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# Potent covalent inhibitors of bacterial urease identified by activity-reactivity profiling

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ABSTRACT

Covalent enzyme inhibitors constitute a highly important group of biologically active compounds, with numerous drugs available on the market. Although the discovery of inhibitors of urease, a urea hydrolyzing enzyme crucial for the survival of some human pathogens, is a field of medicinal chemistry that has grown in recent years, covalent urease inhibitors have been rarely investigated until now. Forty Michael acceptor-type compounds were screened for their inhibitory activities against bacterial urease, and several structures exhibited high potency in the nanomolar range. The correlation between chemical reactivity towards thiols and inhibitory potency indicated the most valuable compound — acetylenedicarboxylic acid, with $K_i^* = 42.5$ nM and $\log k_{GSH} = -2.14$. Molecular modelling studies revealed that acetylenedicarboxylic acid is the first example of highly effective mode of binding based on simultaneous bonding to a cysteine residue and interaction with nickel ions present in the active site. Activity-reactivity profiling of reversible covalent enzyme inhibitors is a general method for the identification of valuable drug candidates.

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Inhibitors binding covalently to the residues present in the enzyme active site constitute a large group of biologically active compounds of significant value.[1,2] Numerous covalent drugs, ranging from aspirin (discovered at the end of nineteenth century) to ibrutinib (approved by the FDA in 2013 for Mantle Cell Lymphoma treatment and in 2014–2016 for various leukemias), are available on the market.[3] Although covalent enzyme inhibitors exhibit numerous crucial advantages resulting from their strong binding to the target, they are often considered less attractive as drug candidates in comparison to non-covalent binding compounds because of drawbacks in the later stages of drug studies; in particular, specificity and toxicity issues are of great concern. These problems are often not predictable and are observed only for some patients.[4]

Michael acceptor-based enzyme inhibitors reacting with a cysteine residue in the active sites of proteins constitute one of the most widely studied groups of covalent drug candidates. Although cysteine proteases can be considered the primary target for this type of molecules,[5] inactivation of non-catalytic cysteine residues is also of interest.[6] Recently, reversibly binding covalent inhibitors have received attention because they are considered to be less prone to off-target modifications.[7–9] However, the main factor governing the nonspecific binding and related toxicity and/or side effects is the chemical reactivity of Michael acceptors towards the –SH group.[10–12]

Urease, an enzyme catalyzing the hydrolysis of urea,[13] is an interesting target for cysteine-binding compounds, although this type of urease inhibitor has been rarely explored. Urease is related to the development of infections caused by pathogenic bacteria, such as *Helicobacter pylori* (gastroduodenal tract) and *Proteus mirabilis* (urinary tract).[14,15] *H. pylori* produces large quantities of both intra- and extracellular urease to increase the pH of its microenvironment by ammonia released during enzymatic urease hydrolysis.[16,17] Some urinary tract colonizing bacteria (e.g., *Proteus mirabilis*) also produce urease, which causes rapid alkalinization of urine and precipitation of inorganic salts, forming stones (struvites and/or apatites).[18–20] The goal of combatting the above-men-

*Abbreviations:* AHA, acetohydroxamic acid; GHS, glutathione; PBMC, peripheral blood mononuclear cells; PI, propidium iodide; SRB, sulforhodamine B; Tol, tollyl.

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tioned pathogens stimulated research on urease inhibitors[21–23] and led to the discovery compounds such as phosphoramidates,[24–28] hydroxamates,[29–32] phosphinates,[33–36] and heterocyclic compounds.[37,38] Currently, only one urease inhibitor available on the market, namely, acetohydroxamic acid,[39] shows significant side effects, including teratogenicity.[40]

The active site of urease contains two nickel ions coordinated by four histidine residues (His137, His139, His249, His275), carbamoylated lysine (Kcx220) and aspartate (Asp363).[41–43] The active site is of relatively small volume (related to the size of urea) and is covered by a movable flap (Fig. S8). This flap contains a cysteine residue that could be targeted by inhibitors.[44] Currently, only a limited number of urease inhibitors possess this mode of action, including benzoquinone, ebselen derivatives, cyclohexanone and cyclopentenone.[45–50] The product of reaction of urease and benzoquinone has been recently characterized structurally.[51]

In this paper, we aimed to establish a general methodology to evaluate the activity-reactivity profile of reversible Michael acceptor-type inhibitors based on studies of urease inhibitors. The various types of Michael acceptors were extensively screened for their inhibitory properties against bacterial urease.

The measured inhibitory activities were correlated with the chemical reactivity (towards a model compound, glutathione) of the studied compounds to find molecules that exhibit favorable profiles related to specific interactions with the enzyme. Moreover, the cytotoxicity (towards mouse fibroblasts BALB/3T3) of the chosen compounds was analyzed. The most important findings were also rationalized by modelling the structures of the inhibitor-enzyme complexes.

A group of forty compounds of non-extended, Michael acceptor-type compounds was selected for screening. These compounds include molecules containing unsaturated functional groups of various geometries: E and Z isomers of substituted double bonds and linear triple bonds or allenes. All groups controlling the chemical reactivity of double/triple bonds contained carbonyl groups but showed significant differences in activating potency. Ketone and ester functional groups are considered to activate strongly, whereas carboxylates are much less reactive. Moreover, analogues of known inhibitors, cyclopentenone (**10a**) and cyclohexanone (**9a**), were also included in the study for comparison.

All compounds were tested against model bacterial urease purified from *Sporosarcina pasteurii* CCM 2056$^{TM}$ with specific activity of 2451 U/mg. The values of the kinetic parameters ($K_M = 4.92 \pm 0.31$ mM and $v_{max} = 4.224 \pm 0.060$ μM $s^{-1}$) of highly purified urease in an enzymatic reaction were determined by fitting the initial reaction velocities measured over a range of urea concentrations to the Michaelis-Menten equation by nonlinear regression. The majority of the assayed compounds exhibited inhibitory activity in the micromolar or nanomolar range (Table 1). The nonlinear progress curves indicated that Michael acceptors produced time-dependent inhibition of urease activity, in which steady-state velocity ($v_s$) was attained slowly with both the initial ($v_i$) and steady-state velocity ($v_s$) decreasing with an increase in inhibitor concentration over the examined ranges. The linear replots of $1/v_i$ and $1/v_s$ showed a slow binding mode of action according to mechanism B, in which the initial *EI* complex undergoes conformational change into the final $EI^*$ complex. The steady state inhibition constants ($K^*_{iLB}$) ranged from 0.00977 to 64.2 μM for compounds **8a** and **3e**, respectively. Importantly, the potency of some of the assayed compounds (**5**, **6a**, **8a**, **8b**, **8c**, **9a**, **9b**, **9c**, **9f**, **10a** and **10b**) exhibited higher activities than the reference inhibitor, acetohydroxamic acid (AHA). All inhibitors demonstrated competitive and reversible type inhibition, as confirmed by Lineweaver-Burk plots and fast dilution assay (data not shown). This mode of action of the studied inhibitors is consistent with the design assumption concerning the reaction with the cysteine residue present at the entrance to the active site. Most probably, the presence of neighboring histidine residue (His323), which could act as a base, makes the covalent modifications of cysteine residue reversible.

There are several interesting structure-activity relationships visible in the study. First, the geometry of the central unsaturated bond has a significant influence on the activity. E isomers of substituted double bond- and triple bond-containing compounds are capable of urease inhibition, whereas Z isomers are completely inactive against the target enzyme (e.g., **3a** and **8a** *versus* **4b** or **3h** and **8c** *versus* **4a**).

Second, carboxylic acids showed similar, or in some cases even higher, activity than analogous alkyl esters (e.g., **1a** *versus* **1b**, **3a** *versus* **3h**, **8a** *versus* **8c**). This finding is of primary importance because carboxylic acid-containing Michael acceptors are generally much less chemically reactive towards –SH groups than analogous esters. Therefore, the high activity of acids is related to their specific interactions with the enzyme (see the following section).

Third, in most cases, the substituents of phenyl groups or changes in ester functional groups have minor influence on the activity (e.g., **1b** *versus* **1d** *versus* **1e** or **3a-3f**). This observation suggests that these groups do not influence inhibitor binding.

Any compound showing high chemical reactivity with functional groups available in biological systems, in particular –SH groups, has to be avoided in medicinal chemistry due to unpredictable influence on other biomolecules. Unspecific toxicity is correlated with high reactivity of Michael acceptors.[10] Therefore, the preferred profile of the Michael acceptor-based active compound is based on a combination of high inhibitory activity and low reactivity towards thiols. The reactivity of representative compounds was assayed using glutathione (GSH) as a model compound with reactive cysteine residues. The modified Ellman method, where the quantification of unreacted GSH was performed using a spectrophotometric-based concentration-response assay, was applied.[52] The reactions of the extremely reactive Michael acceptor compounds (**3a**, **8a**) with GSH are completed within less than 15 min, whereas compound **1b** demonstrates no reactivity, even after 36 h of reaction (examples of progress curves and $^1$H NMR spectra of reaction mixtures are shown in Figs. S3–S5, respectively). The tested compounds spanned nearly six orders of magnitude of reactivity, with $\log k_{GSH}$ values between $-3.138 \pm 0.077$ and $2.588 \pm 0.088$ for ethyl cinnamonate (**1a**) and dimethyl acetylenedicarboxylate (**8a**), respectively (Table S4). The dependence between $pK_i$ and $\log k_{GSH}$, shown graphically in Fig. 1, allows for classification of the studied compounds into three groups. Compounds from the first group show roughly linear dependence between activity and reactivity (**1a**, **8a**, **9a**, **9c**, **10a**, **10b**), which suggests their nonspecific interaction with the enzyme. The second group contains structures with high chemical reactivity and moderate inhibitory activity (**2a**, **2b**, **3a**). In this case, the interactions of the compound with the enzyme are specific but unfavorable due to steric impairment. The third group is of the highest importance because it combines compounds with a preferable profile: high inhibitory potential and low –SH reactivity (**1b**, **3h**, **8c**). In these cases, inhibition of the enzyme is driven not only by –SH reactivity but also specific interactions between the ligand and protein. The specificity of compounds **1b**, **3h**, **8c** was additionally confirmed by complete lack of inhibition of model cysteine-dependent enzyme – papain. On the basis of the discussed graph, compounds of interest are easily indicated; in this particular case, acetylenedicarboxylic acid (**8c**) with $K^*_{iLB} = 42.5$ nM and $\log k_{GSH} = -2.14$ has the most beneficial activity-reactivity profile.

The cytotoxicity towards normal mouse fibroblasts (BALB/3T3) for a group of selected Michael acceptor compounds was

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