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GMP and IMP Are Competitive Inhibitors of CMY-10, an Extended-Spectrum Class C β-Lactamase

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ABSTRACT Nucleotides were effective in inhibiting the class C β-lactamase CMY-10. IMP was the most potent competitive inhibitor, with a K_i value of 16.2 μM. The crystal structure of CMY-10 complexed with GMP or IMP revealed that nucleotides fit into the R2 subsite of the active site with a unique vertical binding mode where the phosphate group at one terminus is deeply bound in the subsite and the base at the other terminus faces the solvent.

KEYWORDS GMP, IMP, β-lactamases, competitive inhibitor

Four classes of β-lactamases (A, B, C, and D), classified based on their amino acid sequence (1), inactivate β-lactam antibiotics. Thus, one of optimal strategies to combat the β-lactamase-mediated drug resistance is the combination of β-lactam antibiotics with β-lactamase inhibitors (BLIs). There are four clinically used BLIs: clavulanate, sulbactam, tazobactam, and avibactam. Clavulanate, sulbactam, and tazobactam are effective only against class A β-lactamases (2–6). Although avibactam inhibits class A, class C, and some class D enzymes (7), there is a report on a KPC-3-producing *K. pneumoniae* strain which is resistant to the recently FDA-approved ceftazidime-avibactam treatment (8). The limited number of clinically applicable BLIs and the emergence of bacterial resistance to them highlight the urgent need of development of novel BLIs. One approach to reach the goal is to secure new chemical scaffolds that fit the active site of β-lactamases in a different way from existing BLIs. If such BLIs were developed, they would escape the rampant resistance mechanism adapted to exiting BLIs. CMY-10 from a virulent clinical strain of *Enterobacter aerogenes* is a plasmid-encoded class C β-lactamase that can hydrolyze imipenem and third-generation cephalosporins as well as penicillins, cefoxitin, and cefotetan (9). Here, we show the inhibitory activity of nucleotides toward CMY-10 and present their unique binding mode in the active site.

The potential inhibition of class C β-lactamases by nucleotides has been suggested by the observation of the adenylylated nucleophilic serine in the active site of a class C β-lactamase, FOX-4 (10). Furthermore, we have reported that adenosine 5′-(P-acetyl) monophosphate (acAMP) is an irreversible covalent inhibitor of class C β-lactamases (11). acAMP inhibits class C β-lactamases through the covalent attachment of its AMP moiety to the nucleophilic serine residue. According to the crystal structures of class C β-lactamases with the adenylylated nucleophilic serine residue (11), the AMP adduct makes extensive interactions with active site residues. It is surprising that class C β-lactamases recognize the nucleotide scaffold that is structurally distinct from its natural β-lactam substrates, which led us to examine whether nucleotides inhibit class C enzymes.

To examine the inhibitory effect of nucleotides, native CMY-10 was purified by using the *m*-aminophenylboronic acid resin as previously described (11) and 0.2 nM CMY-10

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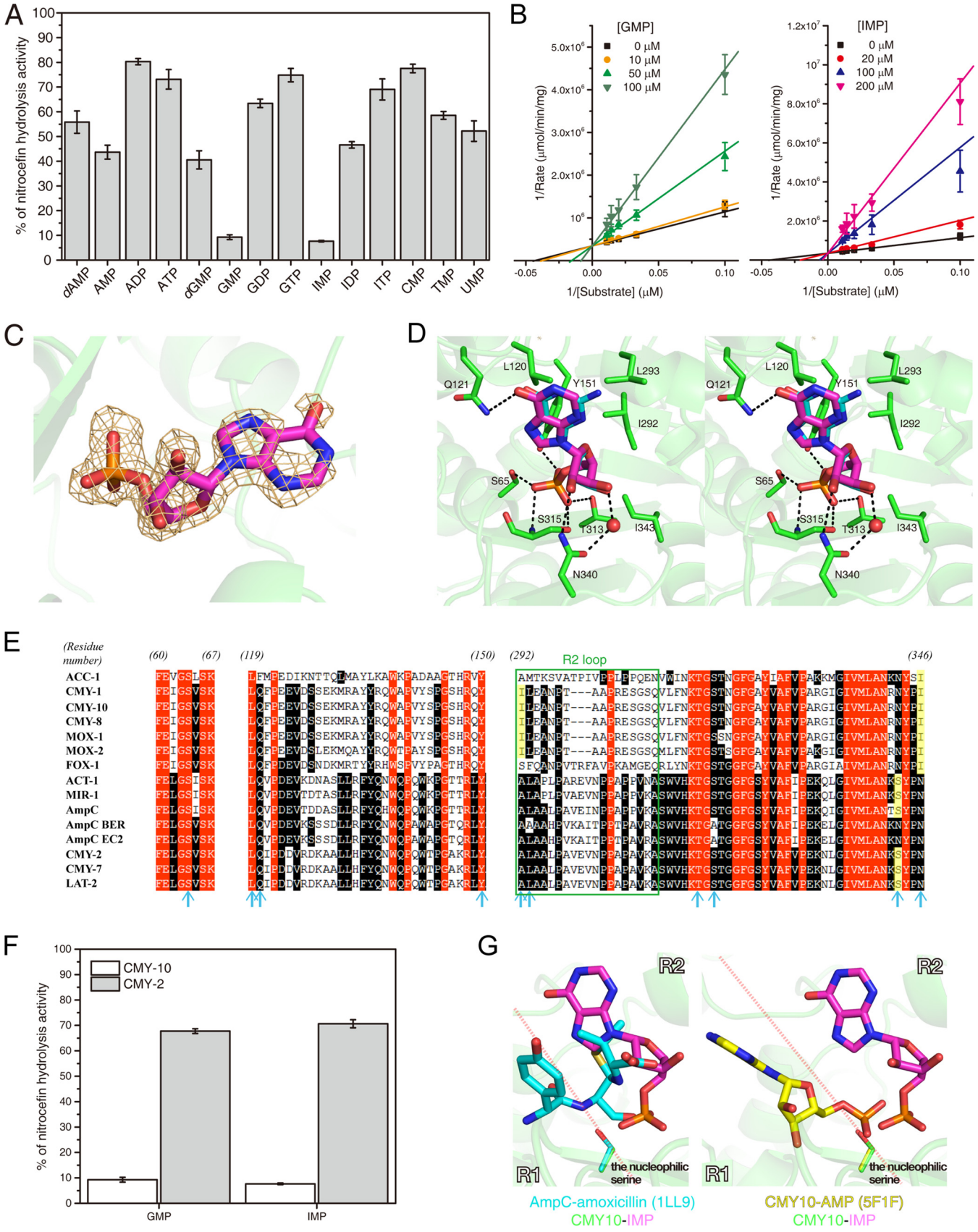

FIG 1 Interactions of GMP or IMP with active site residues of CMY-10. (A) Percent changes in the nitrocefin hydrolysis activity of CMY-10 in the presence of 2 mM nucleotides. dAMP, deoxyadenosine monophosphate; dGMP, deoxyguanosine monophosphate. In all panels, error bars are ± standard deviations

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was mixed with various nucleotides (2 mM) and with 50 mM MES [2-(*N*-morpholino)ethanesulfonic acid] (pH 6.5) at room temperature. The CMY-10 activity was continuously measured by monitoring the hydrolysis of 100 μM nitrocefin at 486 nm (ε = 20,500 M^{-1} cm^{-1}) for 1 h using a SpectraMAX Plus spectrophotometer (Molecular Devices, USA). In reducing the nitrocefin hydrolysis, GMP and IMP were the most effective compounds (Fig. 1A). Subsequently, the steady-state kinetic parameters of GMP and IMP were calculated by fitting the initial nitrocefin-hydrolyzing rates of CMY-10 in the presence of 10 to 200 μM GMP or IMP to the competitive inhibition equation (12). GMP and IMP were revealed to be competitive inhibitors of CMY-10, with K_i values of 20.8 and 16.2 μM (Fig. 1B), respectively.

To delineate the interactions of the two nucleotides with class C β-lactamases at the atomic level, we determined the high-resolution crystal structure of CMY-10 in complex with GMP or IMP (Table 1 and Fig. 1C). The purified CMY-10 with an N-terminal His_6 tag (11) was mixed with 20 mM Tris-HCl (pH 7.0) and with 50 mM GMP or IMP, and then the mixture was concentrated to ~40 mg · ml^{-1} for crystallization. Crystals were grown in a precipitant solution containing 0.1 M cadmium chloride, 0.1 M sodium cacodylate (pH 6.0), and 30% polyethylene glycol 400. The binding modes of GMP and IMP are nearly identical (Fig. 1D); thus, we describe here only the interactions of IMP with CMY-10.

The phosphate moiety is positioned at the bottom of the active site, and the pyrimidine ring of the inosine base is fully exposed to solvent (Fig. 1D). The phosphate moiety is surrounded by four hydroxyl groups of Ser65, Tyr151, Thr313, and Ser315 that are within hydrogen bonding distances (Fig. 1D). The ribose and base moieties lean against the hydrophobic wall lined by Tyr151, Leu120, Ile292, Leu293, and Ile343, with their polar atoms forming hydrogen bonds (Fig. 1D). The 2′-OH of the ribose interacts with the side-chain nitrogen atom of Asn340, and the 3′-OH of the ribose forms water-mediated interactions with the side-chain oxygen atom of Asn340 (Fig. 1D). The N7 of the inosine base makes a hydrogen bond with the side-chain amide oxygen atom of Gln121 (Fig. 1D). The active site residues that interact with IMP are highly conserved or homologously replaced in other class C β-lactamases (Fig. 1E). It is notable that GMP and IMP exhibited weak inhibitory activities against native CMY-2 (Fig. 1F), the progenitor of CMY-10 (11). Compared to CMY-2, CMY-10 has a wide R2 subsite due to the three-residue deletion in the R2 loop (Fig. 1E), which is responsible for its extended-substrate spectrum (9). Consequently, the R2 subsite conformation seems to be related to the inhibition efficiency of GMP and IMP.

We have previously divided the active site of CMY-10 into the R1 subsite and the R2 subsite, with Ser65 at the border between them (9). The R1 subsite designation refers to the region accommodating the R1 side chain at position C7 (or C6) of the β-lactam nucleus, and the R2 subsite interacts with the R2 side chain at C3 (or C2) (Fig. 1G). IMP is bound to the R2 subsite of CMY-10 in a vertical binding mode (Fig. 1G). In general, β-lactam inhibitors and substrates bind to the active site spanning both the R1 and R2 subsites, although they interact mainly with residues in the R1 subsite (Fig. 1G). To our knowledge, the vertical binding mode in the R2 subsite has

FIG 1 Legend (Continued)
of results from triplicate experiments. (B) Lineweaver-Burk plots of CMY-10 inhibited by GMP (left) or IMP (right). (C) The initial maximum-likelihood weighted F_o-F_c electron-density map (gold) contoured at 3σ after molecular replacement for the IMP molecule. In all panels, nitrogen and oxygen atoms are colored in blue and red, respectively, and the active site of CMY-10 is shown in a transparent cartoon (green). (D) Stereo view of the CMY-10/IMP complex. The final model of the CMY-10/IMP complex is superposed onto that of the CMY-10/GMP complex for this panel. Active site residues (green sticks) and IMP (magenta stick) of the CMY-10/IMP complex are presented, but, for clarity, only GMP (cyan stick) of the CMY-10/GMP complex is shown. Red spheres represent water molecules. Dotted black lines indicate polar interactions. (E) Multiple-sequence alignment of class C β-lactamases. Identical residues are highlighted with red backgrounds, and conserved residues are indicated with black and yellow backgrounds. Residues interacting with IMP are indicated by blue arrows. Residues are numbered according to the canonical numbering of *Enterobacter cloacae* P99 AmpC (13). GenBank accession numbers of the exploited β-lactamases are as previously described (12). A green box indicates the R2 loop. (F) Percent changes in the nitrocefin hydrolysis activity of CMY-10 (white bars) and CMY-2 (gray bars) in the presence of 2 mM GMP or IMP. (G) Positional comparisons among IMP, amoxicillin, and AMP in the active site of class C β-lactamases. The final model of CMY-10/IMP complex is superposed onto the crystal structure of the AmpC/amoxicillin complex (left, cyan) or the CMY-10/AMP complex (right, yellow). A dotted red line passing through the nucleophilic serine shows the border between R1 and R2 subsites. R1 and R2 represent R1 and R2 subsites, respectively.

TABLE 1 Crystallographic data collection and refinement

Parameter	Result	
	CMY-10/IMP (5K1F)	CMY-10/GMP (5K1D)
Data collection		
Diffraction source	*PAL-5C*	*PAL-5C*
Wavelength (Å)	1.28270	1.28270
Rotation range per image (°)	1	1
Total rotation range (°)	360	360
Exposure time per image (s)	1	1
Space group	$P2_12_12_1$	$P2_12_12_1$
a, *b*, *c* (Å)	49.698, 59.377, 113.612	60.478, 65.414, 106.115
Resolution range (Å)	50–1.94	50–1.94
Total no. of reflections	653,812	957,372
No. of unique reflections	25,473	24,395
Completeness (%)[a]	99.4 (97.3)	95.7 (88.4)
Redundancy	9.9 (7.4)	9.3 (5.4)
I/σ (I)[a]	57.54 (12.82)	52.81 (9.46)
Overall B factor from Wilson plot (Å^2)	17.1	16.8
R_{sym} (%)[a,b]	8.5 (26.9)	5.7 (17.1)
Refinement statistics		
Resolution range (Å)	45.5–1.94	38.1–1.94
No. of reflections	25,448	24,388
No. of atoms		
Protein	2,691	2,688
IMP	23	
GMP		24
Cadmium	5	5
Water	83	197
B factors (Å^2)		
Protein	18.8	18.3
IMP	32.2	
GMP		26.1
Cadmium	23.4	28.0
Water	18.9	22.7
R[c] (R_{free}) (%)	18.6 (23.1)	18.8 (24.5)
RMS deviations[d]		
Bond length (Å)	0.007	0.007
Bond angle (°)	1.086	1.120

[a]The number in parentheses is for the outer shell.
[b]$R_{sym} = \sum_h\sum_I |I_{h,I} - I_h|/\sum_h\sum_I\sum I_{h,I}$, where I_h is the mean intensity of the i observations of symmetry-related reflections of h.
[c]$R = \sum |F_o - F_c|/\sum F_o$, where $F_o = F_p$ and F_c is the calculated protein structure factor from the atomic model. R_{free} was calculated with 10% of the reflections.
[d]RMS (root mean square) deviations in bond length and angles are the deviations from ideal values.

never been observed in interactions of β-lactam inhibitors/substrates with β-lactamases.

The AMP moiety is located at the R1 subsite of CMY-10 in the crystal structure of the adenylylated CMY-10 (11), which contrasts with the location of IMP at the R2 subsite. Structural superposition clearly shows that the AMP moiety and IMP are positioned at different subsites in the active site (Fig. 1G). It is remarkable that nearly identical chemicals interact with different residues in the same active site, although the structural basis for this unusual observation cannot be presented at this stage.

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