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Identification of Novel Irreversible Inhibitors of UDP-*N*-Acetylglucosamine Enolpyruvyl Transferase (MurA) from *Haemophilus influenzae*

Han, Seong-Gu[1], Won-Kyu Lee[1], Bong-Suk Jin[1], Ki-In Lee[2], Hyeong Ho Lee[1], and Yeon Gyu Yu[1]*

[1]*Department of Chemistry, Kookmin University, Seoul 136-702, Korea*
[2]*Korea Research Institute of Chemical Technology, Daejun 305-600, Korea*

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Uridinediphospho-*N*-acetylglucosamine enolpyruvyl transferase (MurA, E.C. 2.5.1.7) is an essential bacterial enzyme that catalyzes the first step of the cell wall biosynthetic pathway, which involves the transfer of an enolpyruvyl group from phosphoenolpyruvate to uridinediphospho-*N*-acetylglucosamine. In this study, novel inhibitors of *Haemophilus influenzae* MurA (Hi MurA) were identified using high-throughput screening of a chemical library from the Korea Chemical Bank. The identified compounds contain a quinoline moiety and have much lower effective inhibitory concentrations (IC_{50}) than fosfomycin, a well-known inhibitor of MurA. These inhibitors appear to covalently modify the sulfhydryl group of the active site cysteine (C117), since the C117D mutant Hi MurA was not inhibited by these compounds and excess dithiothreitol abolished their inhibitory activities. The increased mass value of Hi MurA after treatment with the identified inhibitor further confirmed that the active-site cysteine residue of Hi MurA is covalently modified by the inhibitor.

Key words: *Haemophilus influenzae*, MurA, high-throughput screening, inhibitor, antibiotics, covalent modification

Haemophilus influenzae is a Gram-negative bacterium that causes clinical diseases such as pneumonia and acute bacterial meningitis in infants and young children. The bacterial cell wall is composed mainly of peptidoglycan, and MurA catalyzes the first step in the biosynthesis of peptidoglycan. MurA transfers the enolpyruvyl group of phosphoenolpyruvate (PEP) to the 3'-hydroxyl group of UDP-*N*-acetylglucosamine (UNAG) to form UDP-*N*-acetylglucosamine (UDP-GlcNAc)-enolpyruvate [6]. MurA is essential for cell growth, since deletion of the MurA gene in *Escherichia coli* or *Streptococcus pneumoniae* is lethal [5, 8]. The indispensability of MurA and its universal presence in bacterial but not in mammalian cells make MurA an attractive target for antibiotic development.

The reaction catalyzed by MurA was initiated by the protonation of PEP and the deprotonation of the 3'-hydroxy group of UNAG, followed by nucleophilic addition at the oxocarbenium ion of PEP in a tetrahedral configuration. Inorganic phosphate was released from the tetrahedral adduct to produce enolpyruvyl-UDP-*N*-acetylglucosamine (EP-UNAG) (Fig. 1) [9, 22]. The crystal structures of MurAs from *Escherichia coli*. *Enterobacter cloacae*, and *Haemophilus influenzae* were highly homologous [21, 23, 25], and the active site was located in the cleft of two domains, and a flexible loop region covered the active site. MurA undergoes a conformational change from an "open" or substrate-free state to a "closed" or substrate-bound state during the catalytic reaction. Upon binding of UNAG to the active site of MurA, the loop region moves toward the active site to form the "closed" state. Studies have shown that substitution of a conserved cysteine residue (Cys117 in Hi MurA) into Ala or Ser completely abolished its catalytic activity [16]; thus, this residue (Cys117 in Hi MurA) in the loop is critical for the catalytic reaction. MurA acts as a general acid to transfer hydrogen to PEP during catalysis [16], and/or it is involved in phosphate release [10]. The cysteine residue in the loop region is highly conserved in MurAs from various bacterial species except a few cases, such as in *Chlamydia trachomatis* or *Mycobacterium tuberculosis* MurA, in which the cysteine is replaced by an aspartate residue [7, 18]. The cysteine residue is also the target of fosfomycin, an antibiotic produced by *Streptomyces* [15]. Fosfomycin forms a covalent adduct to the sulfhydryl group of cysteine to irreversibly inhibit MurA [15, 23]. Although fosfomycin can effectively inhibit the catalytic activity of MurA and the growth of some bacteria, fosfomycin resistance frequently occurs through overexpression or substitution of the MurA active-site cysteine [13, 16]. Additionally, modification or reduced uptake of fosfomycin leads to fosfomycin resistance [1, 4].

**Corresponding author*
Phone: +82-2-910-4619; Fax: +82-2-910-4415;
E-mail: ygyu@kookmin.ac.kr

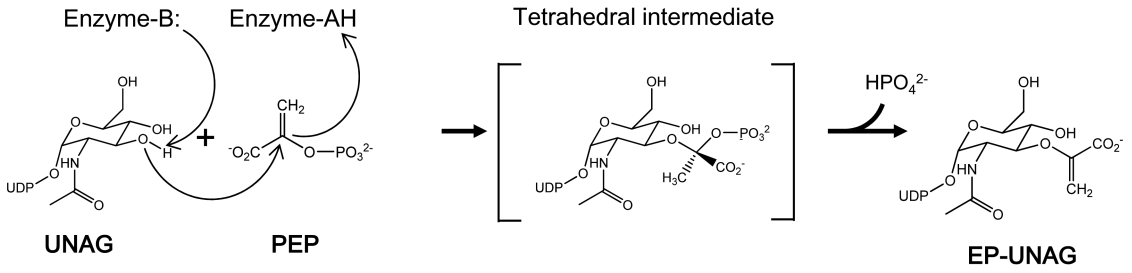

Fig. 1. Enzymatic reaction of MurA.
MurA catalyzes the transfer of the enolpyruvyl group of PEP to UNAG to produce EP-UNAG and inorganic phosphate. Enzyme-AH and enzyme-B: side chains of MurA involved in the transfer reaction. Inorganic phosphate is released from the tetrahedral intermediate to produce enolpyruvyl-UDP-*N*-acetylglucosamine (EP-UNAG).

In addition to fosfomycin, several MurA inhibitors have been reported. Bristol-Myers Squibb compound Cpd1 inhibits *E. coli* MurA with an IC_{50} value in the micromolar range [2], and peptide inhibitors against MurA were identified from biopanning of a phage display library [19]. A derivative of 5-sulfonoxy-anthranilic acid binds to the catalytic loop of *E. cloacae* MurA with a K_i of 16 μM and prevents the transition from the open (unliganded) to the closed (UNAG-liganded) conformation [20]. R.W. Johnson compounds (RWJ-3981, RWJ-140998, and RWJ-110192) showed efficient inhibition against MurA with IC_{50} values in the submicromolar range and minimum inhibitory concentration (MIC) values between 4 and 32 μg/ml against *Staphylococcus aureus* [3]. Although these compounds show inhibition against MurA, none have been developed for clinical application. For example, the R.W. Johnson compounds were shown to inhibit the synthesis of DNA, RNA, and proteins as well as peptidoglycan and failed to protect *S. aureus*-infected mice from death [3]. Hence, potent inhibitory compounds against MurA with distinct structures are required for antibiotic development.

In this study, we identified novel inhibitors against Hi MurA using high-throughput screening of a chemical library from the Korea Chemical Bank. Identified compounds showed a distinct structure, compared with previously known inhibitors of MurA, and IC_{50} values in the submicromolar range. These inhibitors were shown to inhibit Hi MurA through covalent modification of the conserved cysteine residue at the active-site loop region.

Materials and Methods

Materials

The chemical library for high-throughput screening was provided by the Korea Chemical Bank (Daejeon, Korea). Fosfomycin disodium salt, PEP, and UNAG were obtained from Sigma (St. Louis, MO, USA). A pre-packed Ni-NTA column and HiPrep 26/60 Sephacryl S-300 High-Resolution column for ÄKTA FPLC were obtained from GE Healthcare (Little Chalfont, UK). Malachite green oxalate salt and ammonium molybdate tetrahydrate were purchased from Sigma. HRP-conjugated 6× His-tag IgG was obtained from BD Bioscience (Franklin Lakes, NJ, USA). The bicinchoninic acid (BCA) protein assay kit and enhanced chemiluminescence substrate were obtained from Pierce (Rockford, IL, USA).

Preparation of Wild Type and C117D Mutant of Hi MurA

The wild type and C117D mutant of Hi MurA were prepared as described previously [11]. Briefly, *E. coli* Rosetta2(DE3) cells harboring the expression vector of Hi MurA were grown at 37°C in LB media containing 0.1 mg/ml ampicillin and 50 g/ml chloramphenicol, and the expression of Hi MurA was induced by adding 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), when the optical density of the cell culture reached 0.9. Expressed proteins were purified from the cell lysate using Ni-NTA affinity column and size exclusion chromatography.

Enzyme Assay

The activity of Hi MurA was measured as described previously [14]. Briefly, 20 μl of 100 nM Hi MurA in 50 mM Tris-HCl, pH 7.8, was mixed with 20 μl of substrate solution containing 200 μM PEP and 400 μM UDP-GlcNAc and incubated for 30 min at 25°C. For Hi MurA(C117D), 1 μM of Hi MurA(C117D) in 50 mM MES, pH 6.0, was used for the assay. The reaction was terminated by adding 200 μl of malachite green reagent containing 0.045% malachite green and 4.2% ammonium molybdate in 4 N HCl. The amount of phosphate released due to PEP cleavage was estimated by measuring the optical density at 650 nm using the TRIAD multimode detector TRIAD LT (Dynex Technologies, Chantilly, VA, USA). The activity of C117D mutant Hi MurA was measured using the same method used for wild-type MurA, except with 100 mM MES, pH 6.0, as the reaction buffer.

Screening of Chemical Library

To screen the library of bioactive molecules, 1 ml of dimethylsulfoxide solution containing 10 mM of the compound from a 96-well formatted chemical library was preincubated with 20 μl of 100 nM Hi MurA for 20 min at room temperature, and then the residual activity of MurA was measured as described above.

Determination of the Cysteine Reactivity of the Inhibitors

To determine whether the identified inhibitors were reactive towards the sulfhydryl group, each inhibitor was incubated in the absence or presence of dithiothreitol (DTT) in 50 mM Tris-HCl, pH 7.8, at

room temperature for 30 min. Inhibitor activities were then determined as described above. To examine whether the identified inhibitors irreversibly affected Hi MurA, the compounds were incubated with Hi MurA and then extensively dialyzed. Inhibitor activities were then determined as described above.

Mass Analysis

For mass analysis, 10 μM of Hi MurA wild type and mutant were incubated with 0.5 mM of compound 3 for 20 min at room temperature. The mixture was dialyzed twice against 10 mM Tris-HCl, pH 7.5, with a 1,000-fold excess volume of buffer. Before and after dialysis, residual activities were monitored as described above. The total mass of the samples was measured by Probiond Inc. (Seoul, Korea) using the MALDI-TOF method. All the experiments were performed in linear mode using a Bruker Autoflex III time-of-flight mass spectrometer (Bruker, Bremen, Germany), equipped with a Smartbeam laser. Each recorded mass spectrum consisted of the sum of 500 shots at a single sampling position using the positive-ion detection mode. The matrix used in this experiment was sinapinic acid (25 mg/ml, 70% acetonitrile, and 0.2% trifluoroacetic acid).

RESULTS

Identification of Novel Inhibitors Against Hi MurA

To identify novel Hi MurA inhibitors, the compounds deposited in the Korean Chemical Bank (http://www.chembank.org) were tested. A fragment library consisting of 1,841 compounds, which represent unique scaffolds with molecular masses ranging from 300 to 500 Da, were selected and tested for their ability to inhibit Hi MurA (Fig. 2). Compound **1**, which has distinct structural scaffolds compared with previously identified MurA inhibitors, showed more than 80% inhibition of Hi MurA at 50 μM. Approximately 300 compounds that shared common scaffolds with compound **1** were selected from the master library, which consists of

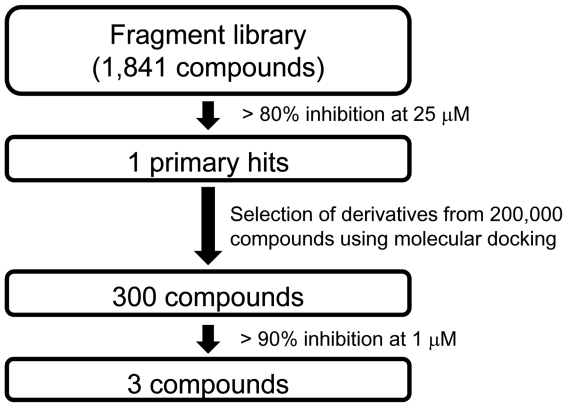

Fig. 2. Screening strategy of HI MurA inhibitors from the Korea Chemical Bank.

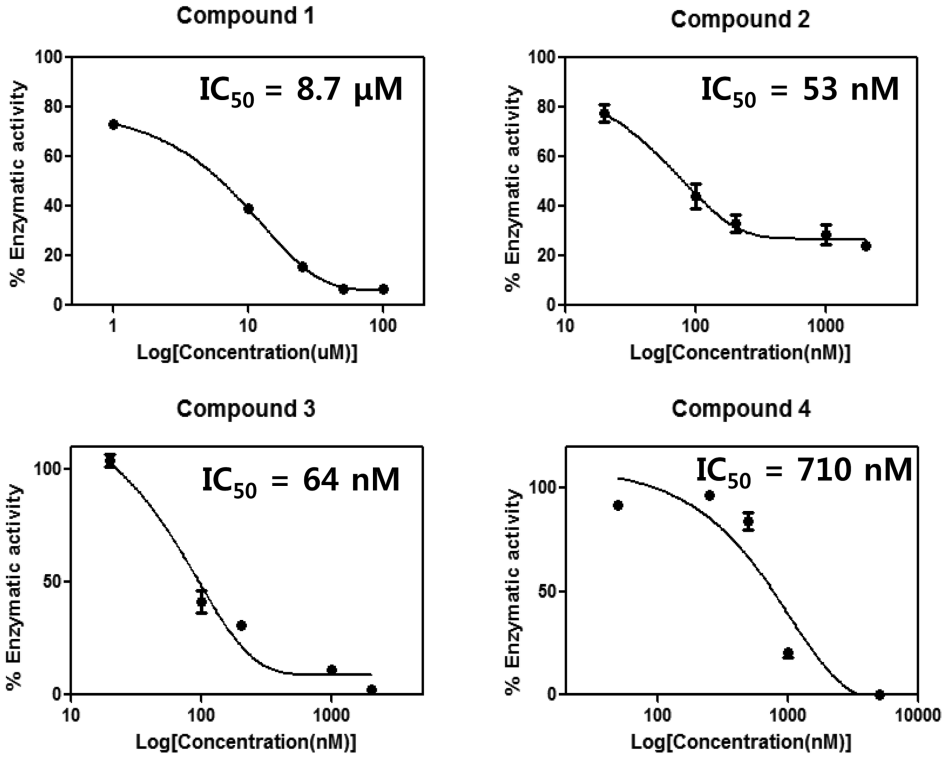

Fig. 3. Concentration-dependent inhibitory activity of isolated compounds.
Inhibitory activities of compounds **1**, **2**, **3**, and **4** were examined by measuring the residual activity of Hi MurA after incubation for 20 min at room temperature. The 50% inhibitory concentration (IC_{50}) values were calculated as 8.7 μM, 53 nM, 64 nM, and 710 nM, respectively.

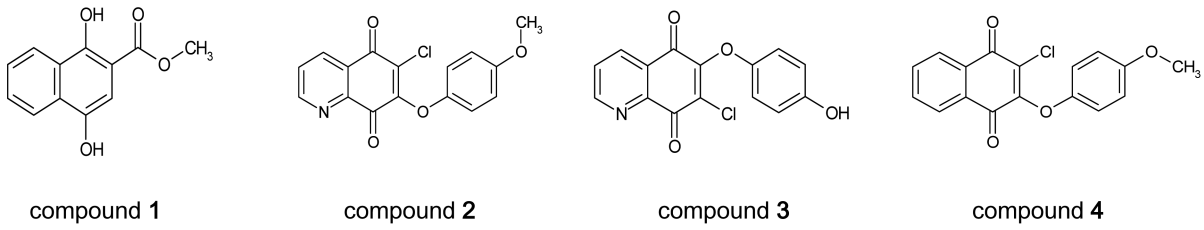

Fig. 4. Structure of identified MurA inhibitors.

approximately 200,000 compounds. The inhibitory activities of the selected compounds were further tested, and 3 compounds (compounds **2–4**) showed approximately 80% inhibition at 1 μM. The concentration-dependent inhibitory activity of compounds **1–4** was measured, and the afferent IC_{50} values of compounds **1–4** were determined as 8.7 μM, 53 nM, 64 nM, and 710 nM, respectively (Fig. 3). Compound **1** is a methylester derivative of naphthohydroquinone (methyl 1,4-dihydro-2-2-naphtholate) and compounds **2–4** are derivatives of quinoline or naphthoquinone (Fig. 4). Inhibitory activities of compounds **2–4** were 10–100 times higher than compound **1**, indicating that the quinone structure rather than hydroxyquinone is important for inhibitory activity. Notably, the IC_{50} value of compound **2** was 14-times lower than that of compound **4**, indicating that the nitrogen in the quinoline moiety is important for inhibitory activity.

Importance of Sulfhydryl Group on Conserved Cysteine Residue of Hi MurA

The cysteine residue in the active-site loop of MurA is critical for catalysis, which is covalently modified by the MurA inhibitor fosfomycin [15, 23]. To determine whether Cys117 of MurA is the target site of compounds **1–4**, the inhibitory activities of these compounds were examined. Substitution of Cys117 with aspartate shifted the optimum pH from 7.8 to 6.0 and decreased the specific activity by 50-fold [11]. As shown in Fig. 5A, all 4 compounds as well as fosfomycin failed to inhibit Hi MurA(C117D), indicating that Cys117 is the inhibitor target site. Furthermore, inhibitor activity towards the sulfhydryl group was examined by measuring residual inhibitory activities of these compounds and fosfomycin after incubation with 10 mM of DTT. DTT treatment reduced the inhibitory activity of fosfomycin by approximately 20%. The activities of compounds **1–4** were completely abolished following DTT treatment (Fig. 5B). These results suggest that Cys117 of Hi MurA is the target of these compounds and that they react with the free sulfhydryl group of the cysteine residue.

Covalent Modification of MurA Cysteine Residues by Compound 3

To test whether compound **3** could irreversibly inhibit Hi MurA, compound **3** was incubated with Hi MurA and its residual inhibitory activity was measured after extensive dialysis. Hi MurA activity was inhibited by compound **3**, and the activity was not recovered after extensive dialysis (Fig. 6). Irreversible inhibition was also observed for fosfomycin, which was known to alkylate Cys117 [15, 23]. This indicates that Hi MurA was irreversibly inhibited by compound **3** through covalent modification. To determine

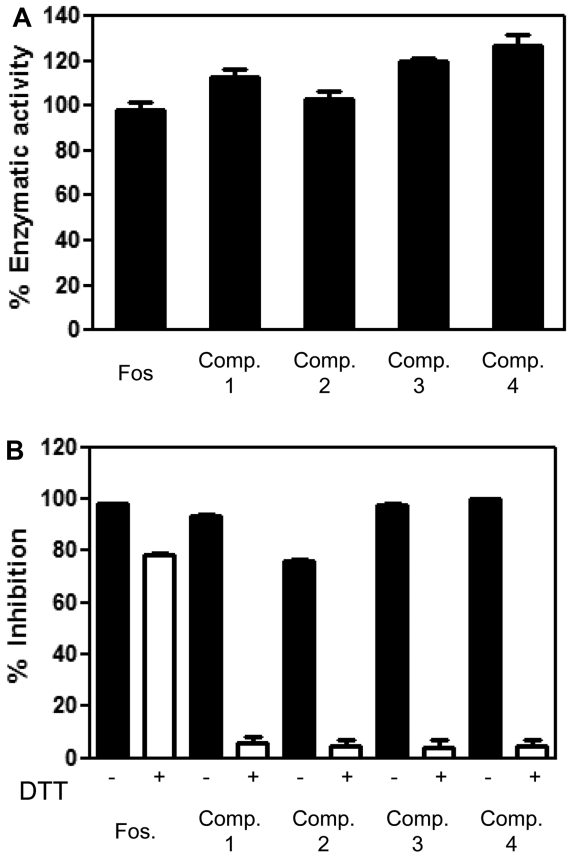

Fig. 5. Sulfhydryl group-dependent inhibitory activity of isolated compounds.
(**A**) The activity of Hi MurA(C117D) was measured in the presence of 100 μM of compound **1** and 10 μM of compounds **2–4**, and the residual activities were compared with that of untreated Hi MurA(C117D). (**B**) Inhibitory activity of fosfomycin (200 μM), compound **1** (100 μM), and compounds **2–4** (10 μM), before and after preincubation with 10 mM DTT for 1 h at room temperature, against Hi MurA was assayed.

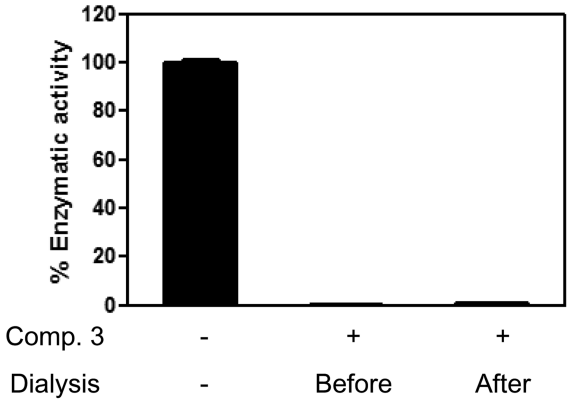

Fig. 6. Irreversible inhibition of Hi MurA by compound **3**.
Residual activities of Hi MurA, treated with 100 μM of compound **3** before and after dialysis against a 1,000-fold volume of buffer with three buffer exchanges, were compared with that of untreated Hi MurA.

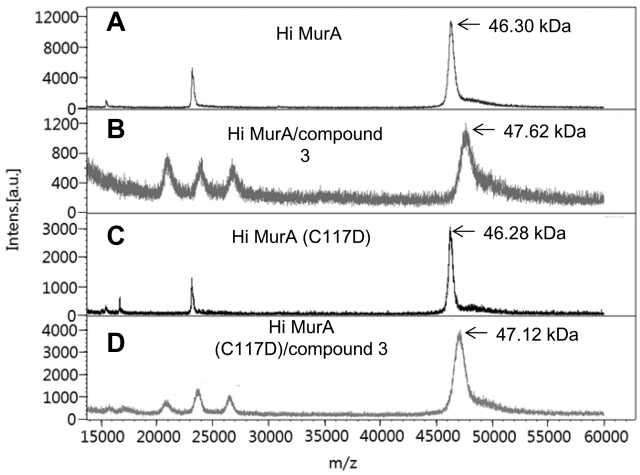

Fig. 7. Mass analysis of Hi MurA and Hi MurA(C117D) after treatment with compound **3**.
The molecular mass of Hi MurA (**A**) and Hi MurA after incubation with compound **3** (**B**) was measured using MALDI-TOF. Additionally, the molecular mass of Hi MurA(C117D) before (**C**) and after (**D**) the treatment of compound **3** was analyzed using MALDI-TOF. Mass values at the center position of molecular ion peaks are indicated.

whether covalent modification had occurred, the mass of Hi MurA was measured before and after treatment with compound **3**. The masses of wild-type and C117D mutant Hi MurA were measured to be 46.30 ± 0.25 kDa and 46.28 ± 0.25 kDa (Figs. 7A and 7C), respectively, which were similar to the calculated values (46,519 Da and 46.531 Da, respectively). The mass of Hi MurA after the treatment with compound **3** was increased 1,320 Da (Fig. 7B). Considering that the molecular weight of compound **3** is 301, approximately 4.4 molecules of compound **3** covalently attached to Hi MurA. When the mass of Hi MurA(C117D) was examined before and after treatment with compound **3**, the mass value was increased 840 Da (Fig. 7D), which corresponds to 2.8 molecules of compound **3** attached to Hi MurA(C117D). These results indicate that compound **3** can covalently attach to MurA cysteine residues, including the conserved cysteine residue in the active-site loop.

DISCUSSION

In this study, we identified novel inhibitors of Hi MurA using high-throughput screening of the Korea Chemical Bank compound library and investigated their inhibition mechanisms. Rather than screening the entire chemical library, which consists of approximately 200,000 compounds, we identified a primary hit, compound **1**, by screening a selected 1,841 compounds, and then examining compounds that share a common structural motif with compound **1**. The identified secondary hits (compounds **2**–**4**) have similar structures to compound **1**, although they have different structures than previously reported MurA inhibitors [18–20, 22]. Structure and activity comparison of the identified compounds indicated that the quinoline or naphthoquinone moiety is more effective in inhibiting Hi MurA than is the hydroxyquinone structure. Additionally, the presence of the nitrogen atom in the quinoline or naphthoquinone ring of compounds **2** and **3**, respectively, is important for inhibitory activity. The covalent addition reaction of quinones to the cysteine residue of the protein has also been reported [17, 24]. This reaction also takes place for the identified compounds. An average of 4.4 molecules of compound **3** attached to Hi MurA during the reaction. Hi MurA possesses 5 cysteines, all of which are predicted to be in the reduced form. These cysteines, including Cys117, are assumed to form covalent adducts with compound **3**.

In conclusion, we identified novel MurA inhibitors containing quinoline or naphthoquinone moieties. Further modification of these compounds to enhance the specificity toward the active-site cysteine, and measurement of antibacterial activity in a culture system are required for the development of a potent antibiotic targeting MurA.

Acknowledgments

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