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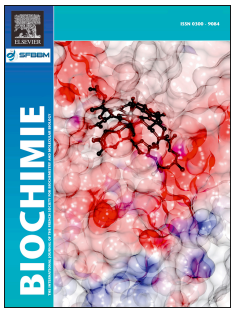

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Novel non-β-lactam inhibitor of β-lactamase TEM-171 based on acylated phenoxyaniline

Grigorenko V.G.[1*], Andreeva I.P.[1], Rubtsova M.Yu.[1], Deygen I.M.[1], Antipin R.L.[1], Majouga A.G.[1], Egorov A.M.[1], Beshnova D.A.[2], Kallio J.[2], Hackenberg C.[2], Lamzin V.S.[2]

[1]Chemistry Department, M.V. Lomonosov Moscow State University, 119991 Moscow, Russia; tel. +74959392727; fax: +74959392742; e-mail: vitaly.grigorenko@gmail.com

[2]European Molecular Biology Laboratory (EMBL), c/o DESY, Notkestrasse 85, Building 25A, 22607 Hamburg, Germany; tel.: +494089902121; fax: +494089902149

[*]Corresponding author, e-mail: vitaly.grigorenko@gmail.com

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Abstract

The microbial resistance to antibiotics is a genuine global threat. Consequently, a search of new inhibitors remains of acute importance due to the increasing spread of multidrug resistance. Here we present a new type of non-β-lactam β-lactamase inhibitor PA-34 based on natural phenoxyaniline, identified using computer-assisted screening of scaffolds related to those of known low-affinity inhibitors. The compound displays reversible competitive inhibition of bacterial β-lactamase TEM-171, with a K_i of 88 μM. Using enzyme kinetics, infra-red spectroscopy, fluorescence quenching and computer docking, we propose that the inhibitor binds at the entrance to the enzyme active site. This is a novel inhibition mechanism compared to binding covalently to the catalytic serine in the active site or non-covalently to the allosteric site. The residues involved in binding the inhibitor are conserved among molecular class A β-lactamases. The identified compound and its proposed binding mode may have a potential for a regulation of the catalytic activity of a wide range of class A β-lactamases. We also hypothesise that the presented route for finding non-β-lactam compounds may be an effective and durable approach for combating bacterial antibiotic resistance.

1. Introduction

Since the discovery of penicillin by Alexander Fleming and its introduction into general medical practice in the 1940s, β-lactam compounds have become one of the three largest classes of antibiotics [1]. They comprise more than half of the antibiotics used worldwide for the treatment of a variety of infectious diseases [2]. These antibiotics represent a broad class

of polar hydrophilic compounds containing a β-lactam ring. They act by inhibiting the synthesis of the peptidoglycan layer of the bacterial cell wall, thereby impairing the structural integrity of the cell [3]. Subsequent drug discovery and development has produced more β-lactam antibiotics, which are, at present, divided into four groups based on their chemical structures: penicillins, cephalosporins, carbapenems and monobactams (Table S1). The driving force behind the search for new antibiotics is the continual ability of bacteria to adapt to earlier and existing generations of antibiotics through a development of resistance mechanisms. This antibiotic resistance, creating a major, widespread problem, has been realised for more than 40 years [4] and has become a serious and growing threat to public health [2, 5-6].

The most prevalent resistance mechanism in Gram-negative bacteria is the production of β-lactamase enzymes, which inactivate the antibiotic by hydrolysing the β-lactam ring [7]. The serine β-lactamases of the molecular classes A, C, and D hydrolyse the β-lactam ring by forming a covalent acyl-intermediate to the catalytic serine in the enzyme active site. The metallo-β-lactamases of molecular class B additionally require one or two zinc ions, and their catalysis proceeds via a transition state involving a zinc-stabilised hydroxide ion [8]. Most attention has recently been attracted by the problem of plasmid-encoded extended spectrum β-lactamases (ESBLs) [9-10]. The term ESBL was initially used to designate the mutant forms of TEM- and SHV-type β-lactamases that are able to hydrolyse oxyimino-cephalosporins, but now it also concerns CTX-M, OXA, AmpC, VEB and other types of β-lactamases [11, 12]. The number of described unique β-lactamases obtained from clinical isolates is estimated to be at least 1,300 [11]. This poses a significant threat to the efficacy of β-lactam antibiotics.

The evolutionary ability of bacteria to adapt to their environment is the driving force for the increasing rates of resistance [13]. As the resistance of bacteria to antibiotics develops indeed rapidly, drug development in addition to a search for new antibiotics is hunting for ways to inhibit β-lactamases. Such inhibitors proved efficient; they target the active site of the enzyme and are co-administered with β-lactam antibiotics – the examples include clavulanic acid with amoxicillin or sulbactam with ampicillin (Fig. S1). However, since the currently used β-lactamase inhibitors contain the β-lactam ring, they are as susceptible to time-limited application as the antibiotics themselves. In addition, the active site of β-lactamases is susceptible to mutations that circumvent the binding of β-lactamase inhibitors; inhibitor-resistant β-lactamases (IRTs) have developed over time, even those that confer resistance to all the marketed β-lactamase inhibitors [14] while preserving the ability to cleave the antibiotics. Dissemination of multiple drug resistance further complicates the task of

inactivating β-lactamases and emphasises the importance of developing novel β-lactamase inhibitors with extended lifetimes [15].

Promising approaches involve the development of non-β-lactam inhibitors, which can be active against a wide range of different β-lactamases. Several inhibitors have already been described [15-17]. These include diazabicyclooctanes (avibactam and MK-7655) and derivatives of boronic acid that are active against class A, C and D β-lactamases. Another type of inhibitor, FTA, 3-(4-Phenylamino-phenylamino)-2-(1H-tetrazol-5-yl)-acrylonitrile, was described and shown to bind to the allosteric site, distinct from the active site of the enzyme [18-19]. Targeting a less frequently mutating area of the enzyme with a widely applicable inhibitor seems, therefore, an intriguing approach to drug design in the field of antibiotic resistance.

The aim of this study was to design a novel non-β-lactam inhibitor, preferably targeting a different binding site of a β-lactamase. Here, we report the synthesis and characterisation of an acylated phenoxyaniline compound, hereafter called PA-34, as a novel type of a non-β-lactam inhibitor. We used recombinant β-lactamase TEM-171 as a model enzyme of class A β-lactamases, which has comparable catalytic parameters to the wild-type β-lactamase TEM-1 [20]. Enzyme-inhibitor interactions were modelled in a molecular docking study and experimentally probed by IR-spectroscopy and fluorescence quenching studies.

2. Materials and Methods

2.1 Reagents

The reagents were purchased from Sigma-Aldrich (http://www.sigmaaldrich.com) and Difco (http://www.bd.com) and used without further purification. Enzymes for DNA amplification, restriction and modification were purchased from Stratagene (http://www.agilent.com), New England Biolabs (https://www.neb.com/) and Thermo Fisher Scientific (https://www.thermofisher.com). Oligonucleotides for sequencing and PCR were purchased from Syntol (http://www.syntol.ru). The plasmid vectors pET-20 and pET-24a(+) and *E.coli* strains DH5α and BL21(DE3) were purchased from Novagen (http://www.merckmillipore.com). The preparative work with DNA was performed using a Qiagen QIAprep Spin Miniprep Kit and a Qiagen QIAquick Gel Extraction Kit (http://www.qiagen.com). Protein electrophoresis (SDS-PAGE) was performed according to Laemmli [21], using a Thermo Fisher Scientific low molecular weight protein kit (SM0431)

as the molecular weight standards. Protein concentration was measured with Sigma-Aldrich BCA test kit.

2.2 DNA manipulation and generation of β-lactamase expression strain

DNA techniques, such as plasmid isolation, transformation of *E.coli*, ligations and restriction analysis were standard methods. *E.coli* strain DH5α was used for cloning and BL21(DE3) for protein expression. The cells were cultivated in lysogeny broth (LB) medium (0.5% yeast extract, 1% peptone, 0.5% sodium chloride and 1.5% bacto agar for solid medium) supplemented with 50 mg/l of kanamycin.

To generate the recombinant β-lactamase TEM-171 overexpression *E. coli* strain, the coding sequence of TEM-171, including a periplasmic signal sequence (www.lahey.org/studies), was amplified with *Pfu* DNA polymerase using β-lactamase TEM-171 encoding plasmid pET-20 as a template, 5'-TTATATTAA*CATATG*CACCCAGAAACGCTGGTGAAAG-3' with *Nde*I site as the forward primer and 5'-TTT*GCGGCCGC*TCACCAATGCTTAATCAGTGAGGC-3' with *Not*I site as the reverse primer (restriction sites are underlined). The PCR product was transferred into the expression vector pET-24a using *Nde*I/*Not*I. The resulting construct pET-bla was verified by DNA sequencing and finally transformed into *E. coli* BL21 (DE3).

2.3 Expression and purification of the recombinant class A β-lactamase TEM-171

Cells were grown under vigorous shaking at 37°C in 300 mL LB medium containing 50 µg/mL kanamycin. When the optical density of the culture medium reached 0.6 (OD_{600}) the expression was induced by the addition of 0.1 mM isopropyl *β-D*-1-thiogalactopyranoside (IPTG). The cells were further grown at 21°C for 50 hrs, harvested by centrifugation (3,600g, 15 min at 4°C) and stored at -20˚C before purification. The periplasmic protein fraction was isolated using osmotic shock, by re-suspending the cell pellet in 10 mM Tris/HCl, pH 8.0, containing 1 mM EDTA and 20% (w/v) sucrose for 15 min at room temperature. Spheroplasts were removed by centrifugation (10,000g, 10 min at 4°C) and the supernatant containing the periplasmic fraction was dialysed against 10 mM Tris/HCl, pH 8.0. The crude extract was applied to a SOURCE^{TM} 15Q (10 cm × 0.75 cm^2) anion exchange column (http://www.gehealthcare.com) and washed extensively with 10 mMTris/HCl buffer, pH 8.0. The recombinant enzyme was eluted with a linear salt gradient in the same buffer, containing about 80 mM NaCl. Fractions containing β-lactamase TEM-171 were pooled and dialysed

against 10 mM Tris/HCl, pH 8.0 for 14-16 hrs at 4˚C. The protein was further purified by size exclusion chromatography using a HiLoad 16/600 Superdex75 pg column (http://www.gehealthcare.com) equilibrated with 10 mM Tris/HCl, pH 8.0. The resulting enzyme-containing fractions were pooled and kept at 4°C for further experiments. The purity of the enzyme was evaluated using SDS-PAGE stained by Coomassie Brilliant Blue. Protein concentrations were determined using Sigma-Aldrich BCA test kit with bovine serum albumin as a standard.

2.4 *In silico* search for new inhibitors

We used the novel ViCi software (www.embl-hamburg.de/vici) for an *in silico* ligand-based drug design. In brief, as search templates we selected two known low-affinity, non-β-lactam inhibitors binding to β-lactamase TEM-1: FTA, 3-(4-phenylamino-phenylamino)-2-(1H-tetrazol-5-yl)-acrylonitrile [18] and CBT, N,N-bis(4-chlorobenzyl)-1H-1,2,3,4-tetraazol-5-amine [18], and two non-β-lactam inhibitors binding to β-lactamase CTX-M-9: 1CE, 3-(1H-tetrazol-5-ylmethyl)-5,6,7,8-tetrahydro[1]benzothieno[2,3-d]pyrimidin-4(3H)-one and F13, 3-fluoro-N-[3-(1H-tetrazol-5-yl)phenyl]benzamide [19]. These inhibitors were used as starting points for screening the ViCi database for compounds of similar shape and charge distribution ([22], Beshnova et al., manuscript in preparation). The top 550 hits from the *in silico* screen were purchased and tested using a high throughput single point assay based on a microtiter plate format. The compounds were solubilised in DMSO and the assay was performed in 20 µl of reaction buffer with the addition of 0.1% BSA or 0.05% of the non-ionic detergent pluronic F-127, and with concentrations of the enzyme, screened compound and the substrate of 6.25 nM, 100 µM and 100 µM, respectively. The potency of the inhibitors was assessed from the endpoint absorbance value at λ=405 nm at a given time point (a 30 minute linear reaction). The obtained data were normalised to the negative control (the same mixture without the inhibitor). From this screen several compounds were identified showing promising inhibitory effect on the β-lactamase TEM-171 activity. From these, the PA-34 compound was chosen for the synthesis and further investigation.

2.5 Synthesis of PA-34 inhibitor

0.093 g of succinic acid anhydride was added to the solution of 0.216 g of 2,4-dichlorophenoxyaniline in 50 ml toluene. The mixture was refluxed for 12 hrs at 110°C and subsequently cooled to room temperature. The resulting precipitate was filtered out, dissolved

in dichloromethane and washed with a saturated solution of ammonium chloride. The organic phase was dried with anhydrous sodium sulphate. After the evaporation of the solvent, we obtained 0.201 g of phenoxyaniline as a white solid with a yield of 67%. The reaction was monitored by thin layer chromatography (TLC) using Merck TLC silica gel 60 F_{254} aluminium-backed precoated plates. The plates were visualised by ultraviolet light at 254 nm. PA-34 was characterised by ^{1}H and ^{13}C NMR, elemental analysis and mass spectroscopy. NMR spectra were recorded using a Bruker 400 MHz spectrometer. The ^{13}C NMR spectra were measured with complete proton decoupling. According to the NMR data, the obtained PA-34 inhibitor was at least 95% pure (Fig. S2). Melting points were measured using a Reichert-Jung Thermovar hot-stage microscope apparatus.

2.6 Determination of kinetic parameters and the inhibition constant

The activity of β-lactamase TEM-171 against CENTA, a chromogenic substrate based on cephalothin, was measured in 50 mM sodium phosphate buffer, pH 7.0 at 25°C using a Shimadzu UV-1602 spectrophotometer. CENTA was prepared as stock solution of 5 mM in 50 mM sodium phosphate buffer, pH 7.0. The total volume of the enzyme assay was 1 ml. The hydrolysis of CENTA was monitored by continuous recording of the absorbance at 405 nm ($\Delta\varepsilon 405 = 6400 M^{-1}cm^{-1}$) [23]. The concentration of β-lactamase TEM-171 in the assay was 0.012 μM. The reaction was initiated by adding CENTA at concentrations of 10, 20, 50, 100 and 200 μM to the solution containing the enzyme. The measurements were made in triplicate. Concentrated inhibitor solution (4 mM) was prepared by dissolving PA-34 in DMSO. The assay was performed in 50 mM sodium phosphate buffer, pH 7.0 at 25°C, with the final concentration of DMSO of 2.5%. The total volume of the inhibition assay was 1 ml. The enzyme was added to the inhibitor solution. The reaction was initiated as described above.

Apparent Michaelis constants (K_{Mapp}) and V_{max} were determined using a weighted Lineweaver–Burk linearisation. The weights were taken as $V_0^4/\sigma^2(V_0)$. The inhibition constant K_I was determined using the weighted linearised dependence of the derived K_{Mapp} values on the inhibitor concentration I_0 according to eq. 1. The weights for K_{Mapp} were set to the inverse of the squares of their estimated standard deviations.

$$K_{Mapp} = K_M\left(1+\frac{[I_0]}{K_I}\right) \qquad (1)$$

2.7 ATR-FTIR spectroscopy for binding studies of PA-34

Attenuated total reflection Fourier transform infra-red (ATR-FTIR) spectra were recorded using a Bruker Tensor 27 spectrometer equipped with a liquid nitrogen cooled mercury cadmium telluride detector. An aliquot of 40 μl of the sample was placed in a Bruker thermostated (22°C) cell BioATR-II with ZnSe ATR-element. The FTIR spectrometer was purged with a constant flow of dry air. In order to prevent minor contamination of spectra we washed ATR-crystal in the following sequence: water, sodium dodecyl sulphate, water, isopropanol, water again and dried with a dry airflow.

As a control of the chamber clearness, a water spectrum was registered and compared to the etalon. ATR-FTIR spectra were acquired from 900 to 4000 cm^{-1} with 1 cm^{-1} spectral resolution. For each spectrum, 100 scans were accumulated at the 20 kHz scanning speed and averaged. Concentrations of β-lactamase TEM-171 and the PA-34 inhibitor were 35 μM and 100 μM, respectively. All measurements were performed against their respective background: for TEM-171 - 50 mM phosphate buffer at pH 7.0; for the PA-34 inhibitor - the same buffer with the addition of 2.5% DMSO. For the enzyme-inhibitor complex, two backgrounds were measured - 50 mM phosphate buffer at pH 7.0, 2.5% DMSO with 1 mg/ml (35 μM) β-lactamase TEM-171 to study the inhibitor state, and the same buffer with 2.5% DMSO and 100 μM PA-34 to study the protein state. Spectral data were processed using the Bruker software system Opus 7.5 which includes linear blank subtraction, straight-line baseline correction and atmospere compensation. Where necessary, seven- or nine-point Savitsky-Golay smoothing was used to remove white noise [24]. Peaks were identified by a standard Bruker peak-picking procedure.

2.8 Fluorescence quenching for binding of PA-34

The measurements were performed in a triangle quartz cuvette (1 ml) with a 10 nm slit width using a Perkin-Elmer LS 50B Fluorescence Spectrometer in 10 mMTris/HCl, pH 7.5, containing 80 mM NaCl at 25˚C. The concentrations of TEM-171 and PA-34 inhibitor were 35 μM and 100 μM, respectively. The spectra were recorded in the 300-400 nm range at the excitation wavelengths of 280 nm and 295 nm. Fluorescence SpectraViewer was used for data visualisation and analysis (https://www.thermofisher.com/ru/ru/home/life-science/cell-analysis/labeling-chemistry/fluorescence-spectraviewer.html).

2.9 Molecular docking of PA-34

The crystal structure of the wild-type β-lactamase TEM-1 determined at 1.9 Å resolution (PDB ID 1xpb [25]) was used for molecular docking studies. The 3D coordinates

of PA-34 were taken from the ChemBridge online chemical store (ID 7030665), and were subject to further energy-minimisation using OpenBabel [26]. The molecular docking was done using AutoDockVina [27]. PDBQT files for ligand and protein were generated using AutoDock tools [28].

3. Results and discussion

3.1 Production of recombinant β-lactamaseTEM-171

To probe the biological activity of inhibitors identified with the ViCi software we used β-lactamase TEM-171 as a model enzyme. It differs from wild-type β-lactamase TEM-1 by one amino-acid substitution, Val84Ile. This substitution has no apparent effect on the catalytic properties, which are similar for both the TEM-1 and TEM-171 enzymes, as shown earlier [20]. We therefore hypothesise that the conformations of the active site and the reaction proton transfer pathway are very similar.

TEM-type β-lactamases contain one disulphide bridge connecting Cys77 and Cys123 residues. To promote the correct formation of this disulphide bond, periplasmic protein expression was chosen for the production of recombinant β-lactamase TEM-171 in *E.coli*, using the pET-bla expression vector that encodes the full-length protein with an N-terminal signal sequence [20]. The total expression level was about 50% of the total protein content. The double bands on the electrophoresis gels indicated the presence of unprocessed (31.5 kDa) and processed β-lactamase TEM-171 (28.9 kDa) in the total bacterial homogenate (Fig. 1A).

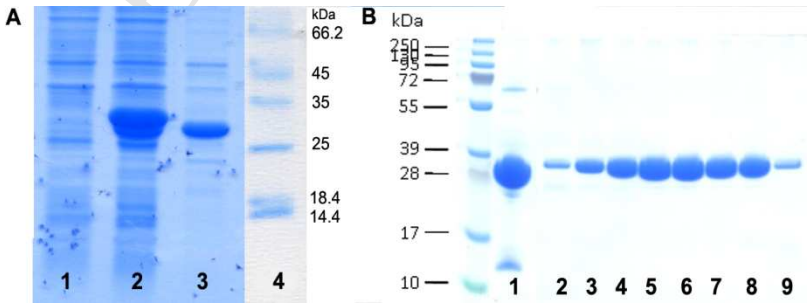


Fig. 1. SDS-PAGE of recombinant β-lactamase TEM-171. Panel A: lanes 1 and 2 –*E.coli* cell lysate before and after the induction with IPTG, respectively; lane 3 – periplasmic fraction; lane 4 – molecular weight standards. Panel B: size-exclusion chromatography: lane 1 – pooled

anion-exchange lactamase fractions; lanes 2–9 - β-lactamase containing fractions after size-exclusion chromatography.

Periplasmic fraction, isolated using osmotic shock, contains only the processed form of β-lactamase TEM-171 (Fig. 1A), indicating correct processing of the signal peptide upon translocation into the periplasm. After subsequent anion-exchange chromatography according to [20], the protein was further purified using size-exclusion chromatography resulting in the estimated purity of the protein of more than 99%. In addition, an increase of the induction-cultivation time to 50 hrs at 21^0C allowed for increasing the yield significantly, to approximately 100 mg of pure protein from 500 ml of the *E. coli* culture (Fig. 1B).

3.2 **Lead discovery and enzyme inhibition with PA-34**

In this study we aimed at identification of a new non-β-lactam inhibitor, which targets either the active or any other binding site of β-lactamases. For this we used a number of non-β-lactam inhibitors as templates. Several newly indentified compounds showed stronger inhibition of the β-lactamase TEM-171 activity compared to the search templates. The selected compound PA-34 is structurally similar to the template inhibitor FTA (Fig. 2 and Table 1) and belongs to the acylated phenoxyaniline group, which is for the first time described in terms of its anti-β-lactamase activity.

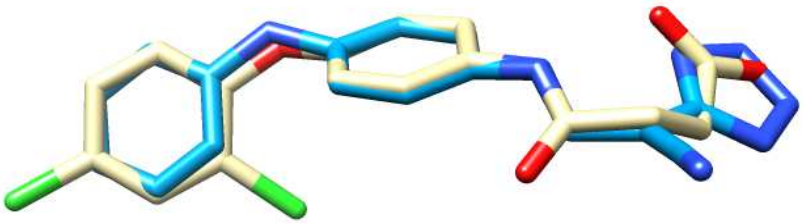


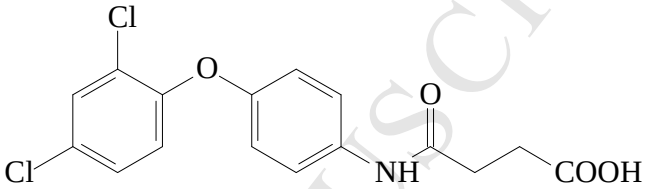
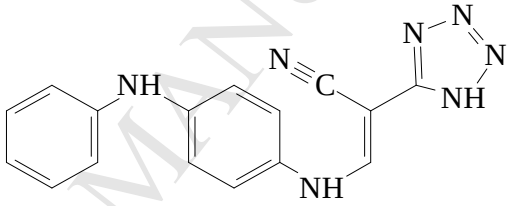
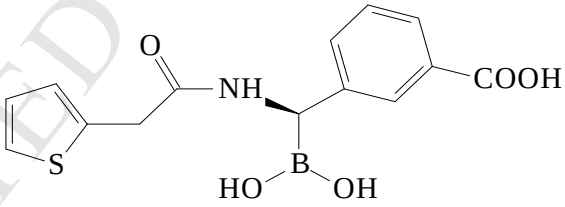
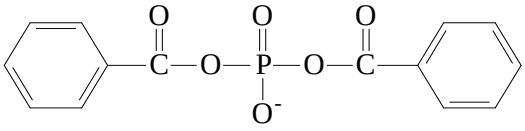
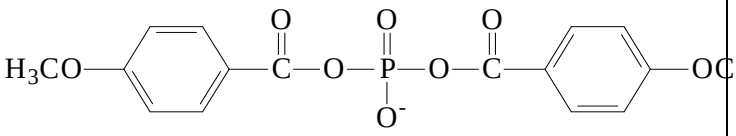
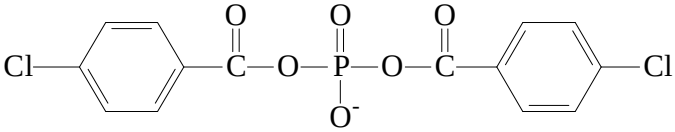
Fig 2. Conformational alignment of the closest search ligand FTA and the novel non-β-lactam inhibitor PA-34.

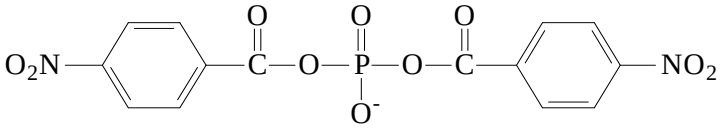
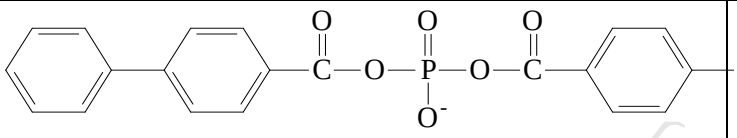
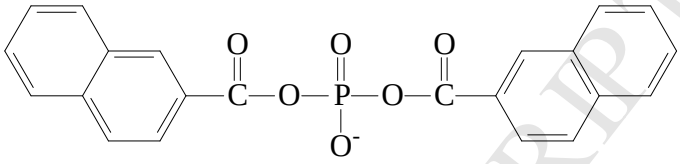
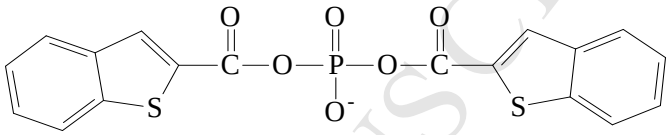
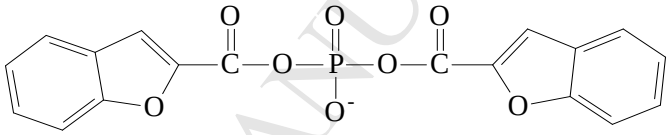
Both the PA-34 and the FTA compounds contain a biaryl fragment. However, in PA-34 this fragment contains chlorine atoms in ortho- and para- positions while FTA contains an

unsubstituted benzene ring. In addition, PA-34 has a carboxyl group at the end of the carbon chain that may be important for binding to the enzyme and its about five times stronger inhibition compared to FTA.

Table 1 also presents some other non-β-lactam structures which target the active site and exhibit inhibition activity against class A β-lactamases including boronic acid, phosphonate derivatives and a cyclic peptide.

Table 1. Structures and inhibition constants of non-β-lactam inhibitors.

Compound	Structure	K_i, μM
PA-34, 4-oxo-4-((4-(2,4-dichlorophenoxy)phenyl)amino)butanoic acid		90±12
FTA, 3-(4-phenylamino-phenylamino)-2-(1H-tetrazol-5-yl)-acrylonitrile) [18]		490±40
Boronic acid *meta*-carboxyphenyl cephalosporin analog [16, 29]		3.9±0.51
Phosphonates [30]		76±6
		0.32±0.04
		2.00±0.18

		190±20
		0.08±0.01
		0.31±0.04
		1.47±0.06
		41±9
cyclic peptide, Pep90 [31]	CysTyrHisPheLeuTrpGlyProCys	333±13

The kinetic measurements of the inhibition with PA-34 indicate that upon a variation of the concentration of the inhibitor, the values of apparent K_{Mapp} change, while the V_m values stay the same (Fig. 3). This is in accordance with the common mechanistic interpretation of a competitive inhibition. From the linear fit according to eq. 1 the derived values of K_M and K_i are 31.5±1.8 and 88±10 μM, respectively (Fig. S3). Some increase in the derived K_M value compared to the one published earlier [20] may be related to the presence of 2.5% DMSO in the buffer. This supports the hypothesis that PA-34 binds to the TEM-171 enzyme in the ratio 1:1 in a competitive and reversible manner.

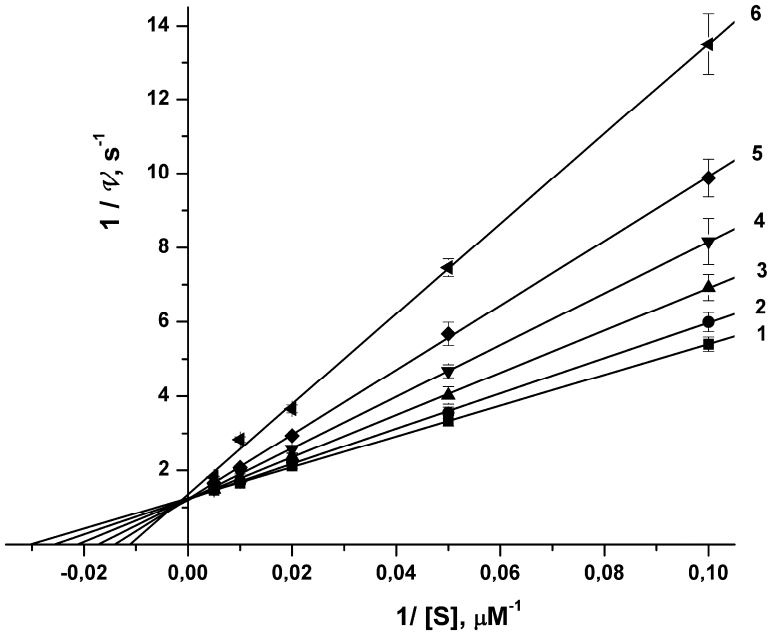


Fig. 3. Lineweaver–Burk representation of the dependence of the initial rate of CENTA hydrolysis by the recombinant β-lactamase TEM-171. The inhibitor PA-34 is present in the following concentrations: 1- 0 μM; 2- 20 μM; 3- 50 μM; 4-75 μM; 5-100 μM; 6- 150 μM. The concentrations of the enzyme and the substrate are as described in section 2.6.

3.3 ATR-FTIR spectroscopy analysis

To characterise the formation of the protein-inhibitor complex we performed ATR-FTIR spectroscopy measurements. According to the equality of location, intensity and structure of bands amide I (1640 cm^{-1}) and amide II (1550 cm^{-1}) in the spectra of protein before and after inhibitor binding (data not shown), we suggest that the binding of the inhibitor does not significantly change the protein state - covalent and non-covalent bonds, degree of hydration, microenvironment and the overall conformation.

According to the literature [32-34], the main absorption bands in the spectra (Fig. 4) can be related to functional groups. The most intensive bands correspond to the oscillations of the aromatic skeleton: C=C (1600 – 1500 cm^{-1}) and C-C (1500-1450 cm^{-1}) which are sensitive to stacking interactions with aromatic ligands [35]. Bands of the carbonyl group (1600 – 1700 cm^{-1}), the carboxyl group (1600 – 1500 cm^{-1}, 1290 – 1250 cm^{-1}), C-N aromatic (1390 - 1350 cm^{-1}) and C-O-C (1098 cm^{-1}) are sensitive to the formation of hydrogen bonds.

The binding of PA-34 leads to significant changes in the spectrum, and a considerable decrease in the intensity is observed (Fig. 4). We exclude a number of possible factors, which could have affected the intensity of the spectra as follows. Specifically, the measurements

chamber was tidied and, as a control of the chamber clearness, we recorded the water spectrum, which was identical to the etalon. We can also exclude possible variations of protein and inhibitor concentrations, as all working solutions were prepared from the appropriate stock solutions in a same manner and the experiments were repeated in triplicate. Finally, we can exclude possible changes of the extinction coefficient due to the fact that each spectrum was registered with 100 scans. Therefore, we propose that the decrease of the intensity is caused by the changes of the functional groups' microenvironment upon the formation of the protein-inhibitor complex. The most dramatic changes were observed for carboxyl group bands (1256 and 1237 cm^{-1} – 80% and 76% consequently), C-O-C band (1098 cm^{-1}, 67%) and C-C aromatic bands (1505 and 1474 cm^{-1}, 60 and 67% respectively) (Table 2).

We propose that the observed changes in the spectra are likely caused by possible stacking interactions of an aromatic ring of the inhibitor with an aromatic protein residue. Tyr105 located at the entrance to the enzyme active site could be regarded as a candidate for such interaction, and this is discussed further in the text. Examples of stacking protein-ligand interaction have been published for other complexes both with biomolecules [35] and small organic molecules [36]. Stacking interactions affect the microenvironment and may cause changes in IR spectra, including a decrease of intensities of some peaks. According to Malevsky [36] and Brendel [37], changes in the intensity of some peaks depend on a dissociation constant of the stacking complex.

Also interesting is the reduction of the intensity for the peaks corresponding to the C-C bond of the aromatic skeleton and the C-O-C bond which links two rings in the PA-34 inhibitor; this may also indicate an interaction of the aromatic skeleton of the inhibitor with the protein.

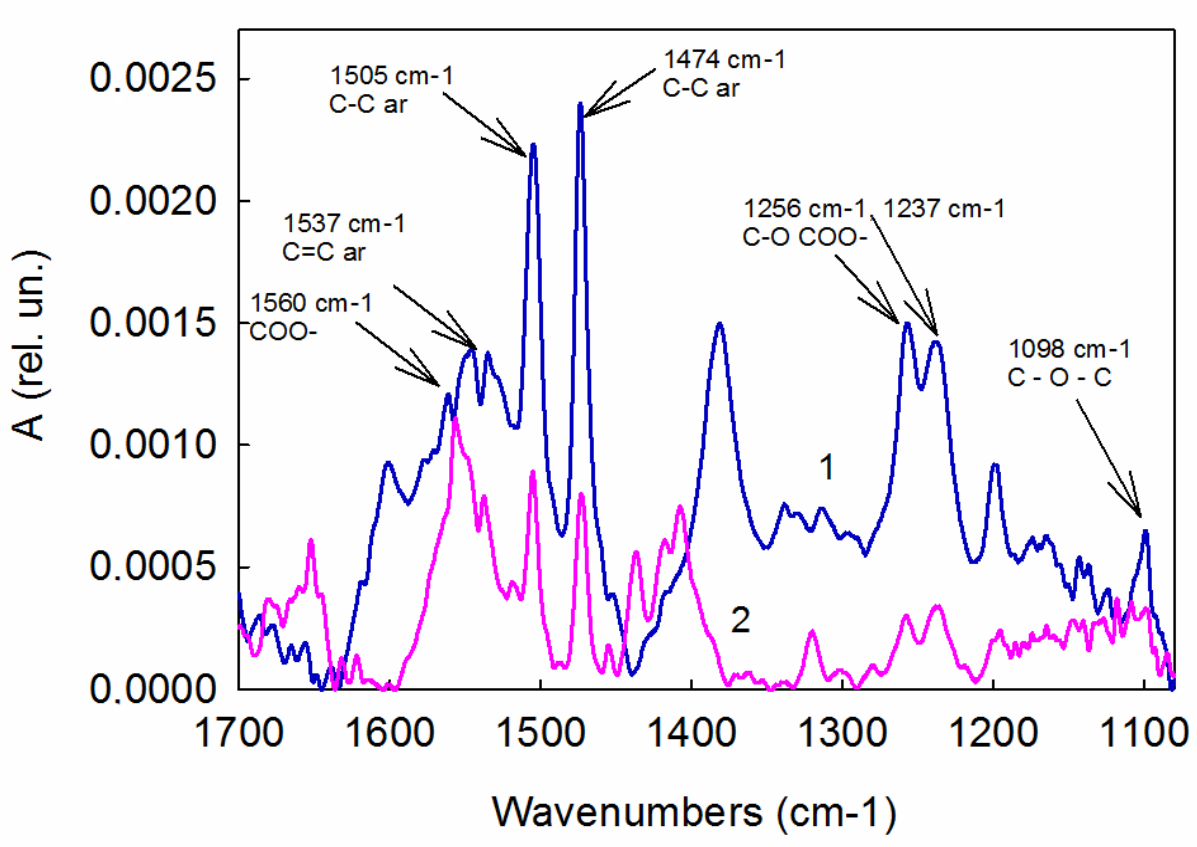


Fig. 4. ATR-FTIR spectra of the inhibitor alone (1, blue) and the protein-inhibitor complex (2, pink). The concentrations of the enzyme and the inhibitor are given in section 2.7. The main peak positions are indicated by the arrows.

Table 2. Intensity of the main absorption bands in the ATR-FTIR spectrum of protein-inhibitor complex.

Functional group	Peak position, cm-1	Peak intensity, a.u., Inhibitor	Peak intensity, a.u. Protein-Inhibitor complex	Intensity reduction (%)
COO-	1560	0.0012	0.0011	8.3
C=C ar	1537	0.0014	0.0008	41.6
C-C ar	1505	0.0022	0.0009	59.8
C-C ar	1474	0.0024	0.0008	66.7
C-O COO-	1256	0.0015	0.0003	80.0
C-O COO-	1237	0.0014	0.0003	75.7
C-O-C	1098	0.0009	0.0003	66.7

3.4 Fluorescence quenching measurements

In order to characterise the complex formation of PA-34 and β-lactamase TEM-171 in more detail, we performed fluorescence quenching experiments. The method uses the intrinsic fluorescence of the aromatic amino acids tryptophan, which is excited at 280 nm and tyrosine, excited at 295 nm [38]. β-Lactamase TEM-171 has four tryptophan residues (165, 210, 229, 290) and four tyrosines (46, 97, 105, 264) according to Ambler numbering [8]. Their fluorescence properties could be affected upon binding of PA-34. We have observed for the enzyme-inhibitor complex a fluorescence quenching of 10% at 295 nm and 26% at 280 nm (Fig. 5). The higher quenching at 280 nm suggests that the potential binding site of PA-34 may be located close to a tyrosine residue.

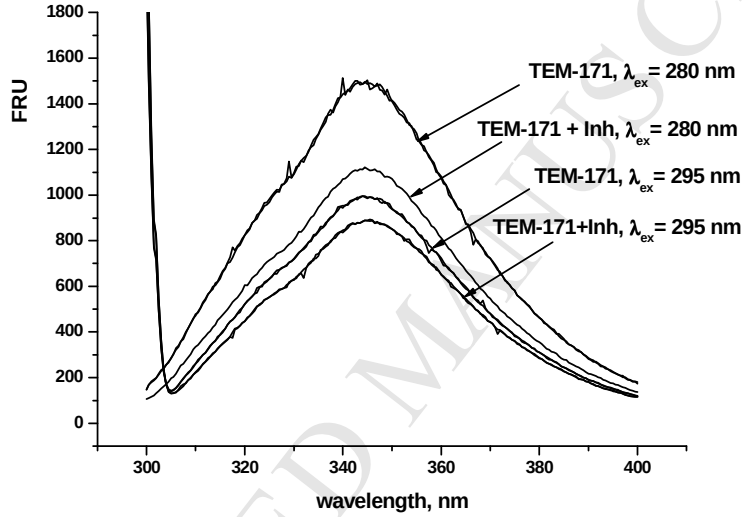


Fig. 5. Fluorescence quenching spectra recorded for β-lactamase TEM-171 and PA-34 inhibitor. The excitation wavelengths are indicated. The concentrations of the enzyme and the inhibitor are given in section 2.8.

In the β-lactamase TEM-1 structure (PDB ID 1xpb) only Trp165 and Tyr105 are located on the surface and are therefore potentially accessible to ligand binding, while the other tryptophan and tyrosine residues are buried in the protein. We therefore propose that the binding site of PA-34 occurs in the vicinity of Tyr105, close to the active site. This conclusion may seem surprising, since the reported binding place of a structurally similar inhibitor FTA, located 16Å from the active site, has a different structural environment with no obvious stacking interactions [18].

The competitive type of PA-34 inhibition observed in our kinetic study, together with the data on ATR-FTIR and fluorescence quenching spectroscopy, albeit indirectly, suggests

that PA-34 is most likely not targeting the FTA allosteric site, but may compete with the substrate at the entrance to the active centre. This conclusion is also in line with five times stronger inhibition of PA-34 compared to FTA

3.5 Proposed ligand binding mode

To elucidate the ligand binding mode and the inhibition mechanism we carried out molecular docking of PA-34 into protein binding sites. Horn and Shoichet have shown that FTA and CBT ligands inhibited the β–lactamase TEM-1 by binding to the allosteric site [18]. Therefore, we docked PA-34 to both the active and the allosteric sites of the 1xpb crystal structure of the wild-type β-lactamase TEM-1. The single mutation, Val84Ile, between TEM-1 and TEM-171 sequences, is located on the protein surface and is about 20Å away from the catalytic Ser70 OH group. The mutated side chain is even farther away, more than 30Å, from the allosteric site. In addition, as noted above, this mutation does not affect the enzyme kinetic properties.

The docking of PA-34 to the allosteric site of the protein could possibly occur if the two helices (residues 218-224 and 274-285) are moved away. Indeed, while in the complex with FTA the shortest distance between these helices is about 8Å, in the native structure of TEM-1, used here for the docking studies, these helices are too close to each other, thus this allosteric binding site is not accessible.

The docking to the protein active site suggests that the binding of PA-34 is governed by the stacking interactions between the dichlorophenoxy ring of the inhibitor and the aromatic ring of the Tyr105 residue (Fig. 6A). Three hydrogen bonds may be important for the stabilisation of this complex (Fig. 6B). Arg275 NH2 and the main chain amide of Gly242 form hydrogen bonds to the COOH group of the inhibitor. The oxygen atom connecting two aromatic rings in PA-34 forms the third hydrogen bond to Asn132 ND2. An interesting feature of the inhibitor binding is a possible formation of a halogen bond between the ligand chlorine atom in the ortho-position and the hydroxyl group of Ser70 with the Cl-O distance of 3.4 Å.

This putative binding mode of PA-34 with β-lactamase TEM-171 is in agreement with the observed changes in the intensity of the absorption bands in the IR spectrum during complex formation (Table 2). The intensity of the absorption bands of the carbon atoms in the aromatic rings is reduced by 60%. The observed reduction of the absorption of C-O-C and the carboxyl group of the inhibitor in the IR spectrum can be explained by the hydrogen bonds to

Arg275, Gly242 and Asn132. We propose that PA-34 acts as an inhibitor by simply blocking the access to the active site as it binds. The fact that PA-34 binds with five times higher affinity compared to FTA could be a reflection of specific interactions between this ligand and the residues located in its binding site. We suggest that PA-34 binds to the entrance of the enzyme active site, hampering the substrate from access to the catalytic Ser70; this fact may account for K_i being somewhat lower compared to other active site inhibitors (Table 1).

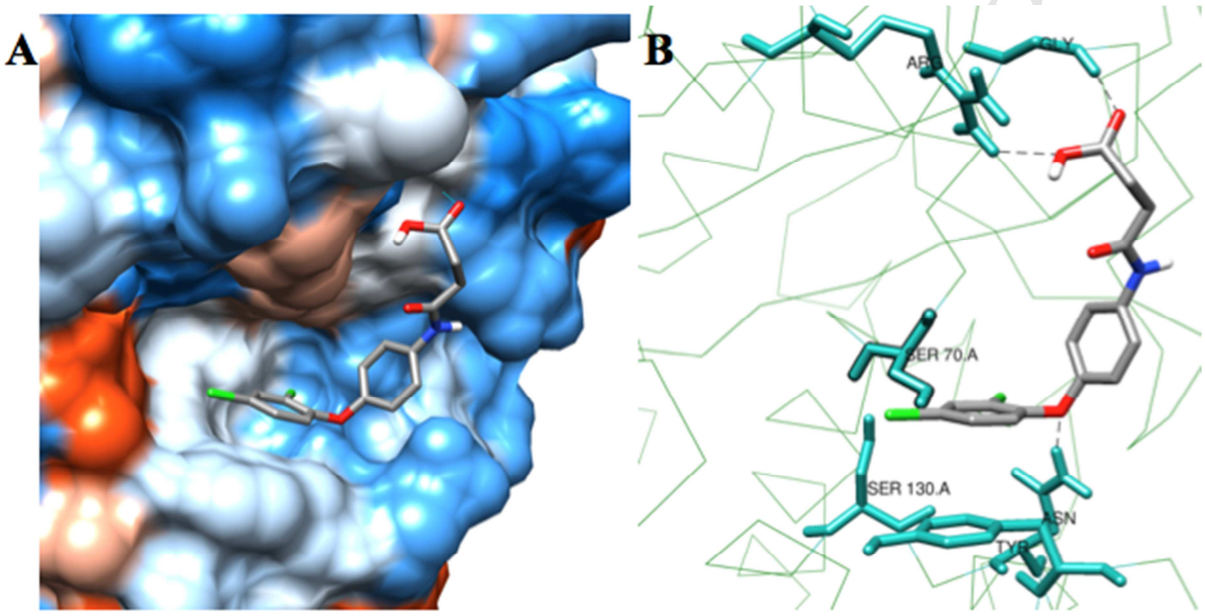


Fig. 6. Docking of PA-34 to the β–lactamase TEM-1 active site. (A) the lowest–energy docking model of PA-34; the protein is shown as electrostatic potential surface with the negative potential in red, neutral in white and positive potential in blue. (B) proposed interactions of PA-34. The hydrogen bonds are shown as dashed lines. This figure was produced with UCSF Chimera package [39].

Sequence alignment shows that Gly242 is conserved among all β-lactamases of molecular class A, involving penicillinases, cephalosporinases, and carbapenemases (TEM, SHV, CTX-M, KPC), and Tyr105 is conserved among all major types of molecular class A β-lactamases (TEM, SHV, CTX-M). Thus, according to the proposed binding mode, the PA-34 inhibitor is coordinated by at least two highly conserved residues. It may be hypothesised that the PA-34 compound can show an inhibitory activity for many clinically relevant β-lactamases of molecular class A.

With this in mind, crystal structures of the enzyme-bound ligands are being sought in order to confirm the proposed binding mode and provide an accurate description of the

conformation of the ligand. Site-directed mutagenesis of the residues of interest (Tyr105, Asn132, Gly242 and Arg275) and chemical modification of the inhibitor itself may also be attempted to illustrate the inhibition mechanism and improve inhibition potency by a rational design approach.

Conclusions

Inhibitors of β-lactamases have been co-administered with β-lactam antibiotics for a number of years, and normally this has improved therapeutic effects. The majority of these inhibitors are themselves β-lactams, with many of them being derived from microbial natural products. Their presence in the biosphere for many years indicates that enzymes, which are naturally resistant to them, may have already evolved. Even if not, the development of resistant species may require only a few mutations and may arise rapidly upon therapeutic use. Due to the great diversity of β-lactamases and their widespread danger, there is a pressing need for novel β-lactamases inhibitors, preferably those which are not based on the established β-lactam paradigm.

We have shown the promise of an approach to finding new potential inhibitors of TEM type β-lactamases with scaffolds distinct from β-lactam-based compounds. The identified inhibitor PA-34 has higher affinity to β-lactamase than the initial search templates. Moreover, the obtained data suggest a new binding mode – blocking access to the active site – that may be of particular interest to a search of stable and widely applicable inhibitors. We propose that binding of the PA-34 inhibitor is assisted by at least two highly conserved residues: Gly242 and the Tyr105. We also propose that the PA-34 compound may be regarded as a potential inhibitor of many clinically relevant β-lactamases of molecular class A. Crystallographic and rational design studies are required to further improve the inhibition power.

There is an increasing number of new drug candidates exhibiting poor solubility in water, which hinders their therapeutic applications. The approaches for solubility enhancement include solid dispersions, chemical modifications, a use of surfactants, etc. [40]. Unknown specificity and toxicity of potential drug candidates may present additional challenges and require the use of advanced drug delivery methods and extensive *in silico*-made predictions.

Although we do not yet have the data about the inhibitory power of PA-34 on other β-lactamases, our results open up new opportunities in a search of broad-spectrum inhibitors

based on the scaffold found, which in turn may help in combating antibiotic resistance more efficiently.

Acknowledgements

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