

1 **High-throughput tandem-microwell assay for ammonia repositions**

2 **FDA-Approved drugs to *Helicobacter pylori* infection**

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24 Running title: Repositioning of old drugs to treat *H. pylori* infection

25

26 **ABSTRACT**

27 To date, little attempt has been made to develop new treatments for *Helicobacter*
28 *pylori* (*H. pylori*), although the community is aware of the shortage of treatments for *H.*
29 *pylori*. In this study, we developed a 192-tandem-microwell-based high-throughput-assay
30 for ammonia that is a known virulence factor of *H. pylori* and a product of urease. We
31 could identify few drugs, i.e. panobinostat, dacinostat, ebselen, captan and disulfiram, to
32 potently inhibit the activity of ureases from bacterial or plant species. These inhibitors
33 suppress the activity of urease via substrate-competitive or covalent-allosteric mechanism,
34 but all except captan prevent the antibiotic-resistant *H. pylori* strain from infecting human
35 gastric cells, with a more pronounced effect than acetohydroxamic acid, a well-known
36 urease inhibitor and clinically used drug for the treatment of bacterial infection. This
37 study offers several bases for the development of new treatments for urease-containing
38 pathogens and to study the mechanism responsible for the regulation of urease activity.

39

40 **Key Words:** Ammonia, High-throughput screening, Antibiotic resistance, Enzyme
41 inhibitor, Urease, Mechanism of action, *Helicobacter pylori*

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43

44 INTRODUCTION

45 Bacteria, fungi and plants, with the exception of animals, contain urease(1). Urease (EC
46 3.5.1.5) is a class of nickel metalloenzyme that hydrolyzes amino acid metabolites to
47 produce ammonia (NH₃) and carbon dioxide(2,3). The active catalytic site of urease
48 consists of two nickel ions, a carbamylated lysine residue, two histidines and an aspartic
49 acid. In addition to the consistent catalytic mechanism, the amino acid sequence of urease
50 has been reported to be highly conserved between different species(4).

51 Bacterial urease is known to be a key virulence factor of some pathogens for a number of
52 diseases(5), e.g., *Helicobacter pylori* (*H. pylori*) for gastritis or gastric cancer, and
53 *Proteus mirabilis* (*P. mirabilis*) for urinary tract infections and urinary stones(6). The
54 pathogens can hydrolyze urea substrates to produce NH₃. The released NH₃ not only
55 helps *H. pylori* to survive in the low pH environment of the stomach but also causes
56 damage to the gastric mucosa, triggering the infection(7). Additionally, NH₃ generated by
57 *P. mirabilis* urease has been demonstrated to form urinary stones and destroy the urinary
58 epithelium in the urinary system(8). Because the human body does not contain urease,
59 bacterial urease has been thought to be an important and specific drug target for
60 combating these pathogens(9).

61 A number of studies have been performed to identify inhibitors of urease(10-12), but only
62 one urease inhibitor, acetohydroxamic acid (AHA), was approved for the treatment of
63 urinary infections and urinary stones in 1983 by the US Food and Drug Administration
64 (FDA)(13,14). Severe side effects, low stability in gastric juice, and a lack of direct

65 evidence for suppressing the growth of pathogens seem to be the limiting factors for the
66 low success rate of these urease inhibitors. Adverse side effects of AHA, including
67 teratogenic effects(15), a low efficiency indicated by the required high dose for the
68 patient (~ 1000 mg/day for adults), and the assumed drug resistance of bacteria, further
69 imply that potent and bioactive inhibitors with new chemical moieties are urgently
70 needed to combat these pathogens. Indeed, the current clinical first-line regimen for the
71 treatment of *H. pylori* [proton-pump inhibitor, clarithromycin, amoxicillin or
72 metronidazole (sometimes tinidazole)](16,17), is unable to completely eradicate *H. pylori*
73 due to the increased antibiotic resistance(16,18).

74 To date, few validated high-throughput assay has been constructed to quantitatively
75 analyze NH₃ and the activity of NH₃-generating enzyme urease, but no high-throughput
76 screening approach has been employed to systematically extend the chemical moiety of
77 urease inhibitors. The current assay to determine the activity of urease mainly relies on
78 colorimetric reactions to determine the concentration of NH₃ using indophenol or
79 Nessler's reaction(19). Recently, a microfluidic chip-based fluorometric assay has been
80 developed to monitor the activity of urease(20,21). In addition, a cell-based assay for *H.*
81 *pylori* urease has been reported lately, and validated by known inhibitors of urease, but it
82 has not been employed to screen new inhibitors for urease yet(22). Overall, the current
83 assay setting and procedures are relatively time-consuming and vulnerable to
84 interference.

85 In this study, we established and validated a new tandem-well-based HTS assay for NH₃

86 and NH₃-generating urease and performed an HTS screening campaign to identify
87 druggable chemical entities from 3,904 FDA or Foreign Approved Drugs (FAD)
88 -approved drugs for jack bean and bacterial ureases. Five clinically used drugs, i.e.,
89 panobinostat, dacinostat, ebselen (EBS), captan and disulfiram, were found to be
90 submicromolar inhibitors of *H. pylori* urease (HPU), jack bean urease (JBU), or urease
91 from *Ochrobactrum anthropi* (*O. anthropi*), a newly identified pathogen with resistance
92 to β-lactam antibiotics(23). Moreover, panobinostat, dacinostat, EBS and disulfiram
93 potently inhibited the infection of *H. pylori*, suggesting that these pharmacologically
94 active moieties or drugs could serve as bases for the development of new treatments for
95 urease-positive pathogens.

96 **RESULTS**

97 **Development of a high-throughput assay and identification of potent inhibitors for** 98 **urease**

99 To construct a high-throughput assay for NH₃-generating urease and prevent the detection
100 interference from substances in the enzyme extraction, we utilized a
101 192-tandem-well-based gas-detection method, which we previously developed to monitor
102 the activity of H₂S-generating enzymes(24,25). The tandem-well design could physically
103 separate the gas product from the enzymatic reaction and enable the specific and
104 real-time detection of the gas-producing enzyme activity (Figure 1A).

105 To construct the HTS assay, we compared three reported protocols for determination of
106 the activity of JBU by using salicylic acid-hypochlorite and Nessler detection reagent, as
107 well as phenol red(20,26,27), which undergo the indophenol and Nessler's reaction with
108 NH₃, respectively. Salicylic acid-hypochlorite and Nessler's reagents could
109 dose-dependently and time-dependently monitor the activity of JBU at various
110 concentrations (Figures S1A and B); however, the phenol red failed to detect it (Figure
111 S1C). We decided to choose salicylic acid-hypochlorite as the detection reagent for the
112 HTS screening assay of JBU (Figure S1A) due to its lower toxicity than Nessler reagent,
113 which contains mercury(26). The absorbance (OD) at 697 nm of the blue complex
114 indophenol generated from salicylic acid was correlated linearly with the concentration of
115 NH₄Cl (19.5 - 625 μM), thus validating the analytic setup for NH₃ quantification (Figure
116 S1D). Moreover, the optimal assay buffer for JBU was found to be phosphate buffer at

117 pH 7.4 (Figure S1E). In contrast, we employed Nessler's reagent to detect the activity of
118 HPU and *Ochrobactrum anthropic* urease (OAU) in subsequent studies since it showed a
119 better sensitivity for the limitation of detection of the activity of HPU than salicylic
120 acid-hypochlorite (Figures S1F and 1G). Collectively, we chose 50 nM of JBU and 25
121 mM urea substrate in the phosphate buffer to perform the assay.

122 Under the assay conditions, AHA showed an IC_{50} of $\sim 160 \mu\text{M}$ (Figure 1B), which was
123 very similar to the previously reported value (IC_{50} of $\sim 140 \mu\text{M}$; ref. (13)), indicating that
124 the newly developed assay for urease was accurate and reliable. However, the IC_{50} of
125 AHA was found to decrease to $33.7 \mu\text{M}$ when using the 50 mM Tris buffer instead of the
126 phosphate buffer in our assay (Table 1). To determine the well-to-well reproducibility, the
127 assay was validated with $200 \mu\text{M}$ AHA ($\sim IC_{50}$) or $800 \mu\text{M}$ (~ 5 -fold IC_{50}) AHA. The
128 tandem-well plate consistently showed distinct differences among the control, the 200
129 μM -AHA-treated and the 800 μM -AHA-treated groups (Figure 1C). The average Z'
130 values of the assay were found to be ~ 0.9 when they were calculated with the 800 μM
131 AHA positive control.

132 To identify novel and potent inhibitors for urease, we screened 3,904 FDA or FAD
133 -approved drugs at $100 \mu\text{M}$. Five potent hits, i.e., panobinostat, dacinostat, EBS, captan
134 and disulfiram, were found to dose-dependently inhibit the activity of JBU with IC_{50}
135 values of 0.2, 1.1, 0.4, 2.3 and $38.9 \mu\text{M}$, which are ~ 800 , 146, 400, 70, 4, -fold more
136 potent than AHA, respectively (Figure 1E and Table 1). Intriguingly, the former two
137 drugs are analogs of AHA. Importantly, all of them seemed to bear significant

138 selectivities for urease since they did not substantially inhibit other gas-producing
139 enzymes, i.e., cystathionine beta-synthase (CBS) and cystathionine γ -lyase (CSE), two
140 H₂S-generating enzymes (Figure 1F). Moreover, the potent inhibitory effects of these
141 inhibitors were likely due to on-target inhibition of JBU rather than the nonspecific
142 reaction with NH₃ or forming an aggregation since they did not react with NH₃ and their
143 inhibition was not attenuated by the detergent (Figures S2A and S2B). In corroborating
144 these findings, EBS and disulfiram have recently been reported to be specific inhibitors
145 of bacterial and plant urease(11,12), respectively, although their mode of actions for
146 inhibiting urease, and their effects on the proliferation or infection of urease-containing
147 pathogens remain little explored.

148

149 **The mode of action study for urease inhibitors**

150 To determine the reversibility of the inhibition by panobinostat, dacinostat, EBS, captan
151 and disulfiram to JBU, various concentrations of the inhibitors and JBU were incubated
152 together for 60 min (Figure 2A). After a 200-fold dilution, the inhibitory effects of
153 panobinostat and dacinostat as well as disulfiram were found to be reversible (Figures 2A
154 and S3C). In contrast, EBS or captan at 100 nM was found to completely block the
155 activity of JBU; this concentration did not affect the activity without the pre-incubation
156 with enzyme (Figure 1E). Additionally, the inhibitions exerted by EBS or captan were not
157 fully recovered (Figure 2A), indicating that both of them were likely to be covalent or
158 slow-dissociation inhibitors for JBU.

159 Surprisingly, the inhibitory effect of disulfiram was found to be dependent on the
160 concentrations of Ni^{2+} ion, the catalytic cofactor for urease (Figure S3C), indicating that
161 it inhibits JBU likely via formation of a complex with the catalytic Ni^{2+} ion and
162 subsequently occupying the active site of JBU. This explanation seems to be plausible
163 since recent findings have revealed that disulfiram inhibits the proliferation of tumor cells
164 by forming a complex with Cu^{2+} (28).

165 Moreover, the inhibitory potencies of panobinostat and dacinostat were found to increase
166 with the pre-incubation time of the compound with urease (Figure 2B). After 2 h
167 pre-incubation, the IC_{50} value of panobinostat and dacinostat were decreased ~ 7.5 folds
168 and ~ 18.8 folds, respectively (Figure 2B). In enzyme kinetics studies for JBU,
169 panobinostat and dacinostat were found to be competitive inhibitors towards urea
170 substrate, with a K_i value of 0.02 and 0.07 μM (Figure 2C and Table 1), which are ~ 105
171 folds and 30 folds more potent than AHA ($K_i \sim 2.1 \mu\text{M}$; Table 1). In consistent with this
172 observation, the inhibition of these two inhibitors doesn't be interfered with Ni^{2+} (Figure
173 S3A). Also, the addition of histidine or cysteine has no effects on the inhibition of
174 panobinostat or dacinostat (Figure S3B). Importantly, the surface plasmon resonance
175 assay demonstrate that these two compounds could physically bind to JBU (Figure 2D;
176 Table 1). The drastic effect seems not only relying on the hydroxamic acid motif that is
177 the known pharmacophore of AHA-derivative inhibitors, but also the hydrophobic ring
178 and secondary amine group, as indicated by that the benzene ring favorably interacts with
179 the His492 residue and/or the nitrogen atom forms an additional hydrogen bond with

180 Asp494 in the modeled inhibitor-JBU complex structure (Figure 2E).

181 In contrast, the inhibition caused by both EBS and captan was found to be prevented by

182 the addition of dithiothreitol (DTT) or free cysteine into the enzymatic reaction, but not

183 that of histidine or Ni^{2+} (Figures S4A-C). Furthermore, the IC_{50} values of the two

184 inhibitors were linear with the concentrations of the enzyme (Figure S4D), an inhibitory

185 feature of the covalent inhibitor(29), confirming that they targeted the enzyme covalently.

186 The inhibition constants for these irreversible inhibitors, i.e., the rate of enzyme

187 inactivation (k_{inact}) and inactivation rate constants (K_{I}), were also determined by

188 nonlinear regression of the time-dependent IC_{50} values (Figure S4E)(29). The k_{inact} and K_{I}

189 for EBS were found to be $2.79 \times 10^{-3} \text{ s}^{-1}$ and $0.73 \mu\text{M}$, which were 4.4 and 2.4-fold better

190 than captan (k_{inact} , $0.63 \times 10^{-3} \text{ s}^{-1}$; K_{I} , $1.76 \mu\text{M}$), respectively. Taken together, the results

191 demonstrated that EBS and captan inhibited JBU by covalently modifying the Cys rather

192 than His residue, the latter of which is known to be the active site of urease (2,3).

193 Interestingly, we observed a synergistic inhibitory effect from the combination of EBS

194 and AHA (Figure 3A), a substrate-competitive inhibitor for urease, implying that EBS

195 targeted Cys residue(s) of another site rather than the active site. Similar experimental

196 results were also obtained for captan. Moreover, the combination of EBS with $2 \mu\text{M}$

197 captan also significantly increased the potency of EBS by 6-fold (right panel, Figure 3A),

198 implying distinct binding sites of the two covalent inhibitors.

199 To corroborate this finding, we performed enzyme kinetics, mass spectrometry and

200 surface plasmon resonance studies (Figures 3B-D). Consistently, EBS or captan displayed

201 a noncompetitive mode for the urea substrate (Figure 3B). Furthermore, tandem-mass
202 spectrometry analysis revealed that Cys313 and Cys406, which were not adjacent to the
203 active site, appeared to be modified by EBS and captan, respectively (Figure 3C). The
204 addition of 274.18 daltons in molecular weight was observed for EBS, demonstrating the
205 breakage of the Se-N bond and formation of the Se-S bond with the Cys residue, a
206 phenomenon that has been reported previously for EBS(30). However, the increase of
207 150.15 daltons suggested that only the isoindole dione moiety of captan modified the Cys
208 residue, accompanied by the release of the trichloromethyl thio moiety [-SC(C1)₃]. This
209 new observation provides a new perspective for the unexplored covalent molecular
210 mechanism of captan.

211 Additionally, a potent and physical interaction between EBS or captan and JBU was
212 observed in the surface plasmon resonance study (Figure 3D). The equilibrium
213 dissociation constant (K_D) for EBS and captan was found to be 89 and 96 nM,
214 respectively.

215 To illustrate the binding mode of EBS or captan, we modeled them into the respective
216 allosteric Cys-containing pocket (Cys313 for EBS, Cys406 for Captan) in JBU by using
217 molecular dynamics simulations (Figure 3E). The carbonyl group of EBS was found to
218 form a hydrogen bond with Lys369, and the phenyl ring interacts with the hydrophobic
219 side chain of Leu308. Additionally, the two carbonyl groups of captan formed four
220 hydrogen bonds with the side chains of Asn517, His542, Tyr544 and Asn688. Taken
221 together, these results implied that these intermolecular weak interactions also

222 substantially contributed to the binding of the covalent inhibitors to the protein, in
223 addition to the covalent interaction.

224 **The inhibitory effect of inhibitors on bacterial ureases**

225 Next, we investigated the effects of panobinostat, dacinostat, EBS and captan as well as
226 disulfiram on the activity of HPU and OAU, two bacterial ureases from *H. pylori* and *O.*
227 *anthropic*, respectively. As expected, these drugs could inhibit the activity of HPU in the
228 crude extracts and showed IC₅₀ values of 0.1 μM, 0.2 μM, 2.8 μM, 3.4 and 8.9 μM,
229 which indicated that they were ~ 259, 130, 10, 8 and 3 -fold more potent than AHA (IC₅₀
230 ~ 25.9 μM; Figure 4A and Table 1), respectively. Moreover, panobinostat, dacinostat,
231 EBS, captan and disulfiram were also found to inhibit the partially purified HPU, which
232 was isolated by size-exclusion chromatography (Figures 4B and S5). Consistently, they
233 also suppressed the activity of OAU at a similar potency to HPU (Figure 4A and Table 1).
234 Compounds **1**, **4** and **6**, which were synthesized in house (Scheme S1), as well as
235 commercially available EBS oxide, also showed a better efficiency than EBS (IC₅₀ ~ 2.8
236 μM) in the *in vitro* HPU-based enzyme assay (Table S1), and **4** displayed a maximum
237 three-fold increase in potency (IC₅₀ ~ 1.1 μM; Table S1). Moreover, we could confirm
238 that panobinostat, dacinostat and EBS as well as EBS oxide, **1**, **4** or **6**, could largely
239 suppress the activity of HPU in culture (Figure 4C). The IC₅₀ values of these inhibitors
240 for inhibiting the urease of the cultured *H. pylori* strain ranged from 5.7 to 23.2 μM
241 (Figure 4C and Table S2).

242 Further, we investigated the effects of panobinostat, dacinostat and EBS, which are the

243 most potent inhibitors for HPU (Figure 4A). The results showed that EBS, but not
244 panobinostat, dacinostat or its analog AHA, has a substantial suppression on the growth
245 of *H. pylori* (Figure 4D). The inability of AHA as well as its derivatives, i.e. panobinostat
246 and dacinostat, on the growth of *H. pylori* as identified above seems to be consistent with
247 the previous finding that AHA doesn't inhibit the growth of *H. pylori*(31). Interestingly,
248 EBS and EBS analogs, as well as disulfiram, could dose-dependently suppress the growth
249 of *H. pylori* and showed a minimum inhibitory concentration (MIC) in a range between 2
250 and 4 µg/ml (right panel of Figure 4D, Figure S6A and Table S2). Importantly, the
251 inhibitory effect of this type of covalent inhibitors lasted for a long period in culture, as
252 indicated by EBS and **1**, which could substantially inhibit HPU even after removal of the
253 inhibitor for 6 h (Figure S6B).

254 **Urease inhibitors prevent *H. pylori* infection in a gastric cell-based bacterial** 255 **infection model**

256 To evaluate the ability of these urease inhibitors to prevent *H. pylori* infection, we
257 constructed a gastric cell-based bacterial infection model using the remaining viable cell
258 number of SGC-7901 adenocarcinoma gastric cells to reflect the virulence of *H.*
259 *pylori*(15). Our results showed that treatment with 30 µM panobinostat, 30 µM dacinostat,
260 20 µM EBS or 20 µM disulfiram could prevent the cell death triggered by *H. pylori*
261 (Figures 5A-B). In sharp contrast, the cells that lacked such treatments were largely
262 sabotaged. Panobinostat and EBS were found to be the most potent agents and almost
263 completely protected from the infection of *H. pylori*. These effects of these drugs seemed

264 to be much more efficient than the effects of 20 μ M AHA or 50 μ M tinidazole, the analog
265 of metronidazole, and one of the two antibiotics in the triple regimens for the treatment of
266 *H. pylori* (16,17). In support of this observation, tinidazole as well as metronidazole
267 hardly suppressed the growth of our *H. pylori* strain, with an MIC value of more than 512
268 μ g/ml in culture (Figure S7A and Table S2), indicating that this strain is resistant to
269 treatment with nitroimidazole-type antibiotics.

270 Since panobinostat, dacinostat, EBS and disulfiram at a concentration up to 100 μ M or 25
271 μ M did not interfere with the proliferation of SGC-7901 gastric cells (Figure S7B), the
272 protective effects in the gastric-cell-based *H. pylori* infection model seemed to be
273 attributed to on-targeting inhibition of the infection transmitted by *H. pylori*. Moreover,
274 all four drugs potentially inhibited the level of ammonia in the cell medium (Figure 5C),
275 indicating that they efficiently suppressed the endogenous urease activity of *H. pylori* in
276 the infection model.

277

278 **The structural basis and inhibitory mechanisms of newly-identified three classes** 279 **urease inhibitors**

280 To identify the active chemical moiety of panobinostat, dacinostat, EBS or captan
281 required for inhibition of urease, we analyzed their structure-activity relationships
282 (Figures 6, Table S1 and S3). The former two inhibitors are hydroxamic acid-based
283 urease inhibitors, and not only their hydroxyamino heads are forming hydrogen bonds
284 with the catalytic Ni²⁺ and residues in JBU or HPU (Asp633 or Ala636 for JBU; Asp362

285 or Ala365 for HPU), but also the acetyl group constitutes one hydrogen bond (His492 for
286 JBU and His221 for HPU; Figures 2E and S8A). Consistent with this observation, the
287 hydroxyamino and acetyl groups of AHA interact with Asp362 or Ala365 and His221 in a
288 co-crystal structure of AHA and HPU(2), respectively (Figure S8A). Compound lacking
289 of this acetyl group, i.e. hydroxylamine, totally abolished the inhibitory effect of this type
290 inhibitor (Figure 6B and Table S3). Apart from these interactions, the hydrophobic
291 benzene ring and secondary amine group of panobinostat were found to be additional
292 pharmacophores (upper panel, Figure 2E), which interact favorably with His492 (JBU) or
293 His221 (HPU) and form an extra hydrogen bond with Asp494 (JBU) or Asp223 (HPU).
294 In supporting this finding, the hydroxamic acid analogs that are lack of the benzene ring,
295 i.e. ricolinostat, ilomastat and pracinostat, are inactive to JBU and HPU (Figure 6B and
296 Table S3). Strikingly, the replacement of benzene with benzimidazole (pracinostat) totally
297 loses the inhibition, suggesting the benzene is critical for maintaining the inhibition.
298 Moreover, the secondary amine group seems to be also important for enhancing the
299 potency of this type inhibitor, since the modification or replacement of it with hydroxyl
300 group or sulfonyl group (dacinostat or belinostat), also weaken ~ 5-fold or 24-fold in IC₅₀
301 values.

302 For EBS analogs, compounds (**2-3**) lacking the Se atom largely lost inhibitory activities
303 toward JBU and HPU (Figure 6B and Table S1). Furthermore, dibenzyl diselenide was
304 also inactive toward both ureases, indicating that the Se-containing benzisoxazole moiety
305 rather than the solo Se atom might be essential for the inhibition. Indeed, Se-containing

306 benzisoxazole (**4**) showed potent inhibition of HPU ($IC_{50} \sim 0.8$ and $1.1 \mu\text{M}$ for JBU and
307 HPU, respectively). The introduction of an electron-donating group to the benzisoxazole
308 moiety apparently strongly reduced the potency (**5**; $IC_{50} \sim 1.4 \mu\text{M}$ for JBU and more than
309 $10 \mu\text{M}$ for HPU; Figure 6B). In contrast, the provision of electron-withdrawing groups to
310 the nitrogen or Se atom of the benzisoxazole moiety, i.e., **6** or EBS oxide, seemed to
311 enhance the potency of JBU by a maximum of three-fold (**6**). Similarly, when weakening
312 the electron-withdrawing effect in the substitution group of the isoindole dione core of
313 captan, the active moiety (Figure 3C), was also found to lead to a decreased potency
314 (Figures 6; Table S1). Taken together, these data indicate that the Se-containing
315 benzisoxazole or the isoindole dione moiety played crucial roles in the potency of these
316 kinds of inhibitors, the Se or N atom of which was subjected to nucleophilic attack by the
317 thiol group of Cys and formed the Se-S or N-S bond.

318

319 **DISCUSSION**

320 In the present study, we could identify that four clinical-used drugs, i.e., panobinostat,
321 dacinostat, EBS and disulfiram, two anti-cancer drugs, an anti -stroke or -bipolar drugs,
322 and an alcohol-deterrent drug, respectively, could protect the gastric cells from the
323 infection at submicromolar concentrations (Table 1 and Figure 5). The efficacy of these
324 drugs substantially exceeded that of AHA, a well-known urease inhibitor and clinically
325 used drug for bacterial infections. They seemed also to be more effective than tinidazole,
326 a metronidazole type antibiotic in the classic triple recipe for *H. pylori* (Figures 5).
327 Moreover, panobinostat, EBS and disulfiram have been administered to humans and do
328 not incur severe side effects(28,32,33). Additionally, these drugs did not affect the
329 viability of mammalian cells at a concentration up to 100 μ M or 25 μ M (Figure S7B),
330 suggesting that they had a rather safe profile in cells and *in vivo*. Taken together, our
331 study armed with the newly-developed HTS assay for urease repositions four clinically
332 used drugs as new advanced leads for the treatment of *H. pylori* infection.

333 The mode of action of panobinostat, dacinostat, EBS or disulfiram was found to inhibit *H.*
334 *pylori* urease and reduce the production of NH_3 in culture (Table 1; Figures S6A, 4B, 4C
335 and 5C), which are well-known bacterial virulence factors(15). Panobinostat and
336 dacinostat are reversible hydroxamic acid-type inhibitors for urease, and displayed more
337 than 250 or 130 -fold potencies than its analog AHA (Table 1). These largely improved
338 inhibitors indeed enhanced the protective effects to the infection of *H. pylori* in the
339 cell-based infection model (Figures 5A and 5C), demonstrating that pharmacologically

340 targeting urease could offer an effective treatment for *H. pylori* and HPU is a validated
341 pharmacological drug target. However, suppression of the urease activity with these
342 potent inhibitors of HPU, could not retard the growth of *H. pylori* in culture, indicating
343 that urease is not crucial for bacterial growths.

344 Moreover, EBS was found to irreversibly inhibit urease by covalently modifying an
345 allosteric Cys residue outside of the active site (Figures 2A and 3). The newly identified
346 covalently allosteric regulation of the activity and stability of urease by EBS and captan
347 may explain why these inhibitors could potently and persistently inhibit urease activity
348 and the growth of *H. pylori* even in the presence of high concentrations of urea substrate
349 (Figure S6B), two merits that are observed for covalent allosteric drugs(34). Indeed,
350 when compared with the reversible inhibitor AHA, EBS displayed an ~ 400 and 10-fold
351 improved potency for JBU and HPU, respectively, and a long-acting inhibitory effect on
352 the endogenous activity of urease and the growth and infection of *H. pylori* in culture
353 (Figures 4C-D, 5B-C and S6B). Importantly, the anti-*H. pylori* MIC value of EBS and its
354 analogs, i.e. EBS oxide, **1**, **4**, **6**, seems to be much effective or at least comparable to
355 metronidazole or clarithromycin, which are the two antibiotics in the classic triple recipe
356 for *H. pylori* (Table S1)(35), indicating these newly-validated chemical moieties for
357 inhibiting the growth of *H. pylori* are promising antibiotics for developing new
358 treatments for urease-containing pathogens. Since the urease activity is dispensable for
359 the growth of *H. pylori* (see our discussions with the mode of action of panobinostat and
360 dacinostat), this finding indicates the effect of EBS-type inhibitor on the growth of *H.*

361 *pylori* is beyond the solo inhibition of urease activity.

362 In summary, we identified five clinical drugs as submicromolar inhibitors for plant or
363 bacterial urease by performing the first HTS campaign of urease. These clinically used
364 drugs panobinostat, dacinostat, EBS and disulfiram inhibit the virulence of *H. pylori* in a
365 gastric-cell-based infection model. This study provides a new HTS assay, drug leads and
366 a regulatory mechanism to develop bioactive urease inhibitors for the treatment of *H.*
367 *pylori* infection, especially antibiotic-resistant strains.

368

369 **EXPERIMENTAL PROCEDURES**

370 **Materials**

371 Jack bean urease (JBU), DMSO, and dithiothreitol (DTT) were purchased from Sigma
372 (Steinheim, Germany). Hypochlorous acid, sodium nitroprusside, salicylate, potassium
373 sodium tartrate, urea, sodium hydroxide, bovine serum albumin, Triton X-100,
374 L-histidine and L-cysteine were purchased from Sangon (Shanghai, China). Nessler's
375 reagent was purchased from Jiumu company (Tianjin, China). Acetohydroxamic acid was
376 purchased from Medchemexpress (Monmouth Junction, NJ). Columbia blood agar plate,
377 liquid medium powder for *H. pylori*, bacteriostatic agent and polymyxin B were
378 purchased from Hopebio company (Shandong, China). RPMI 1640 medium and fetal
379 bovine serum (FBS) were purchased from Gibco (Invitrogen, Gaithersburg, MD). The
380 other materials were purchased from the indicated commercial sources or were from
381 Sigma.

382 **Construction of the high-throughput screening assay for urease**

383 The assay was constructed to measure the activity of urease based on a 192-tandem
384 microwell plate, which we had previously developed to detect the H₂S gas generated by
385 H₂S-generating enzymes(24,25). Phosphate or Tris buffer at various pH values were used
386 to determine the optimal pH for JBU in the presence of 25 mM urea substrate (Figure
387 S1E). The optimal conditions were found to be the 50 mM phosphate buffer and pH 7.4.
388 Moreover, the suitable detection reagent and enzyme concentrations were resolved by
389 testing three types of NH₃ detection reagents with various concentrations of JBU or HPU,

390 i.e., salicylic acid-hypochlorite, Nessler's reagent and phenol red detection reagent
391 (Figures S1A-C). The optimized conditions for the standard assay were found to be with
392 salicylic acid-hypochlorite and commercial Nessler's detection reagents (Jiumu, Tianjin,
393 China) for JBU and HPU, respectively, in the presence of 50 nM JBU or 200-400 nM
394 HPU, 25 mM urea, 100 μ M NiCl₂, and 50 mM phosphate buffer (final concentrations of
395 pH 7.4). The salicylic acid-hypochlorite detection reagent contained 1.6 mM hypochlorite,
396 400 mM sodium hydroxide, 36 mM salicylic acid, 18 mM potassium sodium tartrate and
397 1.6 mM sodium nitroprusside. The assay was performed using multichannel pipettes to
398 add 1 μ l of each compound (solubilized in DMSO or H₂O) and 24 μ l of the enzyme mix
399 (100 nM, 100 μ M Tris, pH 7.9) into the reaction well (Figure 1A), followed by a 30-min
400 incubation. After addition of 50 μ l of salicylic acid-hypochlorite or Nessler's detection
401 reagent to the detection well, 25 μ l substrate solution (50 mM urea, 200 μ M NiCl₂,
402 0.04% bovine serum albumin (w/v)) was mixed with the enzyme in the reaction well. The
403 reaction was monitored at 37 °C, and the absorbance at 697 nm or 420 nm was
404 accordingly measured at the appropriate time points in a microplate reader (Synergy2
405 from BioTek, Winooski, VT).

406 **Primary screening of urease inhibitors using a high-throughput assay**

407 We screened 3,904 compounds of FDA or FAD-approved drugs from Johns Hopkins
408 Clinical Compound Library (JHCCL, Baltimore, MD) or from TopScience Biotech Co.
409 Ltd. (Shanghai, China) at 100 μ M for the inhibition of JBU under standard assay
410 conditions with salicylic acid-hypochlorite detection reagent as described above. The Z'

411 value of the screening assay was calculated from 60 negative samples (2% DMSO) and
412 60 positive samples (800 μ M AHA) and found to be more than 0.9 (36), indicating the
413 assay is an excellent assay. Routinely, 16 negative samples and 8 positive samples were
414 used to determine the assay performance, and screening data with a minimum Z' value of
415 0.5 were accepted.

416 Compounds that show more than 50% inhibition were selected for the further validation.
417 Primary hits were defined as that compound is free of heavy metal atom and shows a
418 more than 50% inhibition at 50 μ M.

419 **Compounds used for follow-up studies**

420 All hits identified from the primary screening and their analogs were reordered in the
421 highest pure powder from commercial sources or synthesized in-house for the following
422 studies: dose-dependent, kinetic studies, biophysical assays, LC-MS/MS analysis, cell or
423 bacteria-based studies. Panobinostat and dacinostat were brought from AdooQ (catalog
424 number: A10518 for panobinostat, A10516 for dacinostat). EBS and captan were
425 purchased from Sigma (catalog number: E3520 for EBS, 32054 for captan). Disulfiram
426 (tetraethylthiuram disulfide) was purchased from TCI Chemicals (B0479). Captafol
427 (1ST21228) was purchased from Alta Scientific Co.,Ltd (Tianjing, China), and dibenzyl
428 diselenide (catalog number: B21278) was purchased from Alfa Aesar (Ward Hill, MA).
429 Abexinostat (catalog number: HY-10990), belinostat (HY-10225), vorinostat
430 (HY-10221), ricolinostat (HY-16026), ilomastat (HY-15768) and pracinostat (HY-13322)
431 were brought from Medchemexpress. The purities of these commercially available

432 primary leads or analogs of leads as well as in-house synthesized EBS derivatives were
433 confirmed to be at least 95% by using HPLC (for details, see below), with an exception
434 for EBS, the purity of which is determined with combustion analysis methods by the
435 supplier. All the HPLC spectra as well as the combustion analysis data for these
436 inhibitors, which were determined either from commercial supplier or by ourself, were
437 included in the Supporting Information (see below).

438 **Determination of IC₅₀ values**

439 The IC₅₀ values of all the hits or their analogs, as well as AHA, on the activity of JBU,
440 HPU or OAU were determined according to the above-described standard assay
441 conditions. Compounds were incubated with the enzyme and assayed at a series of
442 concentrations (at least 7 steps of doubling dilution). Similarly, the IC₅₀ values of these
443 inhibitors for hCBS or hCSE were determined accordingly(24). Sigmoidal curves were
444 fitted using the standard protocol provided in GraphPad Prism 5 (GraphPad Software,
445 San Diego CA). IC₅₀ was calculated by semilogarithmic graphing of the dose-response
446 curves.

447 **Aggregation-based assay**

448 To exclude the mechanism by which inhibitors suppress the activity of urease via
449 colloidal aggregation, we performed an aggregation-based assay in the presence of
450 nonionic detergents(37). Freshly prepared Triton X-100 (Sangon, Shanghai, China) at
451 different concentrations of 0.1%, 0.05%, 0.01%, 0.005%, and 0.001% was first tested for
452 its effects on the activity of JBU under standard assay conditions. Subsequently, the

453 inhibitory effects of panobinostat, dacinostat, EBS, captan and disulfiram, as well as the
454 analogs of EBS in the *in vitro* JBU activity assay, were determined in the presence of
455 0.01% Triton X-100, a concentration that alone has no inhibitory effect on the activity of
456 JBU.

457 **Reversibility assay**

458 To illustrate the mode of action for the inhibitors of urease, we performed the
459 rapid-dilution experiment. After incubation with panobinostat at a concentration of 4 μM ,
460 dacinostat at 10 μM , EBS or captan at 200, 100, 50 or 20 μM for 60 min, JBU (10 μM)
461 was diluted 200-fold in the assay buffer. After a further incubation of 0, 1, 1.5, 2, 3, 4 or 5
462 h, the remaining activity of JBU was accordingly measured (METHODS). The inhibitor
463 concentrations after dilution are indicated in the figure.

464 **Determination of k_{inact} or K_{I} parameters for irreversible inhibitors**

465 The IC_{50} values of EBS or captan for JBU were measured after different preincubation
466 periods with the enzyme, i.e., 5, 10, 20, 30, 40, 45, 60, 70 or 90 min. The k_{inact} and K_{I}
467 values for EBS or captan were obtained by nonlinear regression plotting of the
468 time-dependent IC_{50} data as previously reported(29).

469 **Enzyme kinetics**

470 The reaction rate was determined with JBU at the indicated concentrations of panobinosta,
471 dacinostat, EBS or captan against increasing concentrations of urea substrate (15.625,
472 31.25, 62.5, 125, 250, 500, 1000 mM for panobinosta and dacinostat; 12.5, 25, 50, 100,
473 200 mM for EBS and captan). The data were fitting to the Michaelis-Menten inhibition

474 equation for determination of the competitive and noncompetitive inhibition parameter K_i
475 and αK_i using GraphPad Prism 5 (Table 1, Figures 2C and 3B)(24), respectively. To
476 illustrate the inhibition type, Lineweaver-Burk plots of these inhibitors were drawn and
477 analyzed.

478 **LC-MS/MS analysis**

479 JBU at a concentration of 12.5 μM was incubated with DMSO, 200 μM EBS or 200 μM
480 captan for 120 min at room temperature. Then, three aliquots of 25 μg samples from the
481 inhibitor-treated JBU or purified HPU (fraction 3 in Figure 4B) were digested separately
482 with three proteases, including 0.5 μl trypsin (1 $\mu\text{g}/\mu\text{l}$), 0.5 μl GluC (1 $\mu\text{g}/\mu\text{l}$) or 0.5 μl
483 subtilisin (1 $\mu\text{g}/\mu\text{l}$) overnight. The proteolytic peptides were combined and desalted on
484 C18 spin columns and dissolved in buffer A (0.1% formic acid in water) for LC-MS/MS
485 analysis. The peptides were separated on a 15-cm C18 reverse-phase column (75 $\mu\text{m} \times$
486 360 μm) at a flow rate of 300 nl/min, with a 75-min linear gradient of buffer B (0.1%
487 formic acid in acetonitrile) from 2% to 60%. The MS/MS analysis was performed on the
488 Q-Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA) using
489 standard data acquisition parameters as described previously(38). The mass spectral raw
490 files were searched against the protein database derived from the standard sequence of
491 JBU, HPU or the proteome of *H. pylori* using Proteome Discovery 1.4 software (Thermo
492 Fisher Scientific, San Jose, CA), with a differential modification of 274.18 m/z in the
493 case of EBS and 150.15 m/z in the case of captan.

494 **Surface plasmon resonance assays**

495 The direct interactions between panobinostat, dacinostat, ebselen or captan and JBU were
496 observed by the surface plasmon resonance (SPR) experiment with a BIAcore T200 (GE
497 Healthcare, Uppsala, Sweden). JBU was immobilized on the surface of the CM5 sensor
498 chip via the amino-coupling kit. The working solution used for the SPR assay was PBS-P
499 (10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 2.7 mM KCl, and 140 mM NaCl in presence of 5%
500 DMSO, pH 7.4). To determine the affinity of the inhibitors toward JBU, panobinostat,
501 dacinostat, EBS or captan were diluted to specific concentrations with PBS-P buffer (for
502 panobinostat: 25, 12.5 6.25, 3.125, 1.56 μM; dacinostat: 100, 50, 25, 12.5 6.25, 3.125,
503 1.56 μM; EBS: 1000, 500, 250, 125, 62.5, or 31.25 nM; for captan: 390.6, 195.3, 97.6,
504 48.8, 24.4, 12.2 or 6.1 nM) and subjected to the JBU-coated chips. The K_D values were
505 calculated with BIAcore evaluation software (version 3.1).

506 **Molecular modeling**

507 The crystal structures of ureases were obtained from the Protein Data Bank (PDB code:
508 4GOA for JBU; PDB code: 1E9Y, HPU). The binding modes of panobinostat or
509 dacinostat were gathered by using the CDOCKER module of the Discovery Studio
510 software (version 3.5; Accelrys, San Diego, CA). Alternatively, AutoDock Vina was
511 initially used to dock the EBS or captan to the respective Cys-containing allosteric site of
512 JBU to obtain the appropriate configurations, enabling the reactive motifs of the
513 compounds (the Se-containing benzisoxazole of EBS and the isoindole dione moiety of
514 captan) to fall into the distance restraint of one covalent bond to the sulfur atom of the
515 reactive Cys residue. The Se-S bond or the N-S bond for isoindole dione was then

516 manually incorporated using the Discovery Studio 3.5 software (Accelrys, San Diego,
517 CA). Subsequently, molecular dynamics simulation was performed with AMBER14
518 software and the ff03.r1 force field(39). To relieve any steric clash in the solvated system,
519 initial minimization with the frozen macromolecule was performed using 500-step
520 steepest descent minimization and 2,000-step conjugate gradient minimization. Next, the
521 whole system was followed by 1,000-step steepest descent minimization and 19,000-step
522 conjugate gradient minimization. After these minimizations, 400-ps heating and 200-ps
523 equilibration periods were performed in the NVT ensemble at 310 K. Finally, the 100-ns
524 production runs were simulated in the NPT ensemble at 310 K. The binding modes for
525 these inhibitors were visually inspected and the best docking mode was selected.

526 **Bacterial strains and culture conditions**

527 Bacterial strains of *H. pylori* or *O. anthropic* were obtained from BeiNuo Life Science
528 (Shanghai, China). The strains were maintained on Columbia blood agar plates (Hopebio,
529 Shandong, China) containing 5% defibrinated sheep blood at 37 °C under microaerobic
530 conditions (5% O₂, 10% CO₂ and 85% N₂), which was supplied by an
531 AnaeroPack-MicroAero gas generator (Mitsubishi Gas Chemical Company, Japan). After
532 a culture of 3-5 days in the plate, the bacterial colonies were scratched into the liquid
533 medium for *H. pylori*, containing 10% or 7% fetal bovine serum and an antibacterial
534 cocktail (composed of 10 mg/l nalidixic acid, 3 mg/l vancomycin, 2 mg/l amphotericin B,
535 5 mg/l trimethoprim and 2.5 mg/l polymyxin B sulfate; BeiNuo, Shanghai, China), and
536 microaerobically incubated for another 3 or 5 days. Then, the medium or bacterial cells

537 were collected for subsequent experiments.

538 A single colony of *O. anthropic* was inoculated into Luria-Bertani liquid medium (LB),
539 which was supplemented with 50 mg/l ampicillin, 30 mg/l kanamycin and 10% FBS
540 (Invitrogen) and cultured at 37 °C. After the bacterial culture reached an O.D. of 0.8 at
541 600 nm, the bacterial cells were collected by centrifugation for future experiments.

542 The identification of *H. pylori* and *O. anthropic* strain was carried out by PCR
543 amplification of the urease gene or 16S rRNA with known primers (Table S4),
544 LC-MS/MS analysis of proteins in the extracts, the bacterial urease activity assay or
545 Gram staining.

546 **16S rRNA sequencing**

547 One colony from the *H. pylori* or *O. anthropic* culture plate was suspended in 50 µl of
548 sterile water, and the DNA was liberated by a boiling-freezing method. The 16S rRNA
549 gene was selectively amplified from this crude lysate by PCR using the universal primers
550 27f and 1492r, which have been previously described (Table S4). The PCR products at
551 ~1400 bp were sequenced. The resultant 16S rRNA sequences were compared with the
552 standard nucleotide sequences deposited in GenBank with the BLAST program
553 (<http://www.ncbi.nlm.nih.gov/blast/>). The DNA sequences of 16S rRNA extracted from
554 these strains were confirmed to be from *H. pylori* or *O. anthropic*.

555 **Preparation of crude extracts from the *H. pylori* and *O. anthropic* strains for the** 556 **urease activity assay**

557 For the urease activity assay, *H. pylori* or *O. anthropic* was cultured accordingly in 100

558 ml of broth medium as described above. Bacteria were centrifuged at 5,000 rpm for 30
559 min, and the pellet was washed with phosphate-buffered saline (PBS, pH = 7.4). The
560 pellet was resuspended in 7 ml of PBS in the presence of protease inhibitors
561 (Sigma-Aldrich, Steinheim, Germany) and then sonicated for 30 min of 30 cycles (30 s
562 run and 30 s rest) using the noncontact ultrasonic rupture device (Diagenode, Liege,
563 Belgium). The resultant bacterial lysate was centrifuged twice at 12,000 rpm for 30 min;
564 the supernatant was collected and desalted using a Sephadex G-25 desalting column (Yeli,
565 Shanghai, China). The protein in the fractions was separated by 10% SDS-PAGE, and the
566 corresponding protein band for urease was quantified to determine the concentration of
567 ureases by Coomassie blue R-250 (Sinopharm, Shanghai, China) staining using bovine
568 serum albumin as a standard. The desalted fractions were stored at -80 °C in the presence
569 of 15% glycerol until usage in the activity assay.

570 **Size-exclusion chromatography for the purification of urease from *H. pylori***

571 The crude extract from *H. pylori* was first centrifuged at 12,000 rpm for 30 min. One
572 milliliter of supernatant was loaded onto a gel filtration column (10 mm × 30 cm; GE
573 Healthcare) and eluted with PBS at a rate of 0.5 ml/min on an AKTA Explorer 100 FPLC
574 Workstation (GE Healthcare). The protein peaks observed were collected in Eppendorf
575 tubes in a volume between 0.5 and 1 ml. The collected fractions were separated by PAGE
576 on a 10% Tris-glycine SDS-gel and stained with Coomassie Brilliant Blue R-250 to
577 identify *H. pylori* urease.

578 **Determination of the minimal inhibition concentration and dose-dependent**

579 **growth-inhibition curve for urease inhibitors**

580 The minimal inhibition concentration (MIC) and dose-dependent growth-inhibition curve
581 for the inhibitors on *H. pylori* were determined using the broth dilution method(40).
582 Briefly, *H. pylori* was grown to an OD_{600 nm} of 1.0 in liquid medium supplemented with
583 7% FBS under standard culture conditions. Then, 150 µl *H. pylori* in the diluted culture
584 (OD of 0.1) was incubated with the inhibitors at final concentrations of 1, 2, 4, 16, 32, 64,
585 128, 256, 512 µg/ml or at indicated concentrations for 72 h. The OD_{600 nm} was measured
586 to calculate the percentage of growth inhibition. The DMSO (1% final
587 concentration)-treated *H. pylori* cultures and culture medium in the absence of bacteria
588 were referred as the negative control (0%) and positive control (100%), respectively. The
589 MIC was defined as the lowest concentration of inhibitor that inhibited 100% of bacterial
590 growth. The *H. pylori* strain was found to be resistant to tinidazole or metronidazole and
591 have an MIC of greater than 512 µg/ml.

592 **Bacterial-cell-based assay for measuring the activity of urease in culture**

593 The endogenous activity of HPU in bacterial cultures was determined using the
594 tandem-well-based plate. Briefly, 300 µl of *H. pylori* culture (OD_{600 nm} ~1.0) was treated
595 with panobinostat, dacinostat or EBS as well as EBS analogs for 6 or 24 h at different
596 concentrations (0, 3.125, 6.25, 12.5, 25, 50, 100 or 200 µM). Then, the bacterial cells
597 were centrifuged, washed and resuspended in assay buffer containing 25 mM urea.
598 Finally, the ~100 µl suspension was added to the reaction well of the tandem-well plate
599 and assessed for the activity of urease with Nessler's reagent under standard assay

600 conditions.

601 **Gastric cell infection model of *H. pylori***

602 The cell infection model of *H. pylori* was constructed using the SGC-7901
603 adenocarcinoma gastric cell line and following an established protocol(15). Briefly, *H.*
604 *pylori* was cultured in liquid medium for *H. pylori* at 37 °C for 3-5 days under standard
605 culture conditions (see above). Then, *H. pylori* at a concentration of 1.5×10^6 CFU/ml
606 was treated with the indicated inhibitors for 24 h in culture. The bacterial suspension
607 together with 10 mM urea were subsequently added to the culture medium of SGC-7901
608 cells (MOI = 30), which had been cultured with RPMI 1640 medium plus 10% FBS in a
609 96-well plate for one day, and coincubated with the cells for an additional 24 h. Cell
610 images were obtained at specific time points prior to and one day after addition of the
611 bacterial culture using Image Xpress Micro[®] XLS (Molecular Devices, Sunnyvale, CA)
612 under a 20 × objective lens. The cell numbers in the images were quantified using Image
613 Xpress Software. The protective effects of the inhibitors were calculated by dividing the
614 number of SGC-7901 cells after the 24-h treatment by that prior to the treatment (100%)
615 in the same well.

616

617

618 **DATA AVAILABILITY**

619 All data are contained within the manuscript.

620 **CONFLICT OF INTEREST**

621 The authors declare no conflicts of interest.

622 **ACKNOWLEDGEMENTS**

623 We thank David Sullivan, Jun Liu and Curtis Chong of Johns Hopkins University for
624 providing the Johns Hopkins Clinical Compound Library. We thank Prof. S.C. Tao
625 (Shanghai Center for Systems Biomedicine, Shanghai Jiao Tong University, Shanghai,
626 China) for kindly providing the SGC-7901 cell line. We thank Dr. J.R. Xu (Department
627 of Radiology, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University,
628 Shanghai, China) for assisting with the surface plasmon resonance assay experiment.

629 **Funding**

630 This work was supported by the National Natural Science Foundation of China
631 (31870763, 21834005), the Natural Science Foundation of Shanghai (18ZR1419500), the
632 Shanghai Foundation for the Development of Science and Technology (19JC1413000),
633 and the Research Fund of Medicine and Engineering of Shanghai Jiao Tong University
634 (YG2019QNB27).

635 **AUTHOR CONTRIBUTIONS**

636 F.L., J.Y., J.Y.X., X.Y.W. and F.W. designed the study, and analyzed the data. F.Z.L. and
637 Y.X.Z. synthesized analogs of EBS lead. Y.Y.Z. constructed the assay and performed the
638 high-throughput screening. H.Q.F. and L.J.L. performed the LC-MS/MS analysis. Q.L.

639 and Z.P.X. confirmed the inhibitory activity of compounds. S.S.H performed the
640 molecular simulation. F.L., X.Y.W. and F.W. wrote the paper. All authors reviewed the
641 results and approved the final version of the manuscript.

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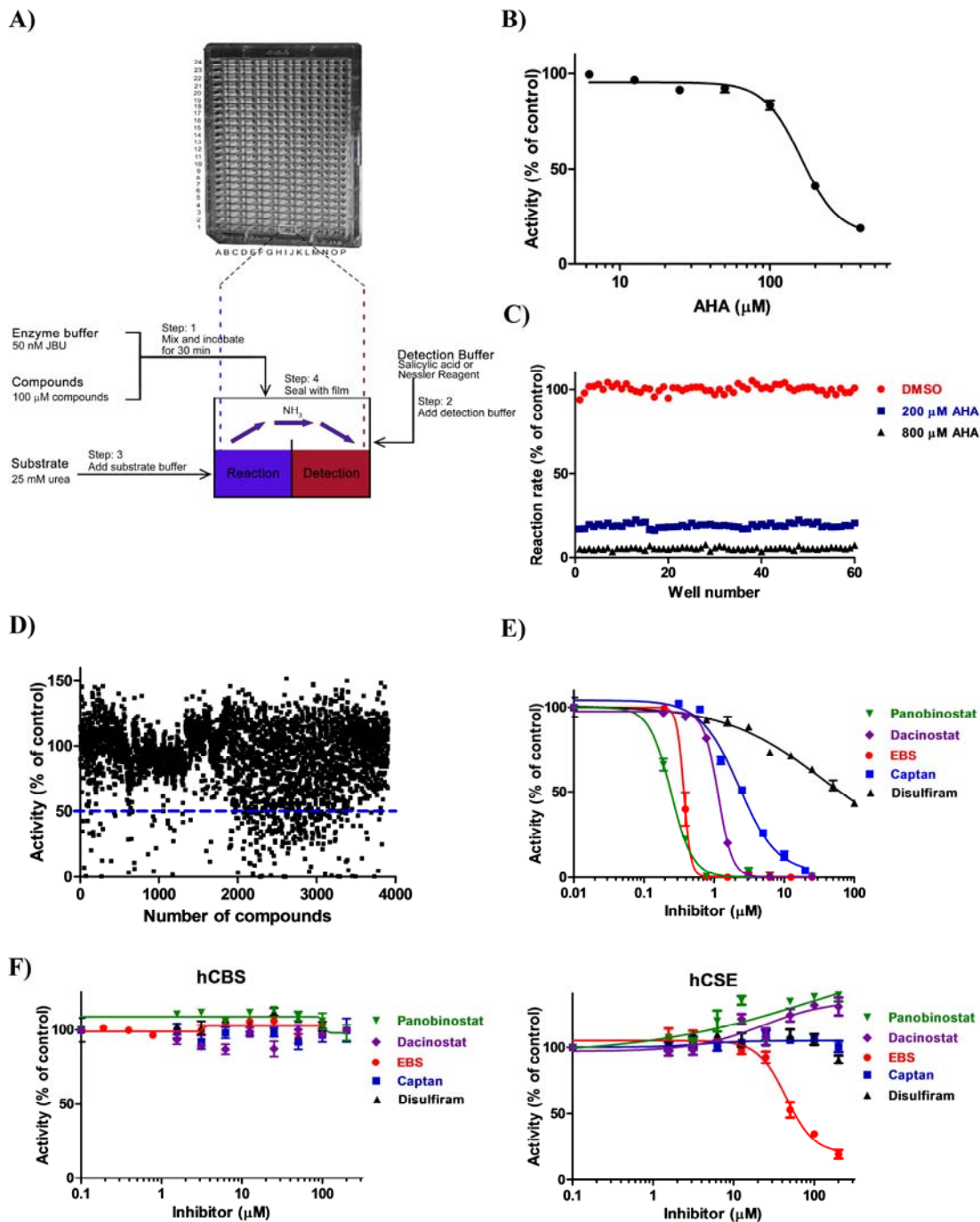
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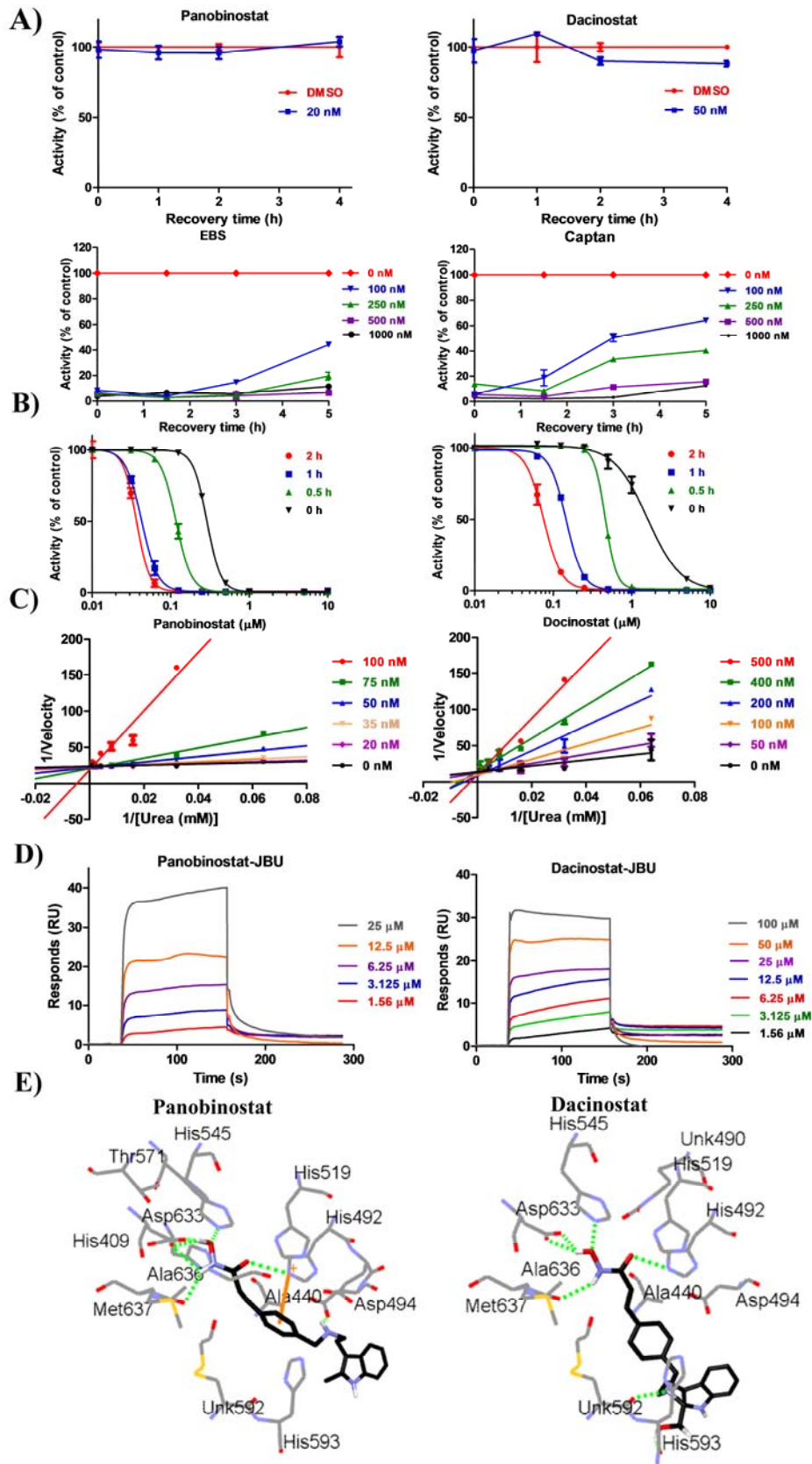
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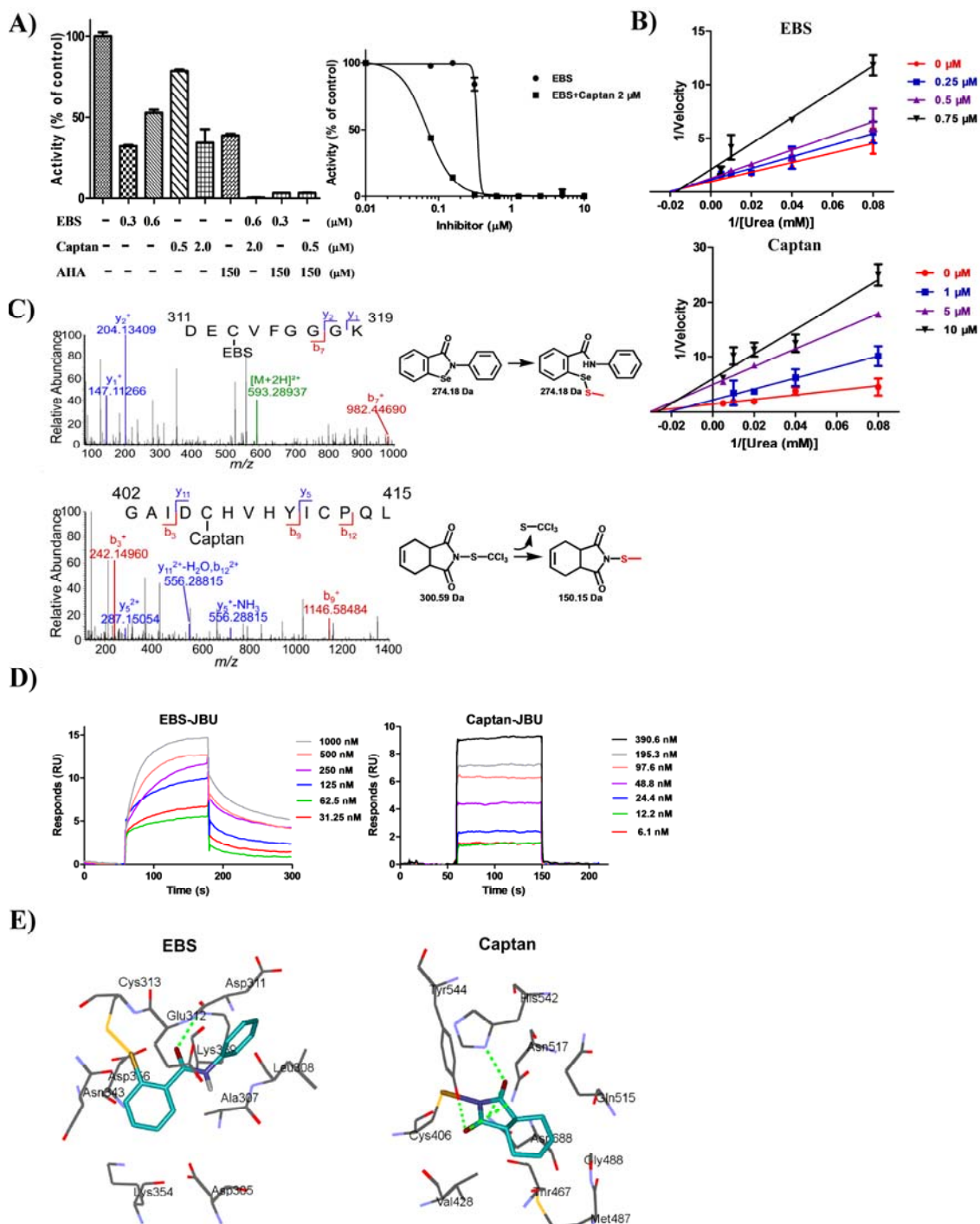
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768 **Figure 1 Development of a new high-throughput assay for urease and the discovery of new**
 769 **urease inhibitors.** (A) Diagram of the tandem-well-based assay for the NH_3 -producing enzyme. The
 770 procedures for the assays and the cross-section of a tandem-well are shown. Blue, the reaction reagent;
 771 red, the detection reagent for NH_3 . (B) Validation of the urease assay with the known inhibitor AHA.
 772 (C) Well-to-well reproducibility of the 192-tandem-well-based assay for urease. ●, 2% DMSO

773 (control, 100%); ■, 200 μ M AHA; ▲, 800 μ M AHA (n = 60). **(D)** High-throughput inhibitor
774 screening for JBU with 192-tandem-well plates. Compound concentration: 100 μ M. **(E-F)**
775 Dose-dependent effects of panobinostat, dacinostat, EBS, captan and disulfiram on the activity of JBU
776 **(E)**, human CBS **(F)** or human CSE **(F)**. Means \pm SDS (n = 3). All experiments except the primary
777 screening **(D)** were independently repeated at least twice, and one representative result is presented.
778
779



781 **Figure 2 Panobinostat, dacinostat, EBS and captan inhibit the activity of JBU.** (A) Panobinostat
782 and dacinostat are reversible inhibitors, whereas EBS and captan are covalent inhibitors or
783 slow-binding inhibitors toward JBU. Means \pm SDs (n = 3). (B) Effects of the incubation period on the
784 IC₅₀ values of panobinostat and dacinostat toward JBU. Panobinostat and dacinostat were
785 preincubated with JBU for the indicated times before performing the standard assay to analyze their
786 inhibitory effects. Means \pm SDs (n = 3). (C) Inhibition of JBU by panobinostat or dacinostat as a
787 function of urea concentration. K_i values for panobinostat and dacinostat, 0.02 μ M and 0.07 μ M,
788 respectively. Means \pm SDs (n=3). (D) Surface plasmon resonance assay analysis of the binding of
789 panobinostat or dacinostat to JBU. K_D were calculated using Biacore evaluation software and listed in
790 Table 1. (E) The putative binding mode of panobinostat or dacinostat in the JBU active site.
791 Panobinostat and dacinostat were docked into the JBU crystal structure (PDB code: 4GOA) using the
792 Discovery Studio software. Residues surrounding the inhibitor within a distance of 3.5 Å are shown in
793 gray; and hydrogen bonds are represented as green dotted lines. The experiments were independently
794 repeated at least twice, and one representative result is presented.
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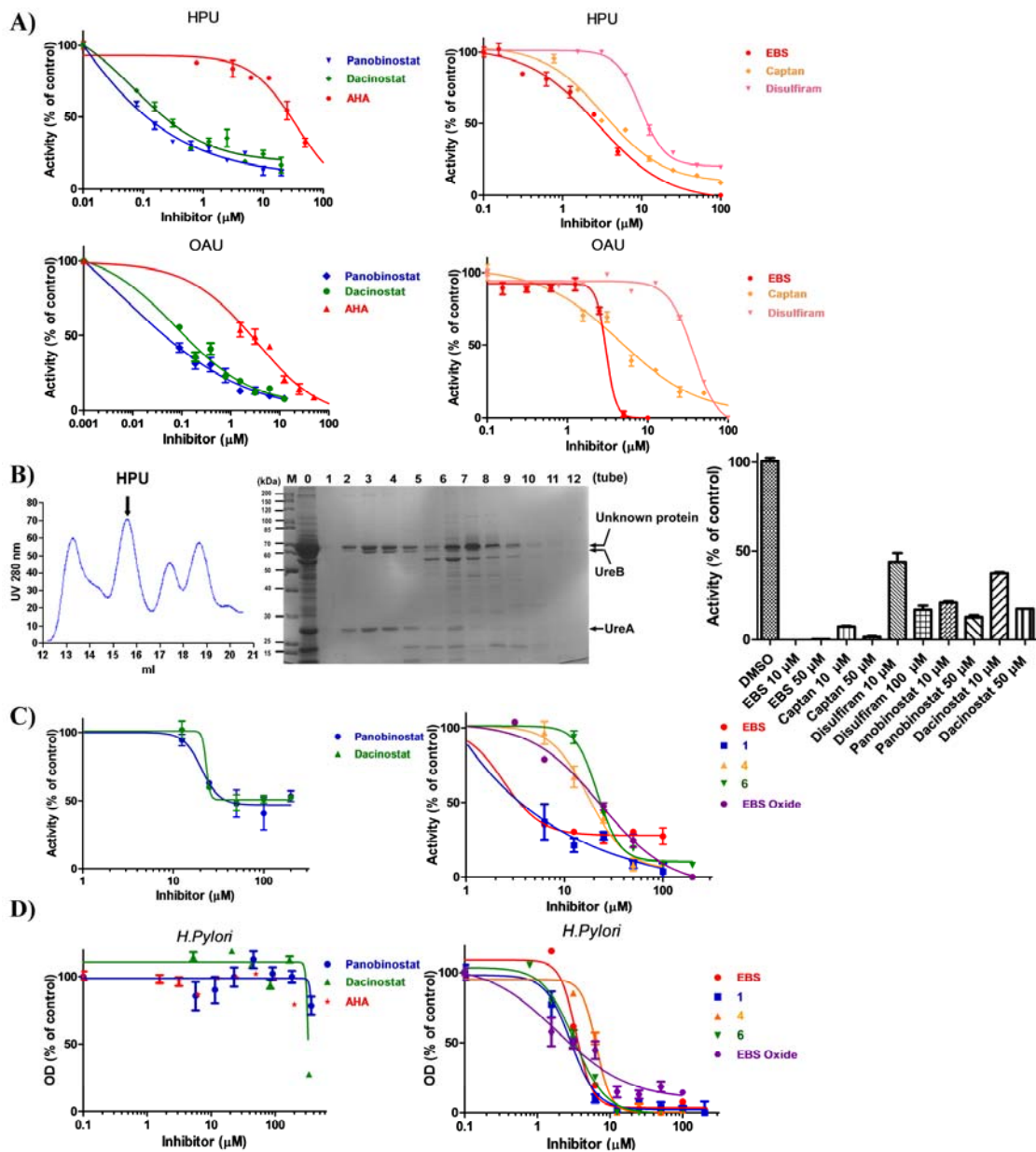
797 **Figure 3 EBS or captan allosterically inhibits the activity of urease by covalently modifying a**
 798 **non-active-site Cys residue.** (A) The synergistic inhibitory effects of the combinations of EBS, captan
 799 or AHA. A dose-dependent synergistic effect of the combination of EBS at the indicated concentrations
 800 with 2 μM captan was observed (right panel). Data are presented as percentages of the controls (DMSO
 801 and 2 μM captan alone in the left panel and right panel, respectively, 100%). Means ± SDs (n=3). (B)

41

802 Inhibition of JBU by EBS or captan as a function of the urea concentration. αK_i for EBS and captan, 0.8
803 μM and 1.1 μM , respectively. Means \pm SDs (n=3). (C) Tandem mass spectrometry analysis of the
804 modification site of EBS and captan on JBU. The Cys modification of EBS and captan on JBU were
805 illustrated in the right panels. (D) Surface plasmon resonance assay analysis of the binding of EBS or
806 captan to JBU. (E) The potential binding modes of EBS and captan in JBU. EBS and captan were
807 modeled into the respective allosteric sites presented in the crystal structure of JBU (PDB code: 4GOA;
808 METHODS). The residues within 3.5 Å surrounding the EBS and captan are shown. Hydrogen bonds
809 are indicated as dashed green lines. The experiments were independently repeated at least twice, and
810 one representative result is presented.

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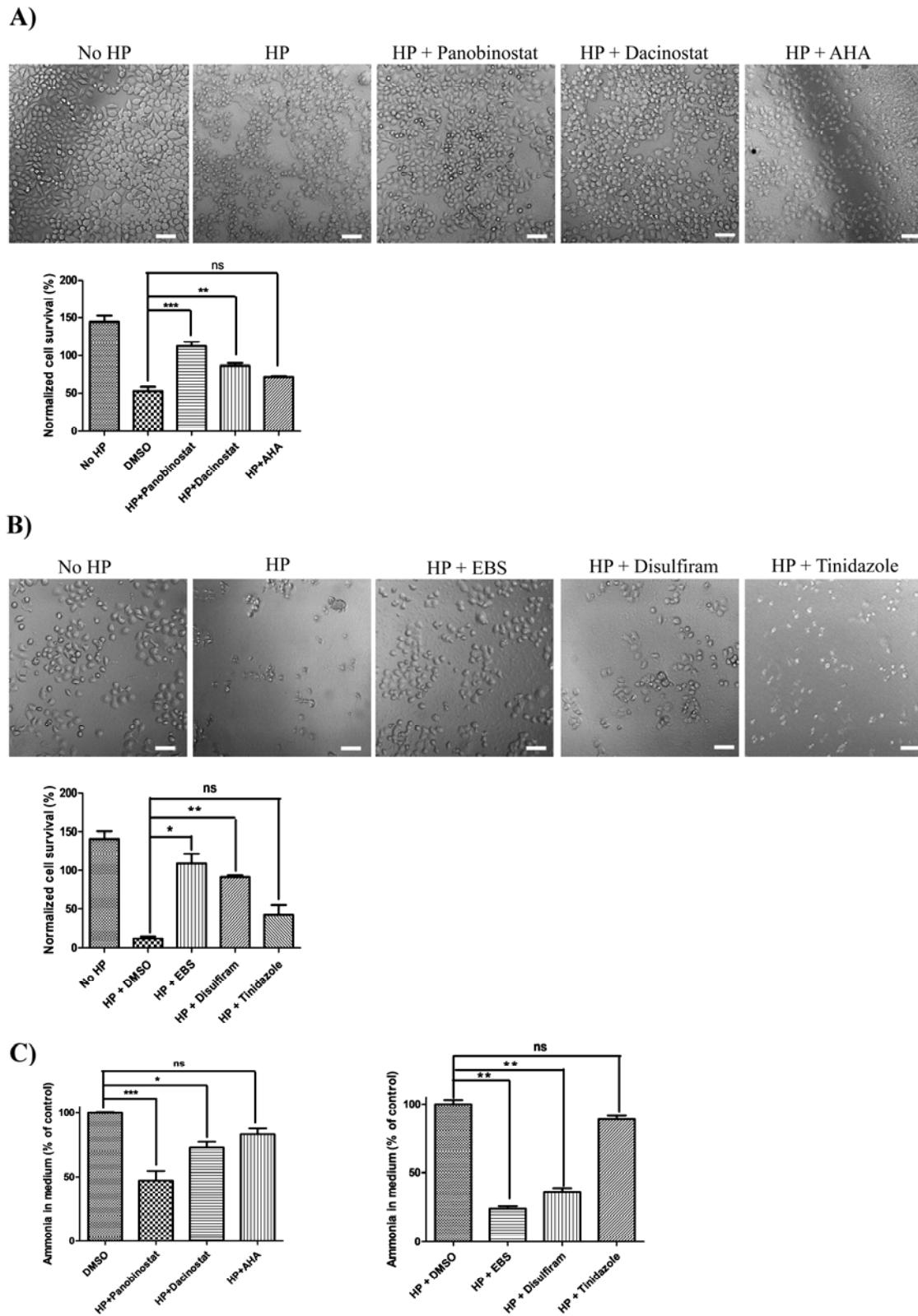
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813

814 **Figure 4 Urease inhibitors suppress bacterial ureases or the growth of urease-containing**
 815 **bacteria.** (A) Dose-dependent effects of panobinostat, dacinostat, EBS, captan, disulfiram and AHA
 816 on the activity of *H. pylori* urease (HPU, upper panel) or *O. anthropic* urease (OAU, lower panel) *in*
 817 *vitro*. (B) Panobinostat, dacinostat, EBS, captan and disulfiram inhibit the activity of purified HPU
 818 from size-exclusion chromatography. Chromatography of the purification is shown in the left panel.
 819 The collected fractions (numbers 1-12) of the peaks (left panel), as well as the crude extract (number
 820 0), were separated by 10% SDS-PAGE and stained with Coomassie Brilliant Blue R-250 (middle
 821 panel). The arrows indicate the peak of *H. pylori* urease (left panel) or subunit A or B of *H. pylori*
 822 urease (middle panel). The collected sample containing the urease (number 3) was tested to evaluate
 823 the inhibitory effects of indicated compounds (right panel). The protein identity of fraction 3 was

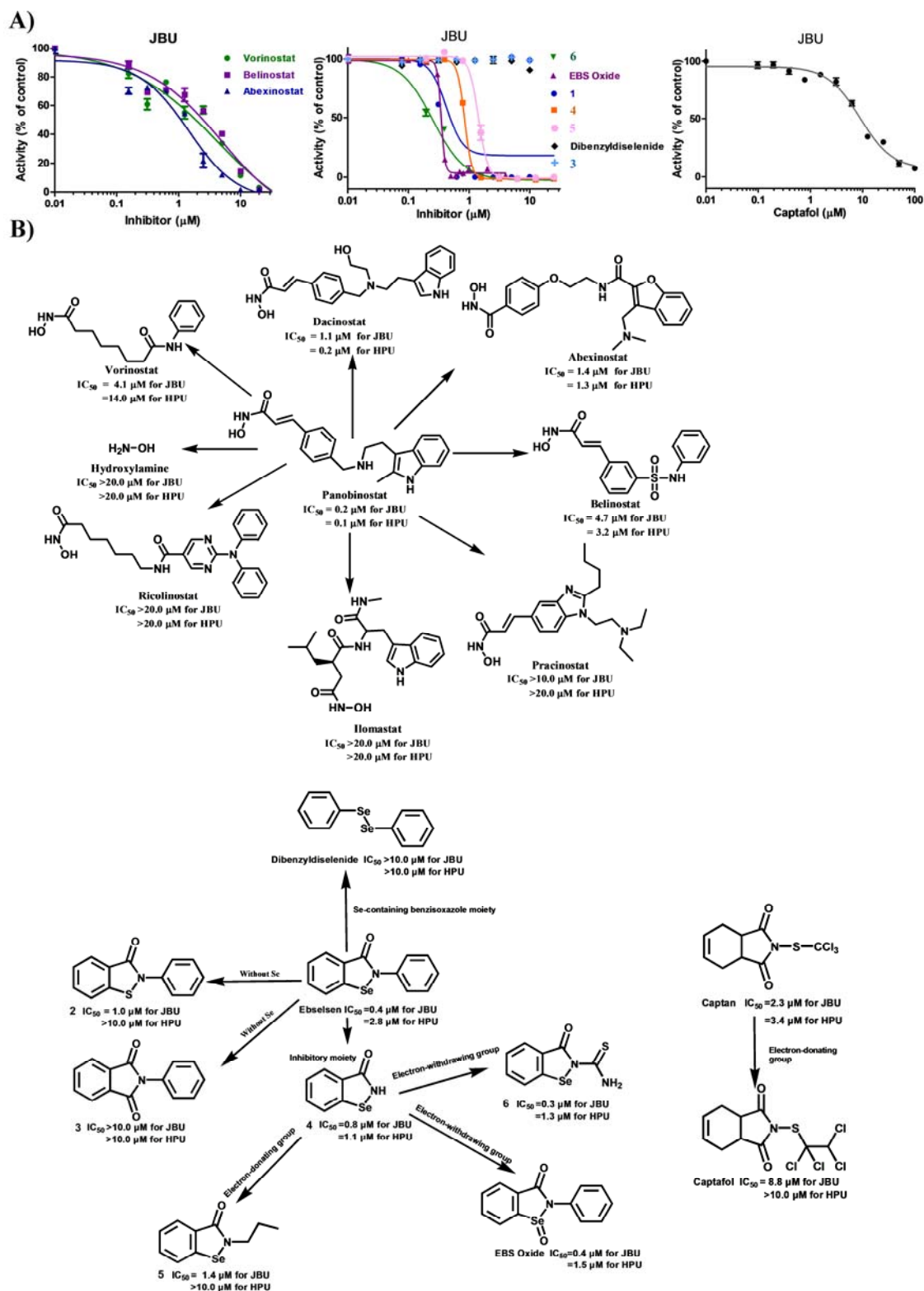
824 analyzed by LC-MS/MS (METHODS and Figure S5). (C) The inhibitory effects of panobinostat,
825 dacinostat and newly synthesized EBS analogs (**1**, **4** and **6**) on the activity of HPU in culture.
826 Inhibitors were incubated with the *H. pylori* bacteria for 6 h. (D) The effects of panobinostat,
827 dacinostat, EBS and its derivatives on the growth of *H. pylori*. Mean \pm SD (n=3). All experiments
828 were independently repeated at least twice, and one representative result is presented.
829
830



831

832 **Figure 5 Panobinostat, dacinostat and EBS inhibits the virulence of *H. pylori* in cultured gastric**

833 **cells.** SGC-7901 cells were infected with HP in the presence of 30 μ M panobinostat (**A**), 30 μ M
834 dacinostat (**A**), 30 μ M AHA (**A**), 20 μ M EBS (**B**), 20 μ M disulfiram or 50 μ M tinidazole (**B**) for 24 h
835 before capturing the images in bright field by Image Xpress Micro® XLS (Molecular Devices,
836 Sunnyvale, CA) under a 20 \times objective lens. A representative image for each treatment condition is
837 shown (n = 3). Scale bars, 100 μ m. The cell numbers before treatment (100%) or after 24 h of
838 treatment were quantified. (**C**) The effects of urease inhibitors on the NH_3 amount of the cell culture
839 medium. After the treatment, the amount of NH_3 in the cell medium of the corresponding samples was
840 quantified with Nessler's reagent, and the data are shown as percentages of the control (DMSO,
841 100%). Means \pm SDs (n=3). Statistical analyses were performed using the raw data by one-way
842 ANOVA with Bonferroni posttests. n.s., no significance; *, p< 0.05; **, p< 0.01; ***, p < 0.001. All
843 experiments were independently repeated twice, and one representative result is presented.
844
845



846

847 **Figure 6. Structure-activity relationships of panobinostat, dacinostat, EBS and captan.** (A) The
848 effects of commercially available analogs of panobinostat and dacinostat, newly synthesized EBS

849 derivatives and commercially available EBS or captan analogs on the activity of JBU. DMSO, 100%.
850 Mean \pm SD (n=3). The experiments were independently repeated at least twice, and one representative
851 result is presented. **(B)** The illustration charts for the structure-activity relationships of hydroxamic
852 acid analogs, EBS or captan.
853
854
855

856 **Table 1 Indication, chemical structure, IC₅₀, αKi, or K_D values of urease inhibitors.**

857

Name	Application	Structure	IC ₅₀ (μM); JBU	IC ₅₀ (μM); HPU	IC ₅₀ (μM); OAU	αKi or Ki (μM) ^a	IC ₅₀ (μM); hCBS	IC ₅₀ (μM); hCSE	K _D (μM)
Panobinostat	Anticancer		0.2 ± 0.006	0.1 ± 0.01	0.07 ± 0.006	0.02 ± 0.01	> 200.0	> 200.0	8.9 ± 0.4
Dacinostat	Anticancer		1.1 ± 0.005	0.2 ± 0.009	0.1 ± 0.01	0.07 ± 0.02	> 200.0	> 200.0	5.3 ± 0.2
Ebselen	Anti-stroke; Anti-bipolar		0.4 ± 0.07	2.8 ± 0.5	3.0 ± 1.0	0.8 ± 0.2	> 200.0	44.3 ± 1.3	0.089 ± 0.005
Captan	Pharmaceutical excipient; Fungicide		2.3 ± 0.2	3.4 ± 0.5	5.8 ± 1.6	1.1 ± 0.2	> 200.0	> 200.0	0.096 ± 0.006
Disulfiram	Alcohol deterrent		38.9 ± 2.7	8.9 ± 1.5	35.0 ± 0.1	-	> 200.0	> 200.0	-
Acetohydroxamic acid	Urinary tract infections		161.8 ± 13.4 33.7 ± 1.0 ^b	25.9 ± 1.2	2.8 ± 0.9	2.1 ± 0.8	> 200.0	> 200.0	-

858

859 ^aFrom the enzyme kinetic study

860 ^bAssay was performed in 50 mM Tris buffer (pH= 7.4).

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1 Supplementary Information

2 **High-throughput Tandem-microwell Assay for Ammonia Repositions**

3 **FDA-Approved Drugs to *Helicobacter Pylori* Infection**

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7

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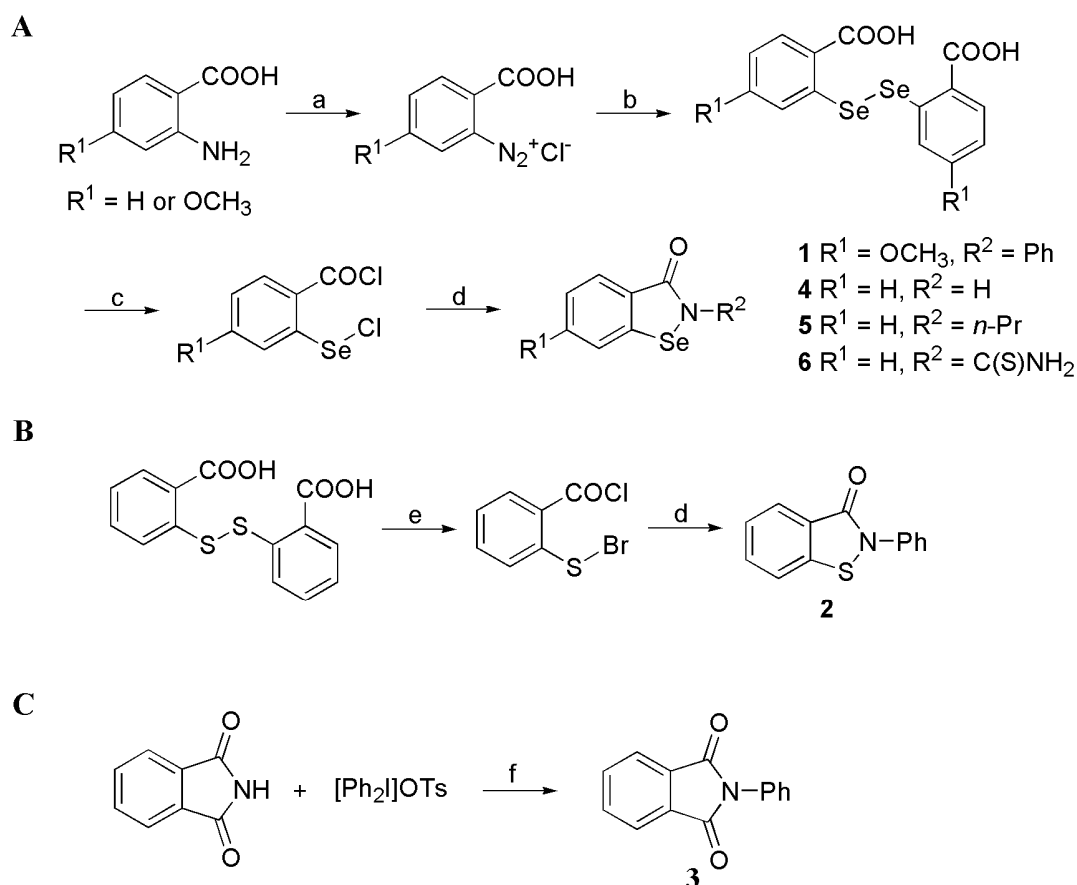
25	Table of Contents	
26	EXPERIMENTAL PROCEDURES.....	S3
27	Figure S1. Development and optimization of the high-throughput assay for urease.....	S12
28	Figure S2. Validation of on-target inhibition of panobinostat, dacinostat, EBS, captan and	
29	disulfiram on JBU.....	S14
30	Figure S3. The mode of action of panobinostat, dacinostat and disulfiram <i>in vitro</i>	S16
31	Figure S4. The mode of action of EBS and captan <i>in vitro</i>	S18
32	Figure S5. The identification of HPU from extracts of <i>H. pylori</i> by LC-MS/MS.....	S20
33	Figure S6. EBS and 1 is a long-acting inhibitor for HPU in culture.....	S21
34	Figure S7. The effects of inhibitors on the cell viability of gastric SGC-7901 cells and	
35	antibiotic resistance of the <i>H. pylori</i> strain.	S22
36	Figure S8. The binding modes of inhibitors in ureases.....	S24
37	Table S1. Chemical structures and IC ₅₀ values of EBS or captan analogs for ureases	
38	S26
39	Table S2. The minimal inhibitory concentration of urease inhibitors or known antibiotics	
40	for inhibiting <i>H. pylori</i> and their IC ₅₀ values in the <i>in cellulo</i> urease assay	S27
41	Table S3. Chemical structures and IC ₅₀ values of hydroxamic acid-based analogs for	
42	ureases.....	S28
43	Table S4. Primer sequences.....	S29
44	Reference	S30

46 EXPERIMENTAL PROCEDURES

47 Synthesis of EBS analogs 1-6

48 Compound **1-6** were synthesized according to literature procedure(1-3), as shown in
49 Scheme S1. The chemical reagents and solvents are purchased from commercial sources,
50 and used without further purification, unless stated otherwise. ^1H NMR spectra for these
51 compounds were recorded with Bruker 400 spectrometer. The chemical shifts of ^1H NMR
52 spectra were referenced to tetramethylsilane (δ 0.00 ppm).

53



54

55 Scheme S1. Synthesis of compounds 1-6.

56 Reagents and conditions: (a) HCl , NaNO_2 , 0°C , 0.5 h; (b) Na_2Se_2 , 60°C , 3 h; (c) SOCl_2 ,

57 85 °C, 3 h; (d) R²NH₂, Et₃N, CH₂Cl₂, rt, 4.5 h; (e) Br₂, CH₂Cl₂, reflux, overnight; (f)
58 Cu(NO₃)₂.xH₂O, Et₃N, toluene, reflux.

59 General procedure for synthesis of Compounds **1**, **4**, **5** and **6** (Route A).

60 The 2-aminobenzoic acid or its derivative was treated with hydrochloric acid (2.50 equiv.)
61 and sodium nitrite (1.06 equiv.) in water (0.7 M) at 0 °C to form the corresponding
62 diazonium salt. Then, the diazonium salt solution was added dropwise to a solution of
63 Na₂Se₂ (0.87 equiv., fresh prepared from selenium powder and NaBH₄ in water) at 0 °C
64 under Argon. The stirring was continued at 60 °C for 3 h. After work-up, crude
65 2,2'-diseleno-dibenzoic acid was obtained. Sequentially, the acid was further converted
66 to 2-(chloroseleno)benzoyl chloride with excess SOCl₂ and one drop of DMF at 85 °C for
67 3 h. After the removal of thionyl chloride, the crude compound was obtained, and which
68 was treated with different amines (1.2 equiv.) and Et₃N (2.0 equiv.) in CH₂Cl₂ (0.1 M)
69 under Argon to afford products **1** and **4-6**, respectively. Silica gel column
70 chromatography was used to purify these compounds, and their HPLC purity was more
71 than 99%.

72

73 2-Phenyl-6-methoxybenzoisosen-3-one (**1**)

74 4-Methoxy-2-aminobenzoic acid and aniline were used to give the compound. ¹H NMR
75 (400 MHz, CDCl₃): δ 8.01 (d, *J* = 8.8 Hz, 1H), 7.62 (dd, *J* = 7.6, 0.8 Hz, 2H), 7.43 (t, *J* =
76 8.0 Hz, 2H), 7.29-7.24 (m, 1H), 7.11 (d, *J* = 2.0 Hz, 1H), 7.01 (dd, *J* = 8.4, 2.0 Hz, 1H),
77 3.92 (s, 3H). MS (*m/z*): 305.0 [M+H]⁺.

78 Benzisosenol-3-one (**4**)

79 *o*-Aminobenzoic acid and ammonia were used to give the product. ¹H NMR (400 MHz,
80 *d*₆-DMSO): δ 9.17 (br, 1H), 8.06 (d, *J* = 8.1 Hz, 1H), 7.81 (dd, *J* = 8.0, 0.8 Hz, 1H), 7.61
81 (td, *J* = 7.6, 1.2 Hz, 1H), 7.42 (td, *J* = 7.6, 0.8 Hz, 1H). MS (*m/z*): 198.9 [M+H]⁺.

82 2-Propyl-benzisosenol-3-one (**5**)

83 *o*-Aminobenzoic acid and *n*-propylamine were used to give the product. ¹H NMR (400
84 MHz, CDCl₃) δ 8.05 (d, *J* = 8.0 Hz, 1H), 7.63 (d, *J* = 7.6 Hz, 1H), 7.58 (td, *J* = 7.6, 1.2
85 Hz, 1H), 7.45-7.40 (m, 1H), 3.83 (t, *J* = 7.2 Hz, 2H), 1.76 (hex, *J* = 7.2 Hz, 2H), 1.00 (t, *J*
86 = 7.2 Hz, 3H). MS *m/z*: 242.0 [M+H]⁺.

87 2-Methylthio-benzisoseno-3-one (**6**)

88 *o*-Aminobenzoic acid and thiourea were used to give the product. ¹H NMR (400 MHz,
89 *d*₆-DMSO): δ 10.21 (d, *J* = 0.8 Hz, 1H), 9.98 (d, *J* = 1.2 Hz, 1H), 8.00 (d, *J* = 8.4 Hz, 1H),
90 7.88 (d, *J* = 8.0 Hz, 1H), 7.71 (td, *J* = 8.0, 1.2 Hz, 1H), 7.45 (t, *J* = 7.6 Hz, 1H). MS (*m/z*):
91 240.0 [M-NH₃]⁻.

92

93 Synthesis of compound **2**.

94 Compound **2** was prepared according to route B (Scheme S1). 2,2'-Dithiobis-benzoic acid
95 was reacted with bromine in CH₂Cl₂ under reflux and Argon, and then treated with
96 aniline and Et₃N in CH₂Cl₂ at room temperature. After purified the crude product by
97 column chromatography, compound **2** was obtained. ¹H NMR (400 MHz, CDCl₃): δ 8.11
98 (d, *J* = 7.6 Hz, 1H), 7.73-7.69 (m, 2H), 7.68-7.65 (m, 1H), 7.51-7.43 (m, 3H), 7.59 (d, *J* =

99 8.0 Hz, 1H), 7.33 (t, $J = 7.6$ Hz, 1H). MS (m/z): 227.0 [M]⁺.

100

101 **Synthesis of compound 3.**

102 Compound **3** was synthesized according to route C (Scheme S1). A Schlenk tube

103 equipped with a stirrer bar was charged with isoindoline-1,3-dione, diphenyliodonium

104 salt (2.05 equiv.) and Cu(NO₃)₂.xH₂O (0.1 equiv.) in dry toluene (0.1 M) under Argon.

105 The mixture was heated to 70 °C, followed by the addition of Et₃N (1.5 equiv.). After

106 stirring at 70 °C for 8.5 h (monitoring by TLC), the resulting mixture was continued

107 stirring at room temperature overnight. Then, the mixture was concentrated and the

108 residue was purified by column chromatography. ¹H NMR (400 MHz, CDCl₃): δ

109 7.99-7.94 (m, 2H), 7.80 (dd, $J = 5.6, 3.2$ Hz, 2H), 7.55-7.49 (m, 2H), 7.47-7.39 (m, 3H).

110 MS (m/z): 223.1 [M]⁺.

111

112 **HPLC method and purity analysis**

113 The purity of compounds **1-5**, ebselen oxide or dibenzyl diselenide was analyzed on a

114 Waters sunfire silica column (4.6×250mm; Waters, Milford, MA), which is coupled to a

115 Waters HPLC system (e2695). 3 μl compound was injected onto the column and

116 separated by a gradient elution [0 min: 95% phase A (hexane), 5% phase B (isopropyl

117 alcohol); 15 min: 60% phase A (hexane), 40% phase B (isopropyl alcohol)] at a flow rate

118 of 0.7 ml/min under room temperature.

119 Similarly, the purity of compound **6** was resolved on a Waters PHERISORB CN column

120 (4.6×250mm, Waters). 5 µl compound **6** was injected onto the column and analyzed at a
121 flow rate of 0.7 ml/min with an isocratic elution of solvent, which is composed of 75%
122 hexane and 25% isopropyl alcohol.

123 The absorbance of the compounds were monitored at a wavelength of 230 nm, and the
124 corresponding spectra were recorded and analyzed for the determination of the purity.

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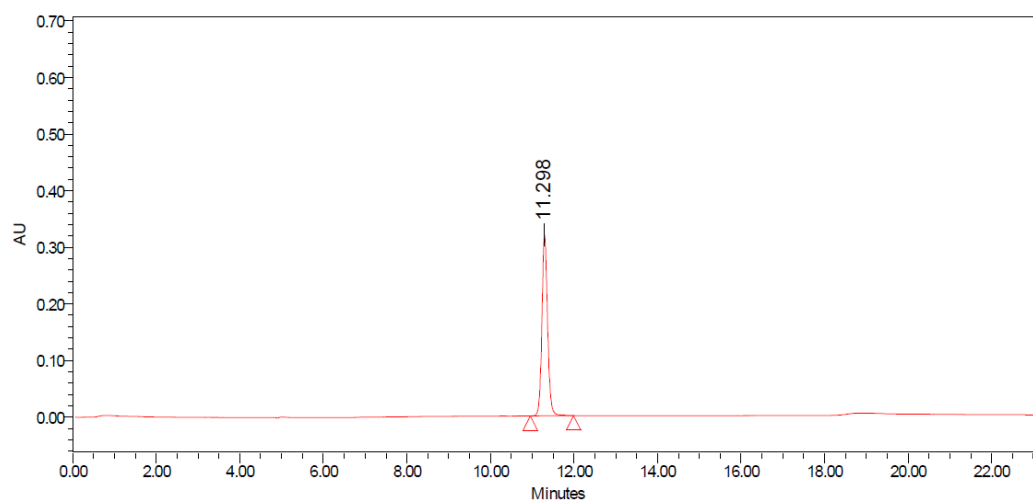
126 **The purity of EBS analogs, which were newly synthesized in house (Compound 1-6)**
127 **or obtained from commercial sources (for Ebselen oxide and dibenzyl diselenide),**
128 **were analyzed by HPLC (for details, see above).**

129

130 **Compound 1**

131 Determined Purity: > 99%; Retention time: 11.30 min

132



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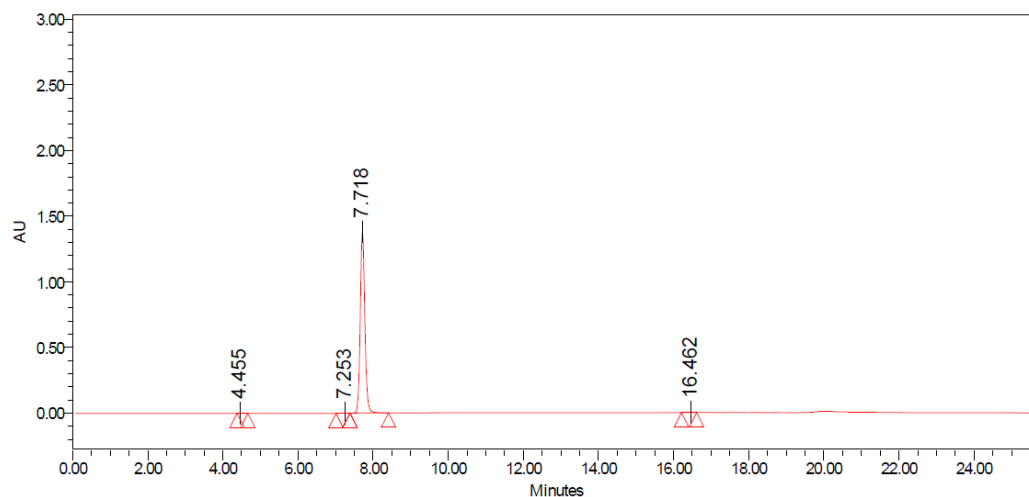
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136 **Compound 2**

137 Determined Purity: > 99%; Retention time: 7.72 min

138



139

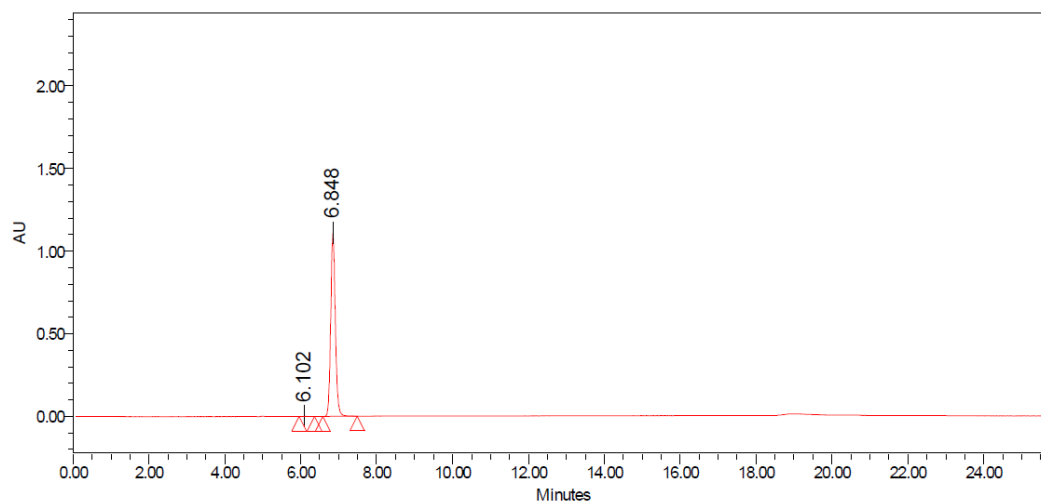
140

141

142 **Compound 3**

143 Determined Purity: > 99%; Retention time: 6.85 min

144



145

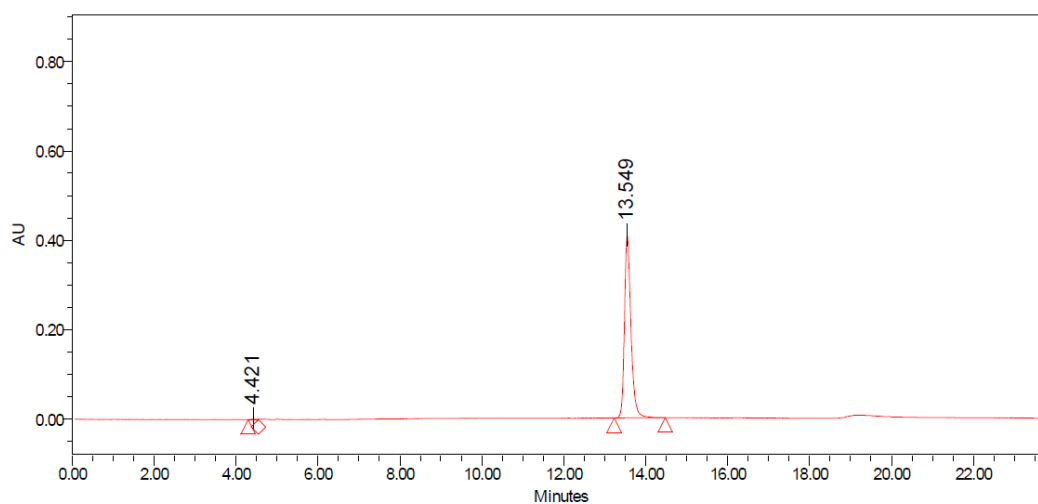
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148 **Compound 4**

149 Determined Purity: > 99%; Retention time: 13.55 min

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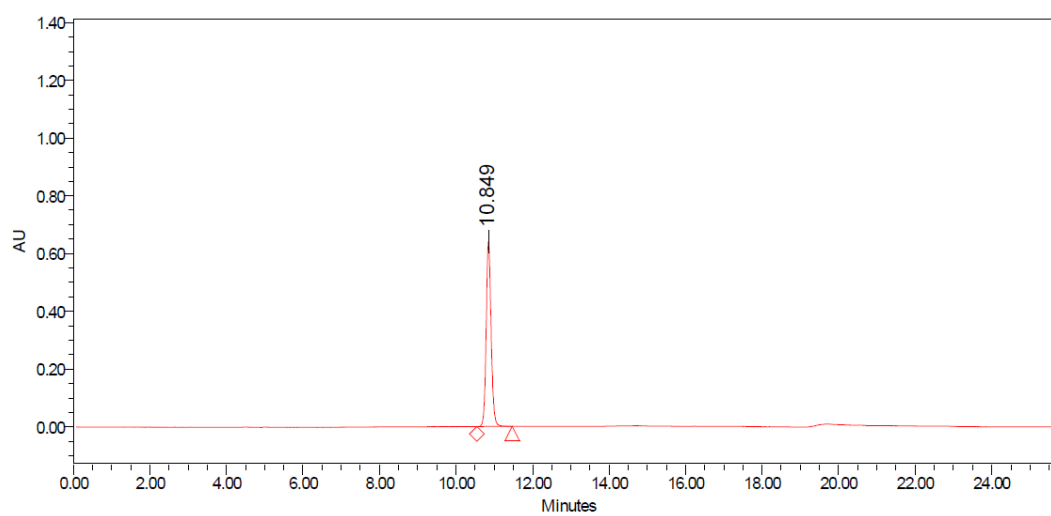
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158

159 **Compound 5**

160 Determined Purity: > 99%; Retention time: 10.85 min

161



162

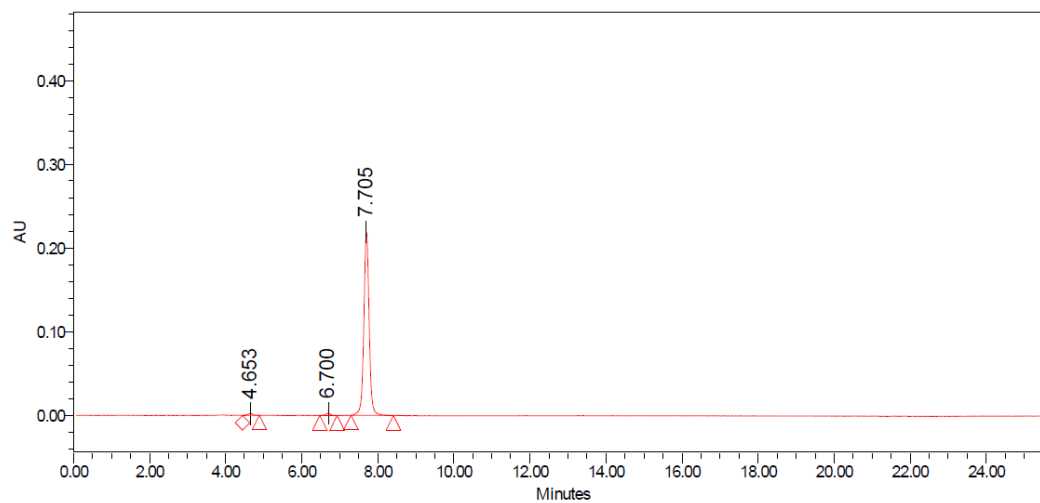
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165 **Compound 6**

166 Determined Purity: > 97%; Retention time: 7.71 min

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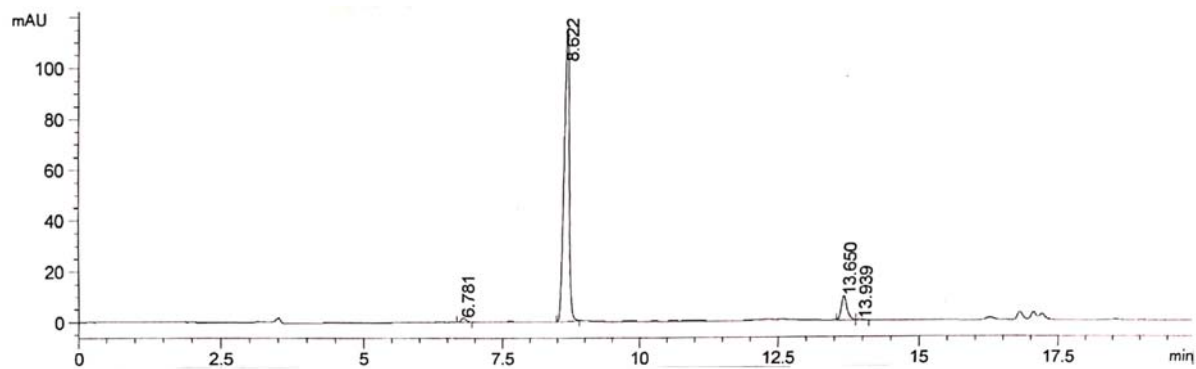
172

173

174 **Ebselen oxide** (Cayman)

175 Determined Purity: 95%; Retention time: 8.62 min

176



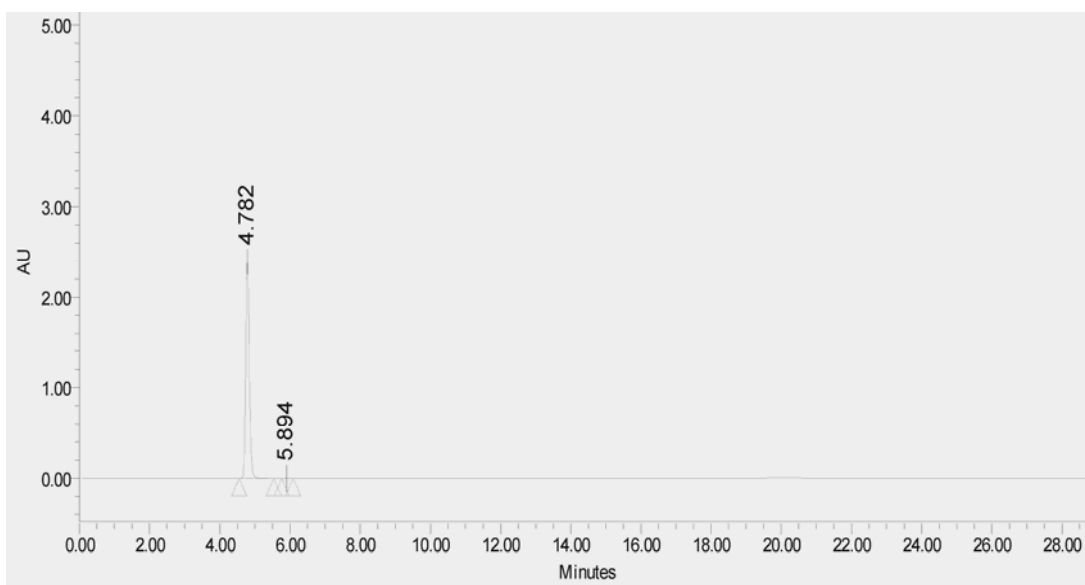
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178

179 **Ddibenzyl diselenide** (Cayman)

180 Determined Purity: > 99%; Retention time: 4.78 min

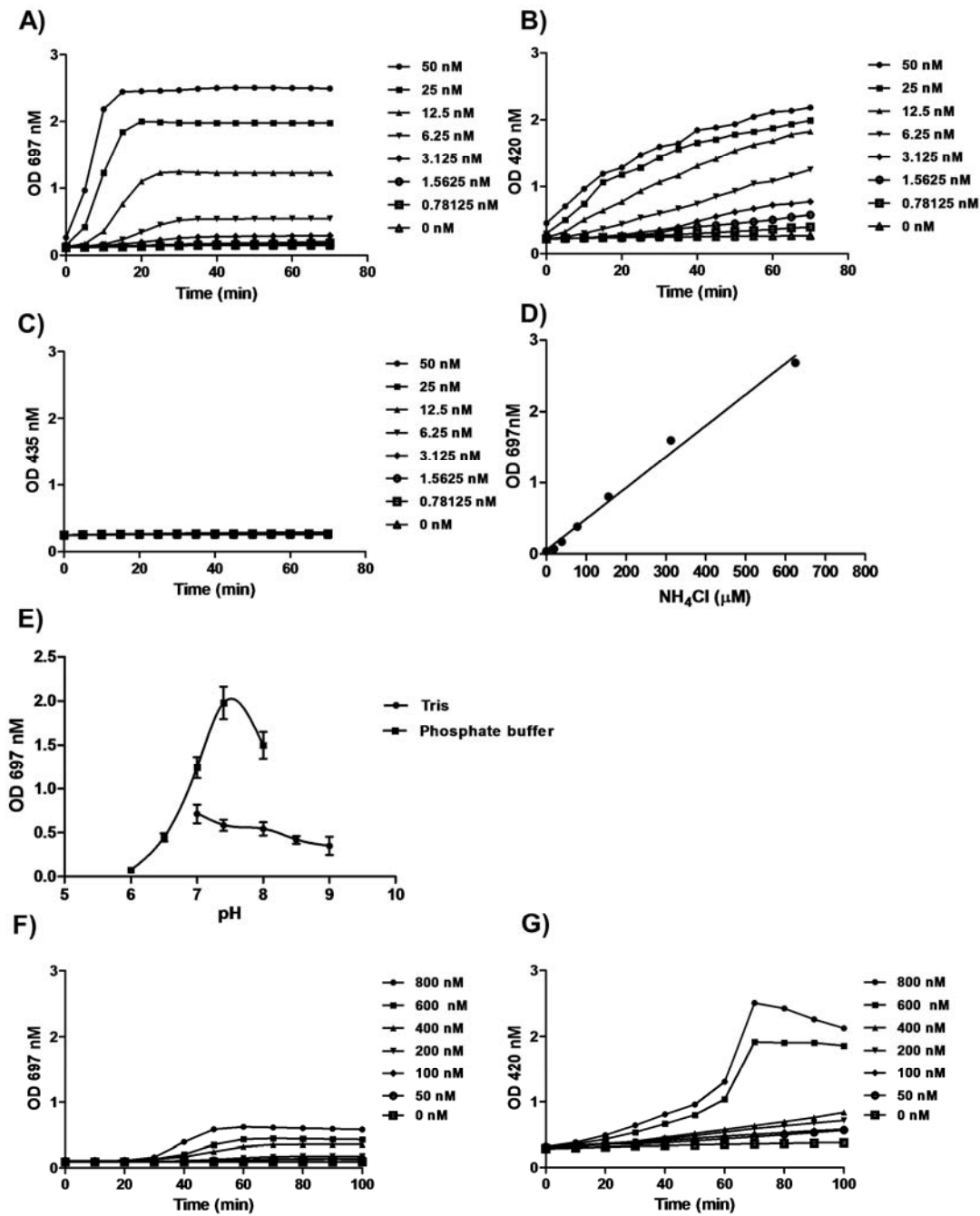
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185

186 **Figure S1. Development and optimization of the high-throughput assay for urease.**

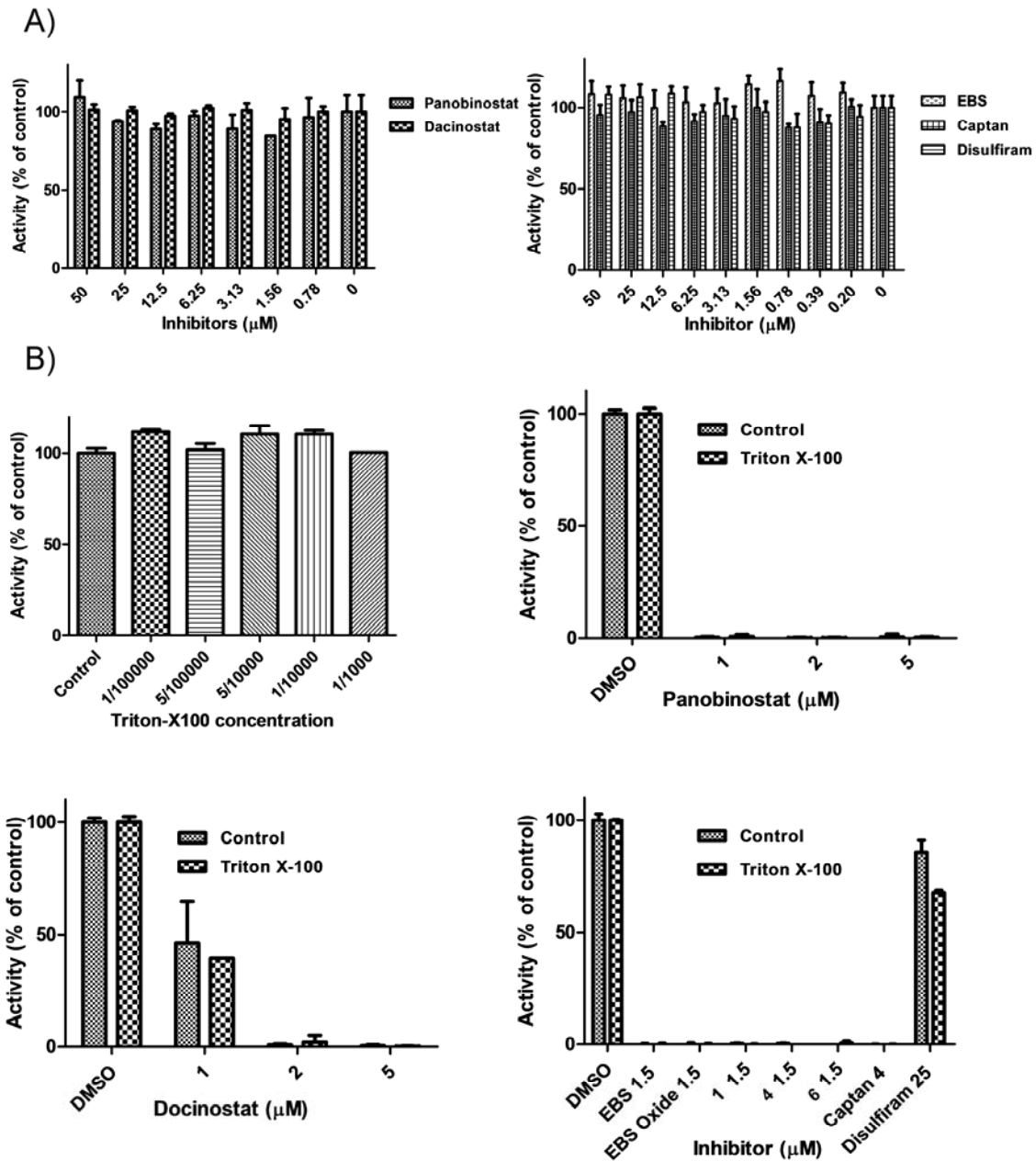
187 Three types of detection reagents, i.e., salicylic acid-hypochlorite (A), Nessler's reagent

188 (B), and phenol red (C), were used to detect the released NH_3 generated by JBU. The

S12

189 assay was monitored in the presence of various concentrations of JBU and 25 mM urea.
190 The absorbance (O.D.) values at 697 nm, 420 nm or 435 nm were recorded accordingly.
191 **(D)** Standard curve of the absorbance of indophenol blue at 697 nm versus the NH₄Cl
192 concentration. Various concentrations of NH₄Cl were mixed with the detection reagent
193 salicylic acid-hypochlorite before measurement of the absorbance at 697 nm in a
194 microplate reader. **(E)** The pH profile of the activity of JBU. The 50 mM phosphate
195 buffer (■) was used to maintain the pH between 6 and 8, and 50 mM Tris-HCl (●) was
196 used for pH 7 to 9. JBU was dissolved in the respective buffers and assayed at a final
197 concentration of 50 nM. **(F-G)** The comparison between salicylic acid-hypochlorite and
198 Nessler's detection reagent for the detection of HPU activity. The assay was performed to
199 detect the urease activity in the extract from *H. pylori* with salicylic acid-hypochlorite
200 (left panel) and Nessler's detection reagent (right panel) in the presence of 25 mM urea.
201 Data are presented as the mean \pm SD (n=3). The curves were fitted to the data points with
202 GraphPad Prism 5. All the experiments were independently repeated twice, and one
203 representative result is presented.

204



205

206 **Figure S2. Validation of on-target inhibition of panobinostat, dacinostat, EBS,**

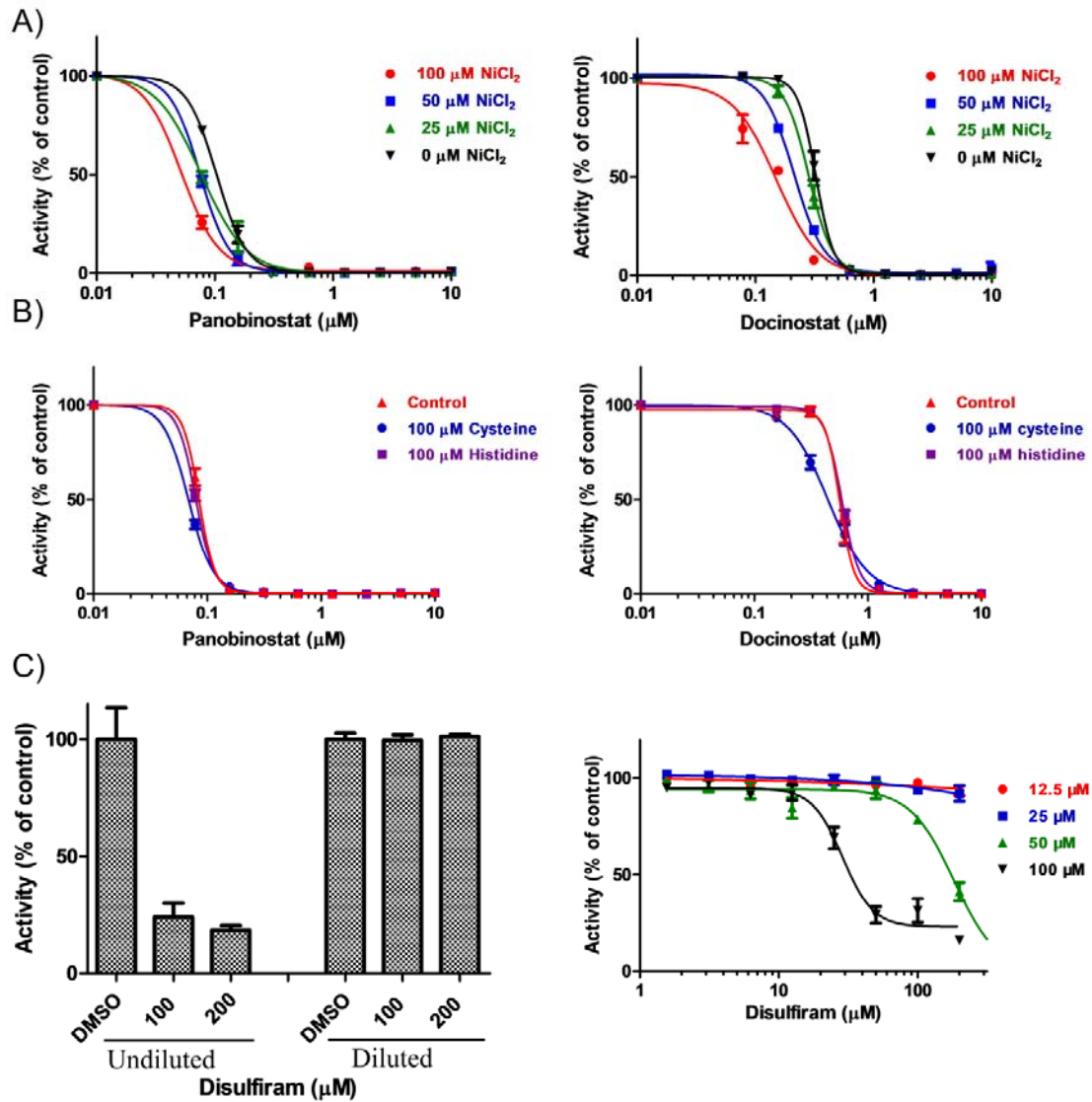
207 **captan and disulfiram on JBU. (A) NH₃ did not interfere with the inhibitors. 5 mM**

208 **NH₃·H₂O was incubated with various concentration of panobinostat, dacinostat, EBS,**

209 **captan or disulfiram in assay buffer. The volatile NH₃ was analyzed with salicylic**

210 **acid-hypochlorite detection reagent (OD₆₉₇ nm). (B) Triton X-100 did not affect either the**

211 activity of JBU or the inhibition potency of panobinostat, dacinostat, EBS, captan or
212 disulfiram as well as EBS analogs. Various concentrations of Triton X-100 were tested for
213 their effects on the activity of JBU. Additionally, the indicated concentrations of
214 panobinostat, dacinostat, EBS, EBS Oxide, captan, **1, 4, 6** or disulfiram were assayed in the
215 presence or absence of 1/10000 Triton X-100 (v/v) to determine whether their inhibitory
216 mechanisms occurred via colloidal aggregation (METHODS)(4). The results are shown as
217 percentages of the respective control (DMSO or H₂O, 100%). Mean ± SD (n=3). All
218 experiments were independently repeated twice, and one representative result is presented.
219



220

221 **Figure S3. The mode of action of panobinostat, dacinostat and disulfiram *in vitro*.** (A)

222 The effect of NiCl₂ on the inhibition of JBU by panobinostat or dacinostat. NiCl₂ at a

223 concentration of 25, 50 or 100 μM was added into the assay that is with the various

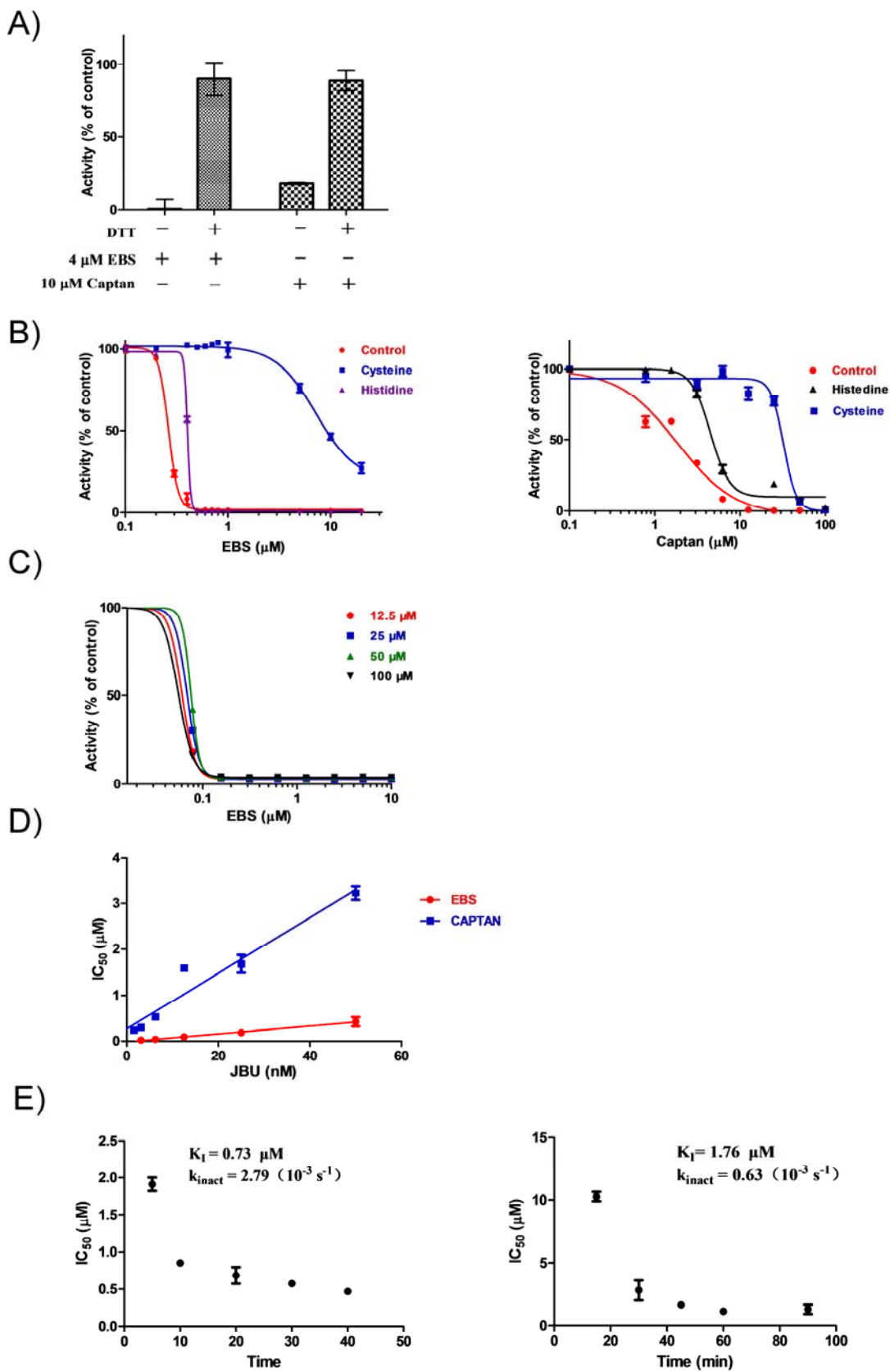
224 concentrations of panobinostat or dacinostat under standard assay conditions. (B) Effects

225 of cysteine and histidine on the inhibition of JBU with panobinostat and dacinostat. The

226 assay samples were incubated with the indicated concentrations of panobinostat or

227 dacinostat in the presence or the absence of 100 μ M Cys or 100 μ M His. The results are
228 shown as percentages of the control (DMSO, 100%). (C) Reversibility of the inhibition of
229 JBU by disulfiram. After incubation with JBU at 200, 100 μ M for 60 min, disulfiram was
230 diluted 200-fold in assay buffer. The diluted concentrations for disulfiram are 1 μ M and
231 0.5 μ M, respectively, which do not inhibit JBU (Fig. 1E). After a further incubation for
232 0.5 h, the remaining activity of JBU was measured accordingly (METHODS). And the
233 effect of NiCl₂ on the inhibition of JBU by disulfiram was shown on the right panel. The
234 results are shown as percentages of the respective control (DMSO, 100%). Mean \pm SD
235 (n=3). All experiments were independently repeated twice, and one representative result is
236 presented.

237



239 **Figure S4. The mode of action of EBS and captan *in vitro*.** (A) Effects of dithiothreitol
240 on the inhibition of JBU caused by EBS and captan. The assay was incubated with 4 μ M
241 EBS or 10 μ M captan in the presence or the absence of 5 mM DTT. (B) Effects of
242 cysteine and histidine on the inhibition of JBU by EBS and captan. The samples were
243 incubated with the indicated concentrations of EBS or captan in the presence or absence
244 of 100 μ M Cys or 100 μ M His. (C) The effect of NiCl₂ on the inhibition of EBS by JBU.
245 NiCl₂ at a concentration of 12.5, 25, 50 or 100 μ M was incubated with the various
246 concentrations of EBS under standard assay conditions. (D) The IC₅₀ values of EBS and
247 captan toward JBU were linearly correlated with the concentrations of JBU. EBS and
248 captan were incubated with various concentrations of JBU, and the IC₅₀ values were
249 determined accordingly. (E) The inhibition constants of K_I or k_{inact} for irreversible
250 inhibitors were determined according to the methods described in ref. (5). Means \pm SDs
251 (n=3). All experiments were independently repeated at least twice, and one representative
252 result is presented.

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UreB :

MKKISRKEYVSMYGPTTGDKVRLGDTDLIAEVEHDYTIYGEELKFGGGKTLREGMSQSNNPSK
EELDLIITNALIVDYTGIIYKADIGIKDGKIAGIGKGGNKDMQDGVKNNLSVGPATEALAGEGLIVTAG
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PTPQPVYYREMFHHGKAKYDANITFVSQAAYDKGIKEELGLERQVLPVKNCRNITKKDMQFNDT
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UreA :

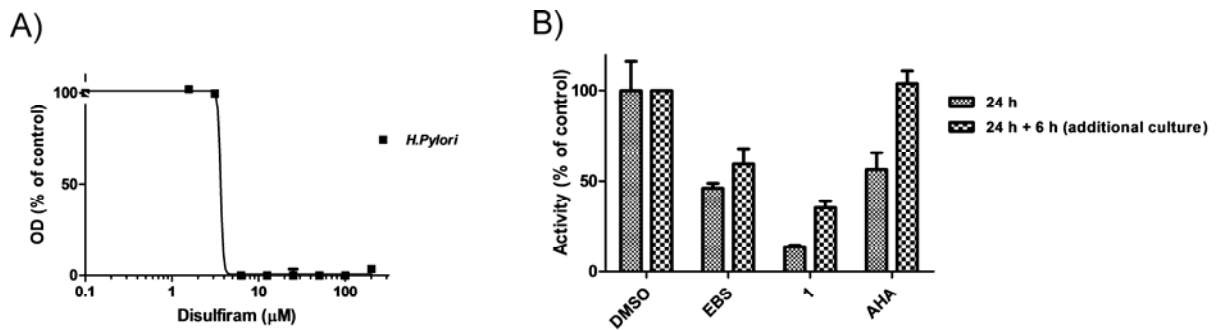
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FGFNALVDRQADNESKIALHRAKERGFHGAKSDDNYVKTIKE

258

259 **Figure S5. The identification of HPU from extracts of *H. pylori* by LC-MS/MS.**

260 Fraction 3 collected by size-exclusion chromatography (Figure 4B) was digested with
261 trypsin, GluC and subtilisin, separated from the C18 reverse-phase column and subjected
262 to analysis with a Thermo Q Exactive Orbitrap (Thermo Fisher Scientific). The peptides
263 in red were identified by LC-MS/MS as subunit A or B of *H. pylori*. The overall coverage
264 of UreB and UreA identified in the analysis of LC-MS/MS was 80.1% and 76.9%,
265 respectively.

266



267

268 **Figure S6. EBS and **1** is a long-acting inhibitor for HPU in culture.** (A) Disulfiram

269 dose-dependently and selectively inhibits the growth of *H. pylori*. Various concentrations

270 of disulfiram were incubated at 37 °C with *H. pylori*. (B) The inhibitory effects of EBS

271 and **1** on the activity of HPU *in cellulo*. EBS, **1** or AHA at a concentration of 100 μM

272 were incubated with *H. pylori* bacteria for 24 h. Additionally, one batch of the treated

273 bacteria was washed, diluted into freshly prepared medium without the addition of the

274 inhibitors, and cultured for an additional 6 h. The *in cellulo* urease activities from the

275 cultured cells under the two treated-conditions were determined accordingly

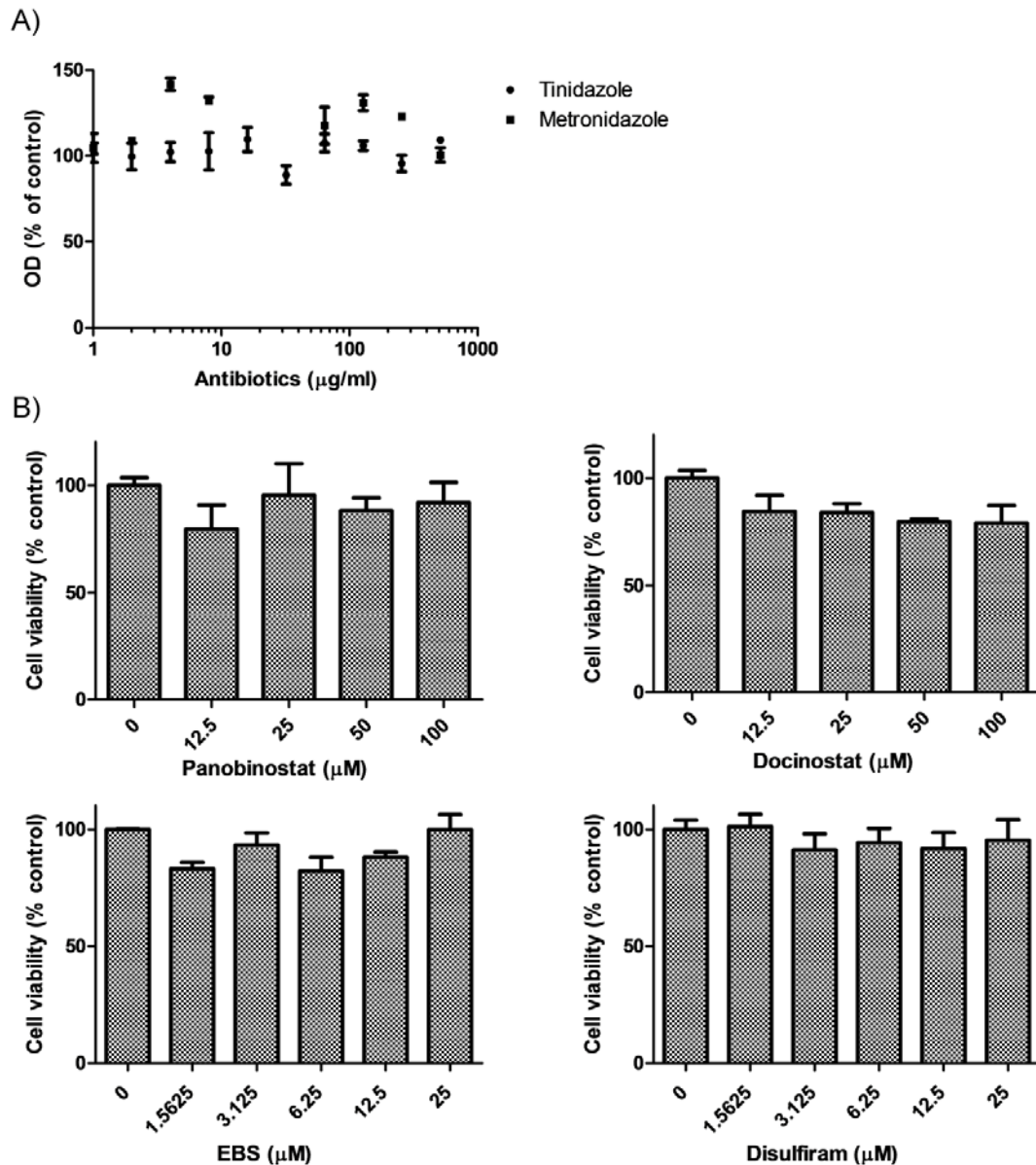
276 (METHODS). The results are shown as percentages of the control (DMSO, 100%). Mean

277 \pm SD (n=3). All experiments were independently repeated at least twice, and one

278 representative result is presented.

279

280



281

282 **Figure S7. The effects of inhibitors on the cell viability of gastric SGC-7901 cells and**

283 **antibiotic resistance of the *H. pylori* strain. (A)** The *H. pylori* strain is resistant to

284 treatment with tinidazole or metronidazole. Various concentrations of tinidazole or

285 metronidazole were incubated at 37 °C with *H. pylori* for 72 h under standard culture

286 conditions, and the OD at 600 nm was recorded using a spectrophotometer to determine

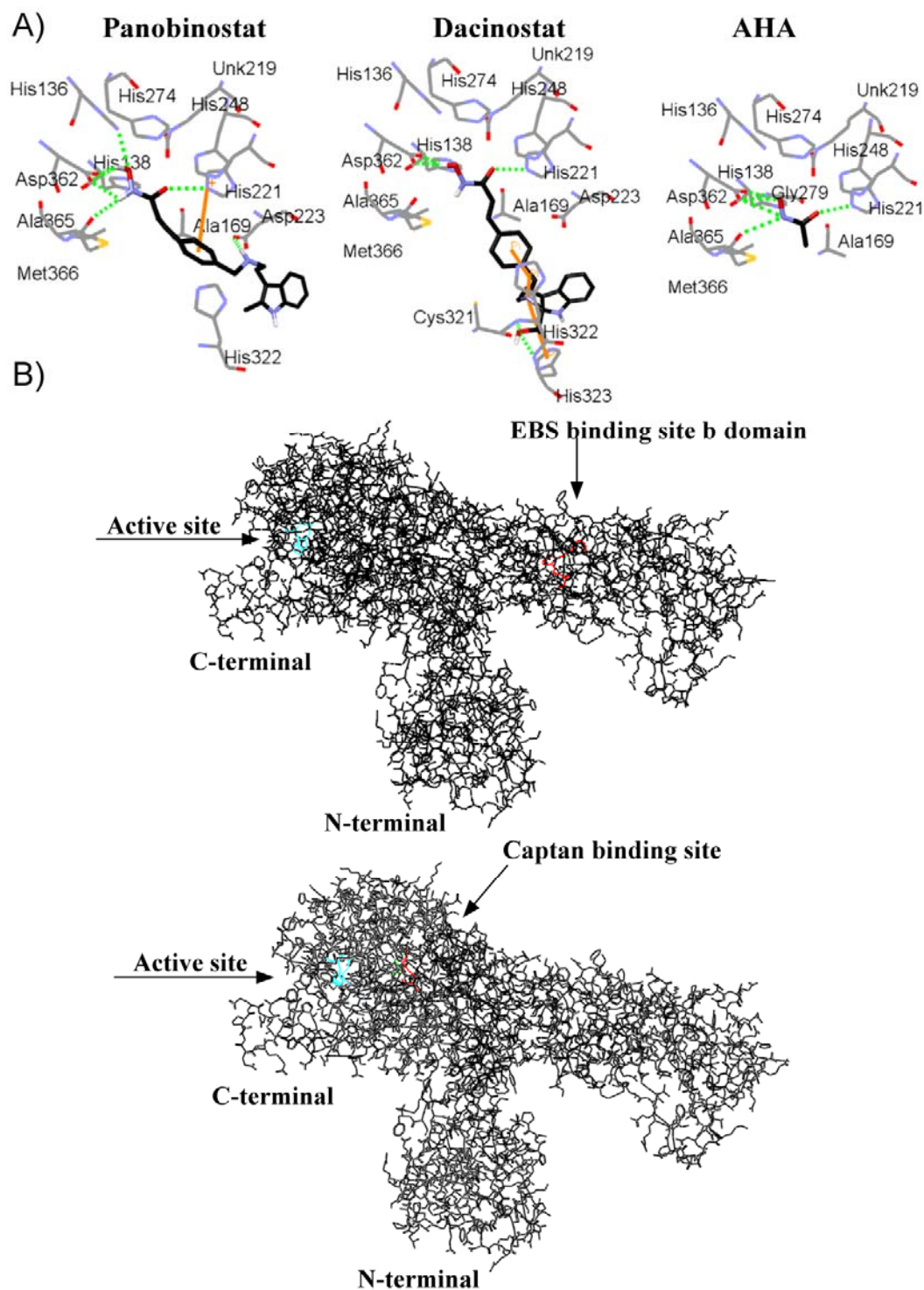
S22

287 the cell growth of *H. pylori* (METHODS). **(B)** The effects of urease inhibitors on the
288 viability of mammalian cells. SGC-7901 cells were incubated with DMSO, the indicated
289 concentrations of panobinosta, dacinostat, EBS or disulfiram for 24 h in a 96-well plate
290 before measurement of cell viability using the CellTiter96® Aqueous One Solution Cell
291 Proliferation Assay (Promega, Madison, WI). The results are shown as percentages of the
292 control (DMSO, 100%). Means \pm SDs (n=3). All experiments were independently
293 repeated at least twice, and one representative result is presented.

294

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298 **Figure S8. The binding modes of inhibitors in ureases.** (A) The putative binding mode

299 of panobinostat (black) or dacinostat (black) in the HPU active site. Panobinostat and

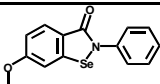
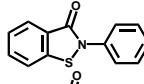
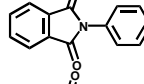
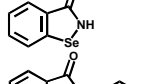
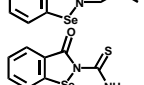
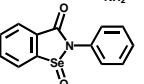
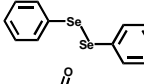
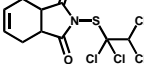

S24

300 dacinostat were docked into the HPU crystal structure (PDB code 1E9Y; ref. (6)) using
301 the Discovery Studio software. Residues surrounding the inhibitor within a distance of
302 3.5 Å are shown in gray or in the default atom color. **(B)** Global view of the binding
303 region of EBS (upper panel) and captan (lower) in JBU. In the modeled EBS or captan
304 and protein complex structure (METHODS and Figure 3E), the protein is shown in black,
305 the key residues (His492 and His519) in the active site of JBU in cyan and the inhibitors
306 as well as its attached Cys residue (Cys313 for EBS, Cys406 for captan; Figure 3E) in red.
307 Hydrogen bonds are represented as green dotted lines.

308

309

310 **Table S1. Chemical structures and IC₅₀ values of EBS or captan analogs for ureases.**

Name	Structure	IC ₅₀ (μM); HPU	IC ₅₀ (μM); JBU	IC ₅₀ (μM); OAU
1		2.0 ± 0.9	0.3 ± 0.007	7.5 ± 0.6
2		> 10.0	1.0 ± 0.002	4.9 ± 1.1
3		> 10.0	> 10.0	> 10.0
4		1.1 ± 0.08	0.8 ± 0.008	2.2 ± 0.1
5		> 10.0	1.4 ± 0.03	5.3 ± 0.9
6		1.3 ± 0.4	0.3 ± 0.04	1.7 ± 0.1
Ebselen Oxide		1.5 ± 0.2	0.4 ± 0.005	3.3 ± 0.1
Dibenzyl diselenide		> 10.0	> 10.0	> 10.0
Captafol		> 10.0	8.8 ± 0.2	9.1 ± 1.1

311

312

313 **Table S2. The minimal inhibitory concentration of urease inhibitors or known**
 314 **antibiotics for inhibiting *H. pylori* and their IC₅₀ values in the *in cellulo* urease**
 315 **assay.**

316

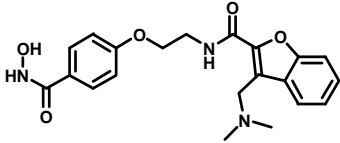
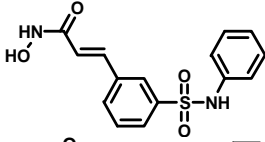
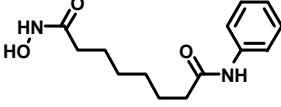
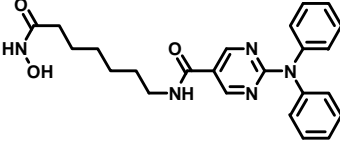
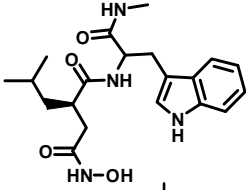
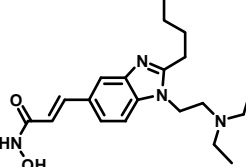
Compound	<i>H. pylori</i> (MIC)		<i>H. pylori</i> (IC ₅₀ values in the <i>in cellulo</i> urease assay; μ M)
	μ g/ml	μ M	
EBS	4	12.5	5.7 \pm 1.3
1	2	6.25	4.7 \pm 1.1
4	2	12.5	18.5 \pm 1.2
6	4	12.5	21.8 \pm 1.0
EBS Oxide	4	12.5	23.2 \pm 1.1
Captan	32	100	29.5 \pm 1.2
Disulfiram	4	12.5	36.3 \pm 1.0
Dibenzyl diselenide	> 64	> 200	> 200.0
AHA	> 16	> 100	-
Tinidazole	> 512	> 2000	-
Metronidazole	> 512	> 3000	-

317 MIC: minimal inhibitory concentration

318

319

320 **Table S3. Chemical structures and IC₅₀ values of hydroxamic acid-based analogs for**
 321 **ureases.**
 322

Name	Structure	IC ₅₀ (μM); HPU	IC ₅₀ (μM); JBU
Abexinostat		1.3 ± 0.2	1.4 ± 0.3
Belinostat		3.2 ± 0.2	4.7 ± 0.5
Vorinostat		14.0 ± 3.9	4.1 ± 1.9
Ricolinostat		> 20.0	> 20.0
Ilomastat		> 20.0	> 20.0
Pracinostat		> 10.0	> 20.0
Hydroxylamine	H ₂ N-OH	> 20.0	> 20.0

323

324

325 **Table S4. Primer sequences.**

No.	Primer	Usage
1	5'- AGAGTTTGATCCTGGCTCAG-3'	5' primer for 16S rRNA
2	5'- AAGGAGGTGATCCAGCCGCA-3'	3' primer for 16S rRNA
3	5'- ATTAATCATTAGATGTATGGCCCTACTACAGGCG-3'	5' primer for UreB
4	5'- AATATACTCGAGCTAGAAAATGCTAAAGAGTTG-3'	3' primer for UreB

326

327

328 **Reference**

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- 345
- 346