibitors with improved potency, especially in area proximal to the active site opening.

The (1H-tetrazol-5-yl)phenyl ring was most frequent among the high scoring candidates in our *in silico* study, suggesting that this functionality is well suited to anchor ligands in the in KPC-2 binding site. The rather weak affinity of the ligands hints that rest of the ligand, i.e. the

| Table 2. *In vitro* interaction between meropenem and synthesized compounds vs *K. pneumoniae* clinical strains. |
|---------------------------------------------------------------|
| **MIC**<sup>[a]</sup> meropenem in combination with synthesized compounds (1:1 molar) <sup>[a, b, c]</sup> (μg/mL) |
| Code | Kpn (C-) | Kpn 99D8 | Kpn 53A8 | Kpn 53A9 |
|------|----------|----------|----------|----------|
| —    | <0.25    | 256      | 256      | 256      |
| 9a   | <0.25    | 256      | 256      | 256      |
| 11a  | <0.25    | 256      | 64       | 256      |
| 32a  | <0.25    | 256      | 128      | 256      |
| 1b   | <0.25    | 256      | 128      | 256      |
| 2b   | <0.25    | 256      | 128      | 256      |
| 4b   | <0.25    | 256      | 256      | 256      |
| 5b   | <0.25    | 256      | 128      | 256      |
| 6b   | <0.25    | 256      | 128      | 256      |

<sup>[a]</sup> Assays were conducted against four *K. pneumoniae* clinical strains isolated from different patients at the Hospital Universitario Son Espases, Palma de Mallorca, Spain. MICs were determined according to EUCAST standards and the presented values are the median of three independent experiments.

<sup>[b]</sup> Compounds were tested alone and showed no activity (MIC > 256) in all cases.

<sup>[c]</sup> Strain C- is control *K. pneumoniae* and meropenem susceptible (MIC < 0.25 μg/ml)

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**1**1a or alternative linkers are best suited remains to be explored. Nevertheless, two promising hit compounds for KPC-2 were retrieved which can serve as starting points to derive more potent inhibitors.

Although a decrease of potency in *in vitro* tests was registered for the designed and synthesized chemical entities, as none of the compounds was able to trigger stronger interactions with the open region of KPC-2 they were meant to target, this study yielded a better comprehension of the catalytic pocket of this enzyme. Our study provided a better understanding of how challenging the target of additional, superficial, binding pockets is and how it could be critical in designing inhibitors with improved potency, especially in area proximal to the active site opening.

The (1H-tetrazol-5-yl)phenyl ring was most frequent among the high scoring candidates in our *in silico* study, suggesting that this functionality is well suited to anchor ligands in the in KPC-2 binding site. The rather weak affinity of the ligands hints that rest of the ligand, i.e. the

![Reagents and conditions](https://doi.org/10.1371/journal.pone.0203241.g013)

Fig 13. Reagents and conditions. a) aryl-sulfonyl chloride (1.2 eq.), pyridine (3 eq.), dry DCM, N₂, r.t, 3 h, 75–95% yield.

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functional groups out of the center phenyl ring and the amide liker, need to be optimized to increase potency. However, introducing a sulfonamide linker was detrimental for potency. We hypothesize that the presence of a sulfonamide instead of an amide led to a rearrangement of the ligand in the binding site to minimize steric hindrance, and thus resulted in the loss of key interactions. These rearrangements can be particularly critical in non-covalent inhibitors like ours that are not stabilized by a covalent interaction with the catalytic serine, as this type of inhibitors are supposed to have lower residence times with respect to covalent β-lactamase inhibitors (Fig 1). In designing larger and more potent inhibitors, additional secondary binding sites which have been found to be critical for affinity improvement need to be considered [45].

The difficulties in optimizing the affinity of hit 11a for its target are also caused by the lack of an accurate 3D structure of the complex. While reliable methods using docking can be obtained, experimental confirmation of the binding modes is desirable to make sure that no wrong tracks are followed [46]. In the case of compound 11a, we are more certain about the position of the tetrazole moiety in the binding site due to the availability of crystal structures of related targets with similar ligands (Table 1) while the placement of the substituent we attempted to optimize is more tentative. Further structural work and medicinal chemistry work is ongoing to significantly increase the potency of the most promising compound 11a in vitro and in vivo and new chemistry is under evaluation for these derivatives, taking advantage of other additional recognition sites in KPC-2.

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Author Contributions

Conceptualization: Raphael Klein, Ruth Brenk, Donatella Tondi.

Formal analysis: Pasquale Linciano.

Funding acquisition: Donatella Tondi.

Investigation: Raphael Klein, Pasquale Linciano, Pierangelo Bellio, Sofia Papaioannou, Jesus Blazquez, Donatella Tondi.

Methodology: Pasquale Linciano, Pierangelo Bellio.

Project administration: Donatella Tondi.

Resources: Giuseppe Celenza, Laura Cendron.

Supervision: Ruth Brenk, Donatella Tondi.

Visualization: Raphael Klein, Pasquale Linciano.

Writing – original draft: Raphael Klein, Pasquale Linciano, Ruth Brenk, Donatella Tondi.
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