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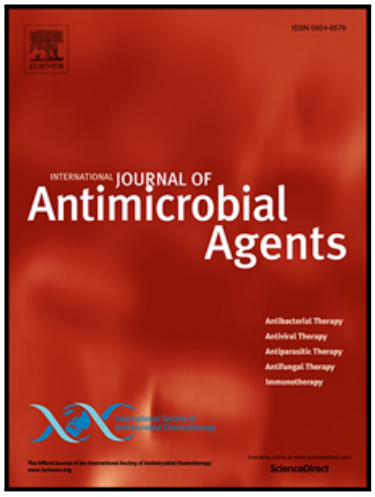

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LY2183240 regioisomers act as competitive and selective inhibitors of class C β-lactamases

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Running Title: LY2183240 regioisomers act as inhibitors of class C β-lactamases

ABSTRACT

The regioisomers of the anandamide-acting drug LY2183240 exhibited specific potent and competitive inhibitory activities against class C β-lactamases. More explicitly, the 1,5- and 2,5-regioisomers inhibited AmpC from *Enterobacter hormaechei* (formerly *Enterobacter cloacae*) with K_i values of 1.8 µM and 2.45 µM, respectively. Structural molecular modelling studies revealed the interaction of the regioisomers with the relevant residues of the catalytic site of cephalosporinase from *E. hormaechei* P99, which included Tyr^{150}, Lys^{315} and Thr^{316}.

Keywords: LY2183240, Tetrazole, β-lactamases, Competitive inhibitor, Antimicrobial resistance

1.0 Introduction

LY2183240 is a tetrazole drug which was initially developed by Eli Lilly as a potent inhibitor of anandamide reuptake and fatty acid amide hydrolase (FAAH), the primary enzyme responsible for degrading anandamide [1]. Nonetheless, functional proteomic screens established several other serine hydrolases that were also inhibited by this compound, indicating that LY2183240 has a wide range of activity against this large and diverse enzyme class [2]. More recently, it was reported that a synthesized regioisomer of this compound, 2,5-LY2183240, behaves similarly to LY2183240 (the 1,5-regioisomer), but with relatively lower pharmacological effects [3], [4]. These reports have stimulated interest to investigate the activities of these isomers against prokaryotic enzymes.

Many β-lactamases are serine hydrolases and a leading cause of antimicrobial resistance towards β-lactam agents [5]. This class of antibiotics are commonly first-line treatments for bacterial infections and consequently resistance to these agents remains a

significant menace to global public health [6]. A proven approach to tackle β-lactamase-mediated resistance is the design of β-lactamase inhibitors capable of protecting β-lactam hydrolysis by these enzymes, reinstating the therapeutic ability of antimicrobial agents [7]. Currently, there are a limited number of β-lactamase inhibitors clinically available, namely clavulanic acid, sulbactam, tazobactam, avibactam, and more recently, vaborbactam and relebactam. Avibactam is a novel and potent inhibitor that inhibits class A, class C, and some class D enzymes, however, there are already reports of cases of ceftazidime-avibactam resistance, which developed during treatment of carbapenem-resistant enterobacterial infections [8]–[10].

The design of an optimal and selective β-lactamase inhibitor still faces a number of challenges and novel strategies are needed. Notwithstanding, whilst not designed for this purpose, several drugs are recognized for their multi-targeting activities [11]. In this context, an alternative tactic is to seek compounds from diverse sources that act on multiple targets and repurpose them for more specific applications. Following on from this concept, in this study, we explored the potential inhibitory activity of LY2183240 regioisomers against different classes of β-lactamases.

2.0 Materials and methods

2.1 Chemicals and enzymes

The regioisomer 1,5-LY2183240 5-([1,1'-biphenyl]-4-ylmethyl)-*N*,*N*-dimethyl-1*H*-tetrazole-1-carboxamide was obtained from Santa Cruz Biotechnology, Inc. (Dallas, Texas, USA) and 2,5-LY2183240 5-([1,1'-biphenyl]-4-ylmethyl)-*N*,*N*-dimethyl-2*H*-tetrazole-1-carboxamide from Cayman Chemical (Ann Arbor, Michigan, USA). Cephalosporinase from *Enterobacter hormaechei* was purchased from Sigma-Aldrich (UK) and purified according to the protocol of Cartwright et al. (1984), TEM-1 was

acquired from Thermo Fisher Scientific, (UK), and NDM-1 from *Klebsiella pneumoniae* NCTC 13443. In addition, AmpC β-lactamases from *Pseudomonas aeruginosa* NCTC 10663 were extracted according to Stapleton et al., (1995). Avibactam and tazobactam were used as reference compounds.

2.2 Enzyme kinetics

Steady-state kinetic parameters were determined with extensive kinetic assays by incubating the β-lactamases TEM-1 (0.25 μM), AmpC P99 (2.5 nM), AmpC from *P. aeruginosa* (9.7 μM) and NDM-1 (supernatant containing the enzyme) with a range of 20μl of nitrocefin concentrations (ε = 20,500 M^{-1} cm^{-1}) (Toku-E Company) (1 – 100 μM) and varying the inhibitor concentrations at room temperature (25ºC) in PBS (up to 1 mL), pH 7.4 [14] Absorbance was measured with a UV-visible spectrophotometer (Thermo Scientific, UK) at 486 nm. All data were collected in triplicate and calculated by fitting the initial nitrocefin-hydrolysing rates of the β-lactamase enzymes to a suitable equation using GraphPad Prism 7 software.

3.0 Results

The IC_{50} and K_i of the LY2183240 regioisomers against different classes of β-lactamases are summarized in the Table. For classes A and B (metallo-β-lactamases), both LY2183240 regioisomers showed no significant effect against TEM-1 and NDM-1. Nonetheless, high inhibitory activity against AmpC from *E. hormaechei* was observed with a K_i of 1.8 and 2.45 μM for the LY2183240 1,5- and 2,5-regioisomers, respectively.

Table

Further investigation also showed that both LY2183240 1,5- and 2,5-regioisomers had potent inhibition against the AmpC beta-lactamase from *P. aeruginosa*, showing IC_{50} values of 1.66 µM and 1.80 µM, respectively, indicating specificity towards class C β-lactamases (Table) [15].

The primary difference between the LY2183240 regioisomers is the position of the carbamoyl moiety (either 1,5- or 2,5-) on the tetrazole heterocyclic group (Fig. 1A and 1B). Additional kinetic analyses using Michaelis-Menten and Lineweaver-Burk equations suggested that both isomers behaved as competitive inhibitors; the inhibitor acted as though it competed with the substrate, in this case nitrocefin, for binding to the enzyme (Fig. 1C and 1D).

Analyses of the data showed that the simple change of position of the carbamoyl group slightly increased the inhibition constant K_i, suggesting an important role in the inhibitory effect of AmpC β-lactamase, as demonstrated by former reports to other pharmacological activities [3], [4]. Additionally, the mechanism of action of LY2183240 towards FAAH proposed by Alexander et al., (2006) involves the carbamoylation of the enzyme's serine nucleophile (S241), corroborating the significance of this functional group for this effect on serine hydrolases.

The K_i and IC_{50} values of both regioisomers were higher than the standard compounds avibactam and tazobactam assessed in this study (Table). These compounds were of course not developed to bind to these types of enzymes, especially from bacterial sources, being originally purposed against mammalian hydrolases.

Progressive inhibition determinations were performed according to the method of Bush et al., (1993). Inactivation of AmpC β-lactamase P99 by the LY2183240 regioisomers showed a clear gradual time- and concentration-dependence (Fig. 1A and 1B). The rate of inhibition rose progressively as more inhibitor was provided. The 1,5-

regioisomer showed complete inactivation of AmpC β-lactamase after 60 minutes of incubation at a molar ratio of 165:1 (Fig. 1A). Similarly, the 2,5-regioisomer exhibited a similar profile, but to a lesser extent, with almost 80% of enzyme inactivation at the same interval (Fig. 1B). A possible explanation for this phenomenon is that the position of the carbamoyl moiety on the tetrazole ring may play an important role in this activity [3], [4]. Whilst the inhibitor-enzyme molar ratio of 7 and 14 showed no significant inhibition effects in the first 120 minutes, after 18 hours of incubation, both concentrations caused more than 50% inhibition.

The kinetic profiles displayed in this work showed similarities with the study conducted using tazobactam against β-lactamases [16]. However, no degree of reversibility was observed in any reaction with the LY2183240 regioisomers, indicating irreversible binding as well as a high affinity towards the enzyme (Fig. 1A and 1B).

Figure 1

To estimate the interactions of the two regioisomers with the AmpC β-lactamase at the atomic level, a molecular docking study was implemented using cephalosporinase PDB ID 1xx2, which represents a standard and clinically-relevant AmpC β-lactamase from *E. hormaechei* P99. The software package iGEMDOCK was selected due to its flexible docking feature, allowing conformational changes to parts of, or the entire molecule, to be made during the best-fit investigation [17]. Furthermore, this package targets the whole protein surface and therefore molecular docking would be entirely objective. This procedure could have biased results if the software restricted the ligand interactions only within the active site.

Figures 1E and 1F illustrate the main interactions of the LY2183240 regioisomers with cephalosporinase; both regioisomers presented significant interactions with residues of the active site of the enzyme, including Tyr^{150}, Lys^{315} and Thr^{316}. These residues play crucial roles as previously reported by many researchers, such as acylation and deacylation processes and substrate recognition [18]–[24]. Moreover, these findings corroborate our kinetics results, where LY2183240 regioisomers compete with the substrate in the catalytic site of the β-lactamase. Additionally, Figure 1G shows the best binding poses predicted for the LY2183240 regioisomers together with nitrocefin. It was observed that when overlaying the conformations, the carbamoyl group of 1,5-LY2183240 occupies the same space as the carboxy group of nitrocefin, interacting specifically with Thr^{316}. This factor may be responsible for the 1,5-LY2183240 regioisomer having higher inhibitory activity when compared to the 2,5-regioisomer. However, this hypothesis needs to be further confirmed with more studies.

As reported by others, LY2183240 demonstrated non-selective inhibitory activity towards mammalian serine hydrolases, especially involved in the endocannabinoid system. Nonetheless, this is the first report of high selective inhibitory activity of LY2183240 regioisomers against AmpC β-lactamases derived from *E. hormaechei* and *P. aeruginosa*, showing that optimum inhibitory activity is dependent upon the position of the carbamoyl moiety (1,5- to 2,5-) on the tetrazole heterocyclic group. Therefore, these compounds may prove useful as a chemical scaffold for the development of new, non-β-lactam, highly selective inhibitors of class C β-lactamases. Efforts to expand this study are in progress, including synthesis of analogues and X-ray single crystal structural analysis of the ligand-enzyme, in order to better comprehend the mechanism of action of this intriguing activity.

Declarations

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Competing Interests: The authors have declared that there are no conflicts of interest.

Ethical Approval: Not required

Sequence Information: Not applicable

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Figure caption

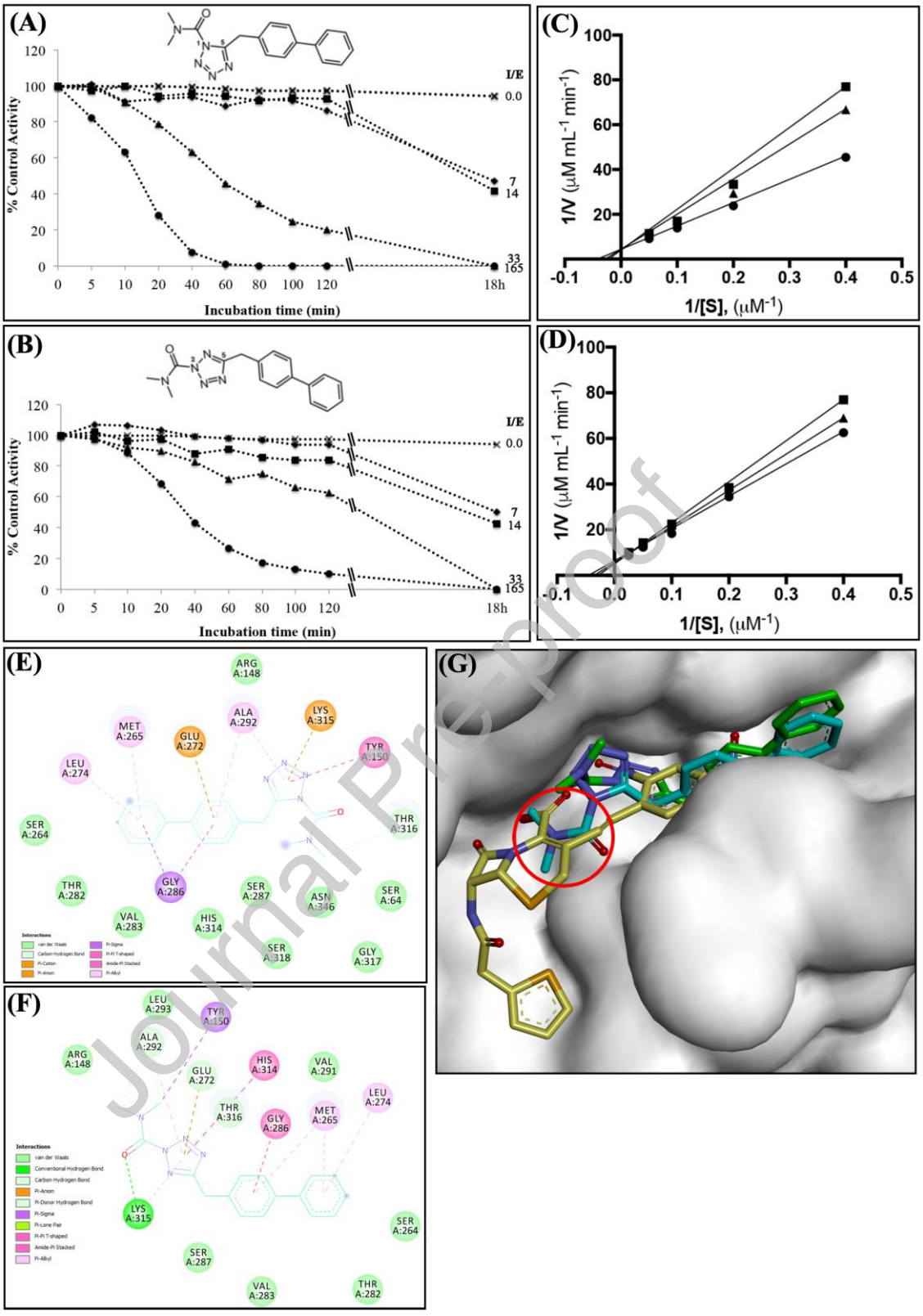

Fig 1. Inactivation of AmpC β-lactamase from *E. hormaechei* by **(A)** 1,5-LY2183240 and **(B)** 2,5-LY2183240. Inactivation mixtures were based on the study of Bush et al. (1993) and contained AmpC β-lactamase (2.5 nM) with 18 (◆), 35 (■), 83 (▲), and

413 (●), nM of the LY2183240 regioisomers and a negative control (×). I/E inhibitor-enzyme ratio. Competitive inhibitory activity of AmpC β-lactamase by **(C)** 1,5-LY2183240 and **(D)** 2,5-LY2183240 (Lineweaver-Burk plots). Final concentrations of both regioisomers were 0 μM (●), 0.5 μM (■), 0.8 μM (▲). Interactions of β-lactamase P99 with **(E)** 1,5-LY2183240 and **(F)** 2,5-LY2183240 in 2D as computed by molecular docking. **(G)** Best poses of molecular docking of LY2183240 regioisomers 1,5- (cyan) and 2,5- (green) with nitrocefin (yellow).

Table. K_i and IC_{50} determinations for inhibitory activities against various β-lactamases by the LY2183240 regioisomers.

	K_i (μM)			
Enzyme	Avibactam	Tazobactam	1,5-LY2183240	2,5-LY2183240
Class A **TEM-1**	0.002[b]	0.020 ± 0.003	>100[a]	>100[a]
Class B **NDM-1**	-	-	>100[a]	>100[a]
Class C ***E. hormaechei*** **P99**	0.004 ± 0.002	0.372 ± 0.06	1.802 ± 0.25	2.453 ± 0.30
P. aeruginosa **10663**	0.180 ± 0.001	-	1.665 ± 0.012	1.801 ± 0.21

[a] No inhibitory activity was detected against these enzymes up to 100 μM.

[b] IC_{50} [15].