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Structure based virtual screening-driven identification of monastrol as a potent urease inhibitor

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ABSTRACT

Virtual screening uses computer based methods to discover new ligands on the basis of biological structures. Among all virtual screening methods structure based docking has received considerable attention. In an attempt to identify new ligands as urease inhibitors, structure-based virtual screening (SBVS) of an in-house database of 10,000 organic compounds was carried out. The X-ray crystallographic structure of *Bacillus pasteurii* (BP) in complex with acetohydroxamic acid (PDB Code 4UBP) was used as a protein structure. As a starting point, ~10,000 compounds of our in-house database were analyzed to check redundancy and the compounds found repeated were removed from the database. Finally 6993 compounds were docked into the active site of BP urease using GOLD and MOE-Dock software. A remarkable feature of this study was the identification of monastrol, a well-known KSP inhibitor already in clinical trials, as a novel urease inhibitor. The hits identified were further evaluated by molecular docking and on examination of the affinity predictions, twenty-seven analogs of monastrol were synthesized by a multicomponent Biginelli reaction followed by their *in vitro* screening as urease inhibitors. Finally twelve compounds were identified as new urease inhibitors. The excellent *in vitro* activity suggested that these compounds may serve as viable lead compounds for the treatment of urease related problems.

1. Introduction

Computational approaches have become a key component of many drug discovery programs both in academia and industry. Over the last few decades, modern computer aided drug design tools established a novel platform for researchers. Time and cost required for chemical synthesis and *in vitro* testing has been dramatically reduced due to these computational technologies [1–4]. Virtual screening (VS) or *in silico* screening, is an increasingly used method for the identification of novel leads from large chemical libraries. Both ligand-based virtual screening (LBVS) and structure-based virtual screening (SBVS) protocols have emerged as popular tools in early drug discovery and development process and among all VS methods, protein-structure-based docking has received significant attention. Structure-based virtual screening (also known as molecular docking) is most commonly employed for the prediction of binding modes and binding affinities of each compound in the dataset by means of high-throughput docking to an X-ray structure or model of the target receptor [5–9].

The hydrolytic enzyme urease (amidohydrolase, EC 3.5.1.5), a nickel containing metalloenzyme, is responsible for the catalytic hydrolysis of urea to volatile ammonia and carbon dioxide. The enzyme releases ammonia and carbamate, which in turn spontaneously generates the products [10–12]. Interestingly, both these molecules (urea and urease) symbolize milestone molecules in initial biological research. The very first organic compound synthesized was urea and the first enzyme which was crystallized in history was urease [13,14]. Furthermore, this enzyme was identified as metalloenzyme as it contains nickel atom [15].

For its enzymatic hydrolysis, urease utilizes an active site containing a binuclear nickel center bridged by a carbamylated lysine and a hydroxide ion as shown by the crystallographic structures resolved for bacterial ureases from *Klebsiella aerogenes*, *Bacillus pasteurii* and *Helicobacter pylori* [16–18]. These ureases have a nearly superimposable active site, which implies that it is common to all ureases, including jack bean urease whose crystallographic structure has not been resolved to date. A number of proposals including computational studies have been made regarding its possible reaction mechanism and consensus has been reached regarding the

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initial coordination of urea in its active site through the urea oxygen attacking the vacant coordination site on nickel atom, however, there are divergent proposals regarding the subsequent steps [19–25].

Urease is involved in human and animal pathogenicity of hepatic encephalopathy, hepatic coma urolithiasis, pyelonephritis, gastric and peptic ulcers, and urinary catheter encrustation are caused by ammonia produced by ureases [10,16]. Urease inhibitors can be considered as a tool to control the damaging effect of ureolytic bacterial infections in humans which occur commonly in the developed countries [26]. There are a number of classes of compounds that are known to show significant inhibitory activity against urease enzyme. Urease inhibitors can be broadly classified into two categories: (1) organic compounds and (2) organometallic compounds. The former category includes hydroxamic acid and its derivatives [27,28], triazoles, coumarines [29–31], isatins, semicarbazones [32], Schiff bases [33,34], urea derivatives [35,36], oxadiazoles [37], and piperidines [38]. The latter category includes organophosphorous/phosphinic inhibitors [26,39], phosphoramides [40] and metal complexes of organotin(IV), vanadium(IV), bismuth(III), copper(II), cadmium(II) copper(II) and zinc(II) [41–46]. Besides, some natural products such as gallocatechin, a naturally occurring flavonoid has been reported as inhibitors of *H. pylori* urease [47].

All available structurally diverse classes of urease inhibitors are not satisfactorily significant to the binding cavity as they cannot interact with the key amino acid residues and the metal ions present in the urease active site. Therefore, the search of new and more effective urease inhibitors to diversify the current urease inhibitors is essential. We have extensively studied the binding modes and QSAR of different new classes (both from natural and synthetic origin) of urease inhibitors in our ongoing drug discovery program. The identified inhibitors were studied for their binding affinities and structure-activity relationships using state-of-the-art computational techniques [48,49]. In continuation to discovering new and diverse classes of compounds as drug candidates, we carried out a SBVS experiment on an in-house database of compounds and identified monastrol, a 3,4-dihydropyrimidine-2-thione derivative, as a potent candidate for urease inhibition. Mayer et al. reported the screening of a large library of compounds where racemic monastrol was identified as KSP inhibitor (IC_{50} = 30 nM) and hence may be considered as a lead for the development of new anticancer drugs [50]. During the drug discovery paradigm it is quite often recommended to start the designing of a drug from a compound that already shows some activity against a given receptor or for a given disease. Since monastrol is a dihydropyrimidine (DHPM) derivative that may also be classed as cyclic thiourea, therefore, it was logical that DHPM scaffold may be a promising candidate for urease inhibition.

2. Results and discussion

2.1. Virtual screening

The three dimensional coordinates of the target protein (PDB ID 4UBP) was retrieved from protein data bank. All water molecules were removed from the protein and the 3D protonation of the protein molecule was carried out using MOE. The protein molecule was subjected to energy minimization using energy minimization algorithm implemented in MOE with default parameters. The minimized protein structure was then used for further study.

With the aim to identify novel and potent urease inhibitors VS of in-house database against urease enzyme was carried out. In a previous study a 3D-structure database of six thousand compounds was developed. In the present report 3D structures of new four thousand compounds were modeled and energy minimized using energy minimization algorithm implemented on Molecular Operating Environment (MOE) and were saved in a separate database. Both these sets of compounds were then added to MOE database.

SBVS experiment was then performed using 10,000 compounds present in our in-house database against urease. Before SBVS experiment, the compounds of the database were checked for redundancy and repeated compounds were removed from the database. Then, Lipinski's rule-of-five (Ro5) was applied on the database for drug-likeness. As a result of this filtering protocol 6993 compounds were found to be drug-like compounds. Finally 6993 compounds were docked by MOE-Dock and GOLD docking programs separately.

2.2. Molecular docking

As a first step, the ability of the docking algorithm was validated to reproduce the co-crystallized pose of three known urease inhibitors (*e.g. bis*-coumarin, imidazol and hydroxamic acid) in the urease pocket. This yielded a good agreement between the docked and crystal structures as these studies of the known inhibitors further confirmed the interaction region of the ligands and a favorable interaction with urease. As the next step, we evaluated whether this approach was agreeable to distinguish between known inhibitors and randomly chosen 3000 drug-like decoys from our in-house database. To this end we docked the 'validation set or decoy set' into the binding site of urease, as a positive control. Two separate docking runs were carried out, one with MOE-Dock and the other with GOLD docking program [51,52]. There were three possible scoring functions in MOE-Dock *i.e.* London dG, affinity dG and ASE, while two scoring functions of Gold were applied. In addition to scoring functions there were several other parameters needed to be set for attaining appropriate binding pose and ranking. For example, it was observed that out of few available placement methods in MOE dock (Triangle Matcher, Alpha Triangle, Alpha PMI, Proxy Triangle, FlexX and pharmacophore) only Triangle Matcher was found satisfactory in order to reproduce the binding mode of one of the known inhibitors *i.e.* acetohydroxamic acid, which was retrieved from Protein Data Bank (PDB ID 4UBP) [53]. Similarly, every scoring function implemented in docking programs was evaluated to analyze the ability to place the correct docking pose in the top rankings. London dG scoring function implemented in MOE dock was able to place all known inhibitors within the top 1% of the whole decoy set tested. Gold fitness function in GOLD docking program also exhibited similar enrichment like London dG in MOE dock.

The next phase of the screening process involved the docking of each molecule in the database into the binding site of the target. This docking process employed the prediction of favorable conformation of the ligand in the target binding site.

The *in silico* successful compounds were docked at the putative urease active site to examine if they fulfilled the requirements presented in the search queries in their docked conformations. Two independent docking experiments were carried out by using default parameters (unless explicitly mentioned) with MOE-Dock and GOLD docking program separately.

Applying the post-docking filtering strategy of both Gold and MOE, 200 compounds were identified as the top ranked hits which led to the identification of few structurally similar compounds ranked in the top list.

All the selected compounds were visually inspected based on our previous dry and wet lab experience for improbable docking orientations in the active site of urease. The resulting compounds were further analyzed for their interactions with the key amino acid residues such as Ala170. His137, His139, Lys220, His249, His275, Gly280, Cys322, His323, His324, Arg339, Ala363, Asp363, thereby, short listing those compounds which showed the known key

Table 1
Scoring functions and IC_{50} of initial hits [29].

S. no.	Compounds	Fitness score		IC50 (μM)
		GOLD	MOE	
1	AAB429a	56.28	−13.4425	110.0 ± 3.06
2	AAB539[a]	41.38	−11.0070	238.5 ± 2.04
3	AAD355[a]	55.93	−15.6482	31.2 ± 0.47
4	8d[a]	60.59	−10.8738	34.10 ± 1.24
5	14i[a]	47.20	−09.0359	150.23 ± 2.10
6	UR A2 (monastrol)	**54.00**	**−13.2499**	**11.76 ± 1.66**
7	FBTZ1	66.00	−09.0144	17.67 ± 1.09
8	FBTZ2	48	−08.3252	45.0 ± 2.43
9	Thiourea	ND[b]	ND[b]	21.0 ± 0.01

[a] Previously reported.
[b] ND: not determined.

interactions with active site residues and Ni798 and Ni799 present in the binding site. As a result, thereof, several compounds were eliminated during docking and post docking filtering strategies [54].

2.3. In vitro urease inhibition studies

The *in vitro* urease inhibitory activity of the rigorously screened twenty candidates was carried out according to literature procedure [55] using thiourea as standard. Seven compounds showed urease inhibitory activity with IC_{50} values ranging from 31.2 to 238.16 μM. The scoring functions and IC_{50} of initial hits are given in Table 1.

The observed activities of compounds AAB429, AAB539, AAD355, 8d and 14i [29,56–58] were 110.0 ± 3.06 μM, 238.16 ± 2.04 μM, 31.2 ± 0.47 μM, 34.10 ± 1.24 μM, and 150.23 ± 2.10 μM respectively. The structures of initial hits are provided as supporting information. Among the eight hits identified AAB429, AAB539, AAD355 were commercially available while the details of other two synthetic compounds (FBTZ1 and FBTZ2) will be reported in a separate publication. This screening resulted in a new class of compounds called dihydropyrimidines and monastrol (URA2) was a member of this important class of heterocycles. We chose UR A2 as the scaffold for further investigation based on following rationale: (1) reasonably active compound (IC_{50} = 11.76 μM) and (2) easy availability of starting materials and (3) one step synthetic derivitization through one pot Biginelli reaction.

2.4. Chemistry

Having identified monastrol as the initial hit in SBVS experiment, a variety of DHPM derivatives were synthesized through one pot Biginelli reaction [59] for exploration of SAR around all the six diversity points of DHPM scaffold. These nonplanar heterocyclic compounds have received considerable attention of the pharmaceutical industry because of their interesting multifaceted pharmacological profiles. Following the Biginelli strategy, we carried out a sonocatalyzed synthesis of three sets of DHPM

Table 2
Inhibition of urease activity by monastrol derivatives of Sets **1**–**3**.

No.	R^1	R^2	R^3	R^4	X	IC_{50} (μM)
Set **1**						
1	H	C_6H_5	OEt	Me	S	61.56
2	H	C_6H_5	Me	Me	S	5.6
3	H	3-Br·C_6H_4	OEt	Me	S	5.36
4	H	3-OMe·C_6H_4	OEt	Me	S	5.4
5	H	3-OH·C_6H_4	OEt	Me	S	11.76
6	H	3-OH·C_6H_4	Me	Me	S	29.93
7	H	3-OMe,4-OH·C_6H_3	OEt	Me	S	31.26
8	H	3-OH·C_6H_4	OEt	OEt	S	NI
9	H	3-OH·C_6H_4	OEt	CF_3	S	NI
10	H	4-OMe·C_6H_4	OEt	Me	S	15.16
11	H	4-NMe_2·C_6H_4	OEt	Me	S	133.03
12	H	3-Thienyl	OEt	Me	S	NI
Set **2**						
13	H	C_6H_5	OEt	Me	O	22.76
14	H	C_6H_5	Me	Me	O	NI
15	H	C_6H_5	Ph	Ph	O	NI
16	H	3-OH·C_6H_4	OEt	Me	O	NI
17	H	3-OMe·4-OH·C_6H_3	OEt	Me	O	NI
18	H	3-OMe·4-OH·C_6H_4	Ph	Ph	O	41.3
19	H	4-NMe_2·C_6H_4	OEt	Me	O	NI
20	H	4-NMe_2·C_6H_4	OEt	OEt	O	58.26
21	H	4-OH·C_6H_4	OEt	OEt	O	425.06
22	H	4-Cl·C_6H_4	OEt	Me	O	NI
23	H	3-Pyridyl	OEt	Me	O	NI
24	Me	C_6H_5	OEt	Me	O	NI
25	CH_2·C_6H_5	C_6H_5	OEt	Me	O	NI
Set **3**						
26	H	4-OMe·C_6H_4	OEt	OEt	–	NI
27	H	3-OMe, 4-OH·C_6H_3	OEt	Me	–	NI

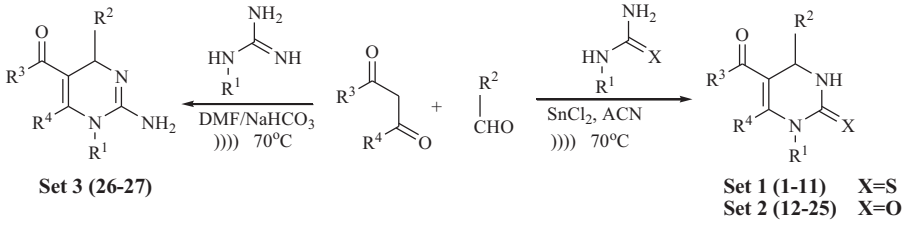
NI: no inhibition observed.

derivatives. The use of ultrasound waves has gradually been introduced in organic synthesis as a green alternative over the last three decades [60]. A large number of organic reactions can be carried out under milder conditions, in shorter reaction times and providing higher yield by ultrasound irradiation. This one pot cyclocondensation of three building blocks of Biginelli reaction led to different elements of diversity at C-2, C-4, C-5 and C-6 as shown in Scheme 1.

2.5. In silico studies

After careful inspection of the initial results, supported by virtual docking experiments, we initially focused on the synthesis of monastrol analogs with different substituents at *meta*-position of the C-4 phenyl ring and measured their capacity to inhibit urease. The results of urease inhibition of these compounds are shown in Table 2.

As mentioned earlier, monastrol (5) was found as one of the initial hits with IC_{50} 11.76 μM in SBVS experiment, however, its synthetic analogs 3 and 4 with 3-bromophenyl and 3-methoxyphenyl respectively, were found to be stronger urease inhibitors with IC_{50} 5.36 μM and 5.4 μM respectively. Moreover, of the two regioisomeric DHPMs 4 and 9, the *m*-isomer (IC_{50} 5.4 μM)



Scheme 1. Synthesis of compounds **1**–**27**.

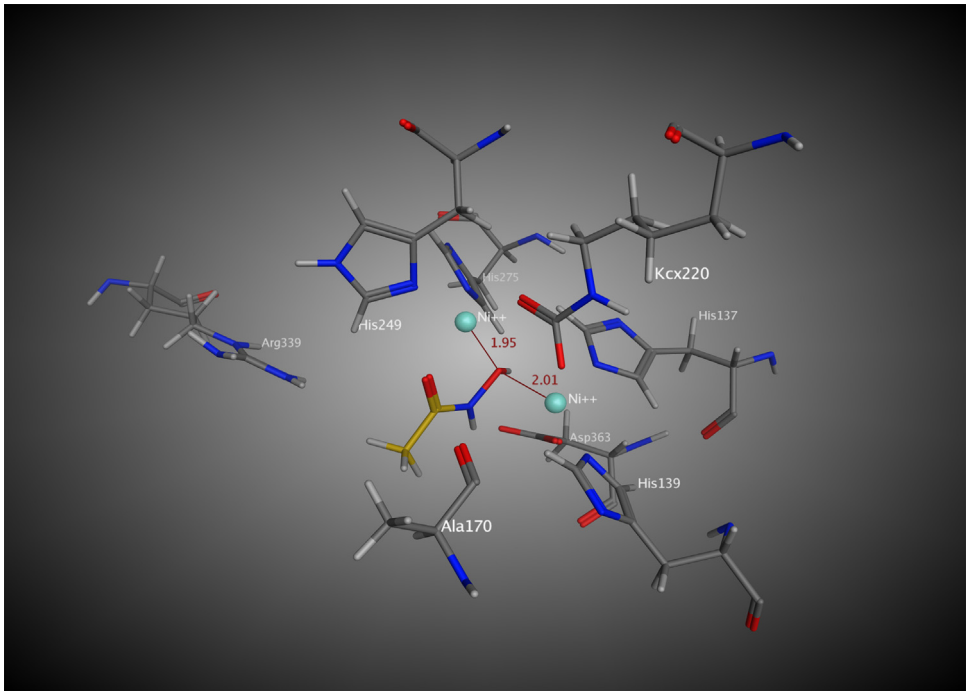


Fig. 1. Modeled mode of binding of acetohydroxamic acid (HAE800) in 4UBP active site.

was found to be more active than the p-isomer (IC_{50} = 15.16 μM). It may, therefore, be generalized that 3-substituted DHPMs with a thiocarbonyl group at C2 position exhibited stronger urease inhibitory activity with the exception of unsubstituted DHPM 2 (IC_{50} = 5.6 μM). These compounds are more potent as compared to the standard *i.e.* thiourea (IC_{50} of 21 μM). However, DHPM-2-ones were found to be far less active than their thione counterparts (Table 1), while 2-amino-4-aryl-1,4-dihydropyrimidines (Set 3) were not found to have any urease inhibitory activity.

All DHPMs that were evaluated for their potential as urease inhibitors were docked in BP urease. *In vitro* urease inhibition studies were carried out using thiourea as standard, however, as its X-ray crystallographic structure in complex with Jack bean urease is not present in Protein Data Bank, therefore, the structure of the complex of acetohydroxamic acid (HAE) and BP urease was used to perform the docking simulation study. The active site of urease is located within the cavity or the crevice in its internal territory in which HAE molecule chelates with two nickel ions *via* hydroxyl oxygen as shown in Fig. 1.

A comparison of ligand–enzyme complex of monastrol (5) with crystallographic complex of HAE revealed a resemblance in the fitting-in mechanism of the two compounds. The coordination pattern of compound 5 *via* its 3-hydroxyphenyl group at C4 mimicked the coordination of HAE, and similar active site residues, as those in HAE, were observed to stabilize the enzyme ligand complex (Fig. 2).

It is well documented in literature that the pharmacological activity of DHPMs is dependent on the absolute configuration at stereogenic center at C-4 and the control of the stereochemistry at C-4 has essential importance as it acts like a molecular gear of chiral regulation during drug-receptor recognition [61,62]. For example, it is exclusively the (S)-monastrol that shows KSP inhibition [63]. The access to enantiomerically pure DHPMs is, therefore, a prerequisite for the development of useful drugs of this structural type.

Since the present study encompasses the synthesis and *in vitro* evaluation of racemic DHPMs as urease inhibitors, exploiting the predictive power of *in silico* tool, we were interested in exploring the probable binding modes of enantiomerically pure (R) and (S) DHPMs into the active site of urease. Therefore, molecular docking studies were carried out on both enantiomers of the synthesized DHPMS using GOLD. It was interesting to note that with only few exceptions, the (S) isomer was found to have a higher GOLD fitness score compared to its (R) isomer. For example, Gold score for S-monastrol was found to be 58.43 compared to its (R) enantiomer *i.e.* 53.95 which reflects a stronger binding of the (S) isomer in the active site of 4UBP due to the formation of a more stable complex with both Ni798 and Ni799 atoms with 3-OH substituent.

Moreover, the orientation of the aryl ring in the active site also seems to play an important role in stabilizing ligand–enzyme complex. A careful glance at the superimposed docking poses of both (R) and (S) monastrol reveals that the phenyl group anchors itself in such a way that enables a stronger interaction of 3-OH group with both Ni798 and Ni799 while similar interactions are not possible in the (R) isomer (Fig. 3).

Parallel studies when conducted on inactive compounds, for example compound 14, revealed that there was no Ni metal ligation in the ligand–enzyme complex which appeared to be essential for stabilizing a ligand–enzyme complex (Fig. 4).

3. Conclusion

This paper presents the first report on the identification of monastrol (a KSP inhibitor) as a strong candidate for urease inhibition in a SBVS experiment. A variety of monastrol derivatives were synthesized which were found to be even stronger urease inhibitors than thiourea. It is, therefore, anticipated that this scaffold may serve as a lead compound and its further modification could give rise to improved inhibitory activity and for developing antiulcer drugs. Having these *in vitro* results, *in vivo* studies on animal models will also be carried out.

4. Materials and methods

4.1. Virtual screening

Recently we have developed our in-house database of ~10,000 compounds. The compounds included in our dataset were either

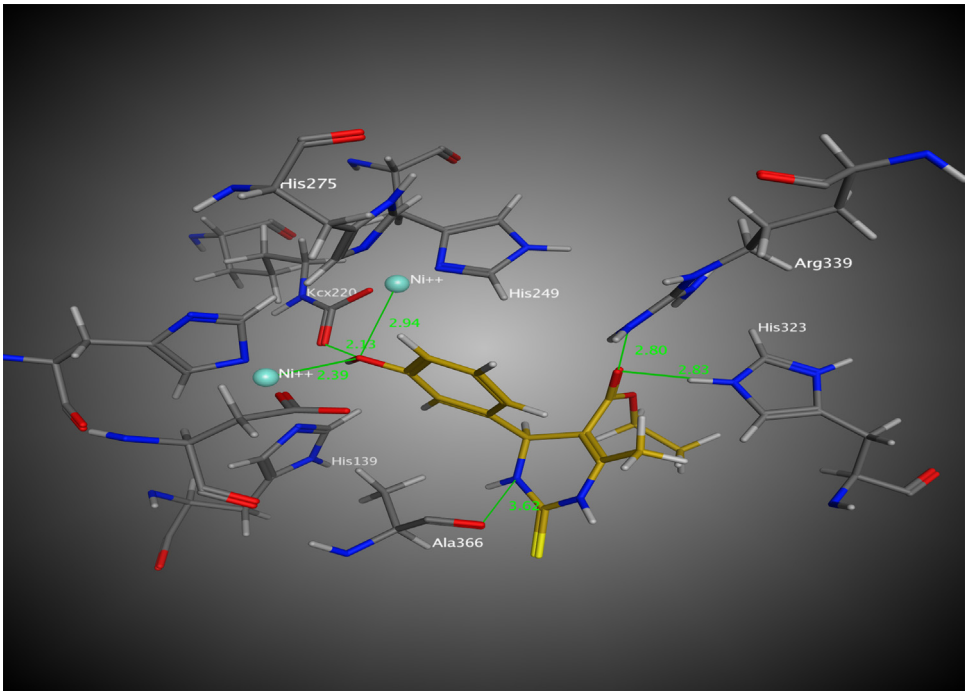


Fig. 2. Molecular level interactions of DHPM 5 (monastrol) in the active site of 4UBP.

of synthetic or natural sources. All these compounds are structurally well elucidated and most of them fulfill the drug-like criteria according to Lipinski rule of five with the exceptions of naturally isolated compounds. In a previous report, a 3D-structure database of six thousands compounds of an in-house database was developed. During present study, two-dimensional structures of over four thousand additional compounds were received from our compound bank initially in the CDX format that was later converted to MOL2 format using Molecular Operating Environment (MOE) software and was saved in a separate database. By using the "washing" module of MOE physiologically relevant protonation state of all the compounds was produced.

A number of X-ray crystallographic structures of urease are available in the Protein Data Bank (PDB). All the structures of urease enzyme were retrieved from the PDB and extensively studied for the selection of suitable structure for the structure-based virtual screening. The criteria used for the selection of best X-ray structure were: (1) highest resolution, (2) complex with potent inhibitors, and (3) explaining the geometry of nickel ions and their interactions with the inhibitor. On the basis of these criteria the X-ray

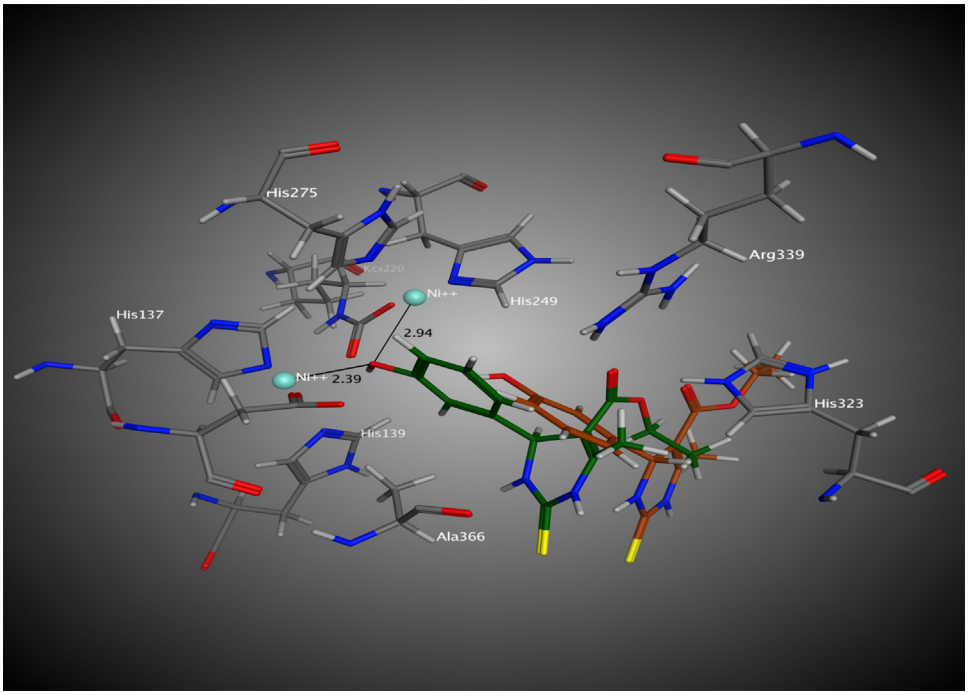


Fig. 3. Superimposition of monastrol (5), S (green) and R (brown) at the putative active site of urease. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

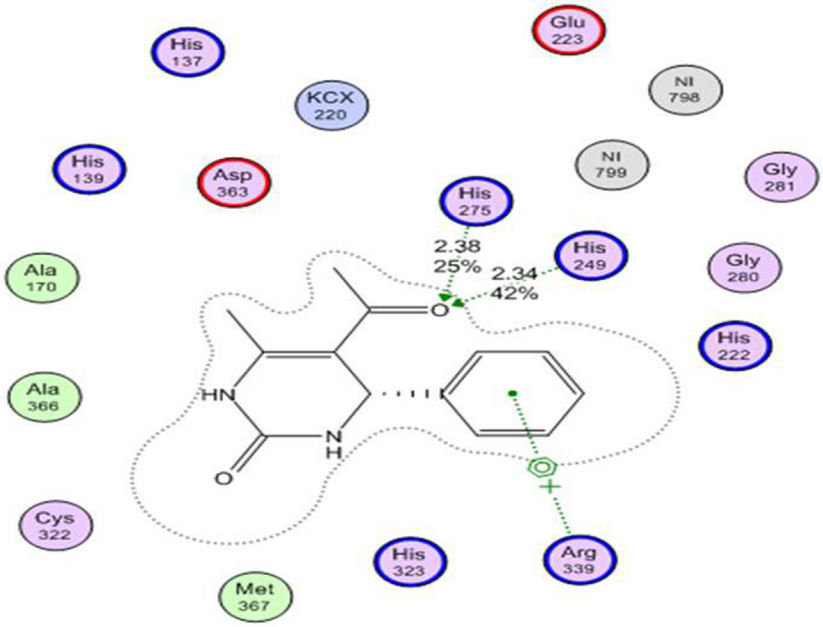


Fig. 4. Binding interactions of DHPM 14 at urease active site.

crystallographic structure of *B. pasteurii* (BP) urease in complex with acetohydroxamic acid was selected for structure-based virtual screening. The resolution of this structure (1.55 Å) was found to be the highest among all the X-ray structure of BPU. This complex structure was prepared for molecular docking by removing solvent molecules followed by 3D protonation and energy minimization using MOE.

4.2. Molecular docking

MOE-Dock tool was employed to explore suitable binding conformations between small compounds and a macromolecular protein. It uses a Monte Carlo Simulated Annealing method for docking a ligand in the binding site of a receptor molecule. The program forms a 3D grid encompassing the center of the docking site. The target compounds were built using the builder interface of the MOE program and subjected to energy minimization. The crystal structure of protein complex urease with HAE was obtained from PDB (PDB ID 4UBP). The edited crystal structure after removing water molecules was imported into MOE and all hydrogen atoms were added to the structure with their standard geometry followed by their energy minimization using MOPAC 7.0. The resulting model was subjected to systematic conformational search at default parameters with RMS gradient of 0.01 kcal/mol using Site Finder. The enzyme was searched for its active site and dummy atoms were created from the resulting alpha spheres. The backbone and residues were kept fixed and the energy minimization was performed. Root mean square deviation (RMSD) values were used to compare the ligand between the predicted and its corresponding crystal structure. The resulting docked poses with RMSD less than 1.5 Å were clustered together and the lowest energy minimized pose was used for further analysis. All DHPMs were docked following the same protocol. Ten different conformations were selected for each ligand. All other parameters were maintained at their default settings. The best conformation of each of the ligand–enzyme complex was selected based on energetic grounds. The resulting ligand–enzyme complex model was then used for calculating the energy parameters using MMFF94x force field energy calculation and predicting the ligand–enzyme interactions at the active site.

The software GOLD uses a genetic algorithm (GA) to explore the full range of ligand conformational flexibility with the partial flexibility of protein. The GOLD scoring function includes the terms for hydrogen-bonding, vdW, and intramolecular energies. The vdW interactions for the protein-ligand complex are described by 8–4 potential. A 12–6 potential was used for the ligand steric energies that also includes the torsional energies and the active site with a 10 Å radius sphere was defined. For each independent GA run, a maximum number of 100,000 GA operations were performed on a single population of 100 individuals. Operator weights for crossover, mutation, and migration were set to 95, 95, and 10, respectively. To allow for poor nonbonded contacts at the start of each GA run, the maximum distance between hydrogen donors and fitting points was set to 4.0 Å, and non-bonded vdW energies were cut-off at 2.5 Å. Finally twenty top ranked poses were saved.

4.3. Chemistry

All the reagents and anhydrous solvents were purchased from standard commercial vendors and were used without any further purification.

Sonication was performed in Elma E 30 H (Germany) ultrasonic cleaner with a frequency of 37 KHz and a nominal power of 250 W. ^{1}H and ^{13}C NMR spectra were recorded in DMSO-d_6 on a Bruker spectrometer at 300 and 75 MHz respectively using tetramethylsilane (TMS) as internal reference. Chemical shifts are given in δ scale (ppm). Mass spectra were recorded on Agilent technologies 6890N Gas chromatography (GC) and an inert mass selective detector 5973 GC–mass spectrometer. Melting points were determined in open capillaries using Gallenkamp melting point apparatus (MP-D). The progress of all the reactions was monitored by TLC on 2.0 cm × 5.0 cm aluminum sheets precoated with silica gel 60F_{254} with a layer thickness of 0.25 mm (Merck).

4.3.1. General method for the synthesis of 3,4-dihydropyrimidine-2-ones and 3,4-dihydropyrimidine-2-thiones

A mixture of an aldehyde (10 mmol), a diamino compound (12 mmol), a dicarbonyl compound (10 mmol, mL), $SnCl_2{\cdot}2H_2O$ (10 mol%) and acetonitrile (10 mL) was mixed in a pyrex tube. The

mixture was then irradiated in ultrasonic bath at 70–75 °C. The reaction was monitored by TLC. After the completion of the reaction, the resulting precipitate was filtered and crude product was recrystallized from an appropriate solvent or purified through column chromatography.

4.3.1.1. 5-(Ethoxycarbonyl)-6-methyl-4-phenyl-3,4-dihydropyrimidin-2(1H)-thione (1). **1** was prepared according to the general procedure by using benzaldehyde, thiourea, ethyl acetoacetate. Yield: 74%. m.p. 217 °C [64,65]. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.10 (t, 3H, J = 7.2 Hz, OCH_2CH_3), 2.21 (s, 3H, CH_3), 4.01 (q, 2H, J = 7.2 Hz, OCH_2), 5.18 (d, 1H, J = 3.3 Hz, *CH*), 7.20–7.37 (m, 5H, *Ar-H*), 9.63 (br s, 1H, *NH*), 10.32 (br s, 1H, *NH*). ^{13}C NMR (75 MHz, DMSO-d_6): δ 14.3 (OCH_2CH_3), 18.5 (CH_3), 54.8 (*C*H-Ar), 59.9 (OCH_2), 100.4 (*C*-COOEt), 126.0–129.0 (C-aromatic), 147.4 (CH_3C=C), 164.2 (*C*OOEt), 181.6 (C=S); EIMS calculated for $C_{14}H_{16}N_2O_2S$ ($M^{+\bullet}$) 276.

4.3.1.2. 5-Aceto-6-methyl-4-phenyl-3,4-dihydropyrimidin-2(1H)-thione (2). **2** was prepared according to the general procedure by using benzaldehyde, thiourea, acetylacetone. Yield 69%. m.p. 183 °C [66]. ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.20 (s, 3H, CH_3), 2.29 (s, 3H, CH_3CO), 5.10 (d, 1H, J = 3.3 Hz, *CH*), 7.0–7.6 (m, 5H, *Ar-H*), 8.87 (br s, 1H, *NH*), 10.00 (br s, 1H, *NH*).). ^{13}C NMR (75 MHz, DMSO-d_6): δ 18.4 (CH_3), 28.7 (CH_3CO), 54.6 (*C*H-Ar), 101.4 (*C*-COMe), 125.0–129.0 (C-aromatic), 147.8 (CH_3C=C), 180.2 (C=S), 194.4 (*C*OMe); EIMS calculated for $C_{13}H_{14}N_2OS$ ($M^{+\bullet}$) 246.

4.3.1.3. 4-(3-Bromophenyl)-5-(ethoxycarbonyl)-6-methyl-3,4-dihydropyrimidin-2(1H)-thione (3). **3** was prepared according to the general procedure by using 3-bromobenzaldehyde, thiourea, ethyl acetoacetate. Yield 60%. m.p. 182 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.93 (t, 3H, J = 6.9 Hz, OCH_2CH_3), 2.20 (s, 3H, CH_3), 3.98 (q, 2H, J = 7.3 Hz, OCH_2), 5.18 (d, 1H, J = 3.3 Hz, *CH*), 6.61 (s, 1H, *Ar-H*), 6.67 (d, 2H, *Ar-H*), 7.12 (t, 1H, *Ar-H*), 9.60 (br s, 1H, *NH*), 10.52 (br s, 1H, *NH*). ^{13}C NMR (75 MHz, DMSO-d_6): 14.9 (OCH_2CH_3), 18.1 (CH_3), 53.4 (*C*H-Ar), 59.7 (OCH_2), 101.4 (*C*-COOEt), 119.7–144 (C-aromatic), 124.2 (C-aromatic-Br), 147.1 (CH_3C=C), 164.1 (*C*OOEt), 179.8 (C=S); EIMS calculated for $C_{14}H_{15}N_2O_2SBr$ ($M^{+\bullet}$) 355.

4.3.1.4. 5-(Ethoxycarbonyl)-4-(3-methoxyphenyl)-6-methyl-3,4-dihydropyrimidin-2(1H)-thione (4). **4** was prepared according to the general procedure by using 3-methoxybenzaldehyde, thiourea, ethyl acetoacetate. Yield 64%. m.p. 153 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.09 (t, 3H, J = 7.2 Hz, CH_3), 2.27 (s, 3H, CH_3), 3.80 (s, 3H, OCH_3), 4.10 (q, 2H, J = 7.1 Hz, OCH_2CH_3), 5.18 (d, 1H, J = 3.3 Hz, *CH*), 6.40 (s, 1H, *Ar-H*), 6.45 (d, 1H, *H*-aromatic), 6.50 (d, 1H, *Ar-H*), 9.37 (br s, 1H, *NH*), 10.25 (br s, 1H, *NH*). ^{13}C NMR (75 MHz, DMSO-d_6): δ 14.9 (OCH_2CH_3), 18.1 (CH_3), 53.7 (*C*H-Ar), 56.8 (OCH_3), 59.8 (OCH_2), 99.3 (*C*-COOEt), 119.6–140.0 (C-aromatic), 148.2, (CH_3C=C), 150.9 (C-aromatic-OCH_3), 165.1 (*C*OOEt), 182.3 (C=S); EIMS calculated for $C_{15}H_{18}N_2O_3S$ ($M^{+\bullet}$) 306.

4.3.1.5. 5-(Ethoxycarbonyl)-4-(3-hydroxyphenyl)-6-methyl-3,4-dihydropyrimidin-2(1H) thione (5). **5** was prepared according to the general procedure by using 3-hydroxybenzaldehyde, thiourea, ethyl acetoacetate. Yield 79%. m.p. 185 °C [67]. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.12 (t, 3H, J = 7.3 Hz, OCH_2CH_3), 2.27 (s, 3H, CH_3), 4.05 (q, 2H, J = 7.3 Hz, OCH_2), 5.09 (d, 1H, J = 3.3 Hz, *CH*), 6.67 (s, 1H, *Ar-H*), 6.86 (d, 2H *Ar-H*), 7.11 (t, 1H *Ar-H*), 9.61 (br s, 1H, *NH*), 9.96 (br s, 1H, OH) 10.29 (br s, 1H, *NH*). ^{13}C NMR (75 MHz, DMSO-d_6): δ 14.1 (OCH_2CH_3), 18.4 (CH_3), 54.1 (*C*H-Ar), 59.4 (OCH_2), 99.4 (*C*-COOEt), 122.7–141.0 (C-aromatic), 145.4 (CH_3C=C), 157.4 (*C*-OH), 164.5 (*C*OOEt), 182.6 (C=S); EIMS calculated for $C_{14}H_{16}N_2O_3S$ ($M^{+\bullet}$) 292.

4.3.1.6. 5-Aceto-4-(3-hydroxyphenyl)-6-methyl-3,4-dihydropyrimidin-2(1H)-thione (6). **6** was prepared according to the general procedure by using 3-hydroxybenzaldehyde, thiourea, acetylacetone. Yield 69%. m.p. 191 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.20 (s, 3H, CH_3), 2.29 (s, 3H, CH_3), 5.34 (d, 1H, J = 3.3 Hz, C*H*), 6.67 (s, 1H, *Ar-H*), 6.86 (d, 2H *Ar-H*), 7.11 (t, 1H, *Ar-H*), 9.68 (br s, 1H, *NH*), 9.96 (br s, 1H, OH), 10.42 (br s, 1H, *NH*). ^{13}C NMR (75 MHz, DMSO-d_6): δ 18.1 (*C*H$_3$), 29.4 (CH_3CO), 54.1 (*C*H-Ar), 101.0 (*C*-COMe), 123.0–143.5 (C-aromatic), 147.9 (CH_3C=C), 157.4 (*C*-OH), 181.7 (C=S), 194.4 (*C*OMe); EIMS calculated for $C_{13}H_{14}N_2O_2S$ ($M^{+\bullet}$) 262. CHNS Analysis calculated for $C_{13}H_{14}N_2O_2S$, C = 59.52; H = 5.38; N = 10.68; O = 12.20; S = 12.22 Found: C = 59.59%; H = 5.41%; N = 10.54%; O = 12.21; S = 12.25.

4.3.1.7. 5-(Ethoxycarbonyl)-4-(4-hydroxy-3-methoxyphenyl)-6-methyl-3,4-dihydropyrimidin-2(1H)-thione (7). **7** was prepared according to the general procedure by using vanillin, thiourea, ethyl acetoacetate. Yield 68%. m.p. 210 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.03 (t, 3H, J = 7.2 Hz, CH_3), 2.28 (s, 3H, CH_3), 3.70 (s, 3H, OCH_3), 4.18 (q, 2H, J = 7.5 Hz, OCH_2CH_3), 5.30 (d, 1H, J = 3.6 Hz, *CH*), 6.40 (s, 1H, *Ar-H*), 6.45 (d, 1H, *H*-aromatic), 6.50 (d, 1H, *Ar-H*), 9.71 (br s, 1H, *NH*), 10.45 (br s, 1H, *NH*), 10.60 (s, 1H, *OH*). ^{13}C NMR (75 MHz, DMSO-d_6): δ 14.9 (OCH_2CH_3), 17.9 (CH_3), 53.1 (*C*H-Ar), 56.7 (OCH_3), 60.4 (OCH_2), 100.1 (*C*-COOEt), 119.7–139.0 (C-aromatic), 144.3 (C-aromatic-OH), 148.2, (CH_3C=C), 151.2 (C-aromatic-OCH_3), 165.3 (*C*OOEt), 180.6 (C=S); EIMS calculated for $C_{14}H_{18}N_2O_4S$ ($M^{+\bullet}$) 322.

4.3.1.8. 5-(Ethoxycarbonyl)-4-(3-hydroxyphenyl)-6-ethoxy-3,4-dihydropyrimidin-2(1H) thione (8). **8** was prepared according to the general procedure by using 3-hydroxybenzaldehyde, thioures, diethylmalonate. Yield 71%. m.p. 213 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.08 (t, 6H, J = 7.1 Hz, OCH_2CH_3), 4.18 (q, 4H, J = 7.1 Hz, OCH_2), 5.25 (d, 1H, J = 3.3 Hz, *CH*), 6.62 (d, 2H, J = 8.5 Hz, *Ar-H*), 7.05 (d, 2H, J = 8.5 Hz, *Ar-H*), 9.64 (br s, 1H, *NH*), 9.75 (s, 1H, OH) 10.40 (br s, 1H, *NH*). ^{13}C NMR (75 MHz, DMSO-d_6): 183.1 (C=S), 166.2 (C=O), 158.0 (*C*-OH), 156.7 (C=N), 129.6 (C-aromatic): EIMS calculated for $C_{15}H_{18}N_2O_4S$ ($M^{+\bullet}$) 322.

4.3.1.9. 5-(Ethoxycarbonyl)-4-(3-nitrophenyl)-6-(trifluoromethyl)-3,4-dihydropyrimidin-2(1H)-thione (9). **9** was prepared according to the general procedure by using 3-nitrobenzaldehyde, thiourea, ethyl trifluoroacetoacetate. Yield 71%. m.p. 221 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.12 (t, 3H, J = 7.2 Hz, CH_3), 4.03 (q, 2H, J = 7.2 Hz, OCH_2CH_3), 5.24 (d, 1H, J = 3.3 Hz, *CH*), 7.61 (m, 2H, *Ar-H*), 7.76 (br s, 1H, *NH*), 9.05 (br s, 1H, *NH*), 8.09 (m, 2H, *Ar-H*). ^{13}C NMR (75 MHz, DMSO-d_6): 14.7 (OCH_2CH_3), 52.6 (*C*H-Ar), 60.4 (OCH_2), 98.1 (*C*-COOEt), 119.7–144.1 (C-aromatic), 139.0 (C-CF_3), 146.0 (*C*-NO_2), 148.9, (CH_3C=C), 164.1 (*C*OOEt), 180.1 (C=S); EIMS calculated for $C_{14}H_{12}F_3N_3O_4S$ ($M^{+\bullet}$) 375.

4.3.1.10. 5-(Ethoxycarbonyl)-4-(4-methoxyphenyl)-6-methyl-3,4-dihydropyrimidin-2(1H)-thione (10). **10** was prepared according to the general procedure by using anisaldehyde, thiourea, ethyl acetoacetate. Yield 77%. m.p. 153 °C [68]. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.14 (t, 3H, J = 7.1 Hz, OCH_2CH_3), 2.29 (s, 3H, CH_3), 3.72 (s, 3H, OCH_3), 4.04 (q, 2H, J = 7.1 Hz, OCH_2), 5.17 (d, 1H, J = 3.3 Hz, *CH*), 7.22 (d, 2H, J = 8.6 Hz, *Ar-H*), 7.35 (d, 2H, J = 8.6 Hz, *Ar-H*), 9.61 (br s, 1H, *NH*), 10.39 (br s, 1H, *NH*). ^{13}C NMR (75 MHz, DMSO-d_6): δ 14.8 (OCH_2CH_3), 18.4 (CH_3), 54.2 (*C*H-Ar), 56.4 (OCH_3), 60.1 (OCH_2), 99.8 (*C*-COOEt), 119.0–141.0 (C-aromatic), 149.0, (CH_3C=C), 150.7 (C-aromatic-OCH_3), 165.4 (*C*OOEt), 182.1 (C=S); EIMS calculated for $C_{15}H_{18}N_2O_3S$ ($M^{+\bullet}$) 306.

4.3.1.11. 5-(Ethoxycarbonyl)-6-methyl-4-(4-N,N-dimethylphenyl)-3,4-dihydropyrimidin-2(1H)-thione (11). **11** was prepared according to the general procedure by using 4-(N,N-dimethyl)benzaldehyde, thiourea, ethyl acetoacetate. Yield 78%. m.p. 226 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.11 (t, 3H, J = 7.1 Hz, OCH_2CH_3), 2.25 (s, 3H, CH_3), 3.67 (s, 6H, $N(CH_3)_2$), 4.03 (q, 2H, J = 7.1 Hz, OCH_2), 5.34 (d, 1H, J = 3.3 Hz, *CH*), 7.12 (d, 2H, *Ar-H*), 7.60 (d, 2H, *Ar-H*), 9.63 (br s, 1H, *NH*), 10.36 (br s, 1H, *NH*). ^{13}C NMR (75 MHz, DMSO-d_6): 180.6 (C=S), 165.2 (C=O), 119–136 (C-aromatic), 44.2 (NCH_3): EIMS calculated for $C_{16}H_{21}N_3O_2S$ ($M^{+\bullet}$) 319.

4.3.1.12. 5-(Ethoxycarbonyl)-6-methyl-4-(3-thienyl)-3,4-dihydropyrimidin-2(1H)-thione (12). **12** was prepared according to the general procedure by using thiophene-3-carboxaldehyde, thiourea, ethyl acetoacetate. Yield 56%. m.p. 256 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.08 (t, J = 7.5 Hz, OCH_2CH_3), 2.25 (s, 3H, CH_3), 4.18 (q, 2H, J = 7.0, OCH_2), 5.18 (d, 1H, J = 3.3 Hz, *CH*), 6.66 (d, 1H, *Ar-H*), 7.00 (d, 1H, *Ar-H*), 9.58 (br s, 1H, *NH*), 10.42 (br s, 1H, *NH*). ^{13}C NMR (75 MHz, DMSO-d_6): δ 14.5 (OCH_2CH_3), 18.3 (CH_3), 54.6 (*C*H-Ar), 59.8 (OCH_2), 99.7 (*C*-COOEt), 121.0–138.0 (C-aromatic), 148.6, (CH_3C=C), 164.7 (*C*OOEt), 180.6 (C=S); EIMS calculated for $C_{12}H_{14}N_2O_2S_2$ ($M^{+\bullet}$) 282.

4.3.1.13. 5-(Ethoxycarbonyl)-6-methyl-4-phenyl-3,4-dihydropyrimidin-2(1H)-one (13). **13** was prepared according to the general procedure by using benzaldehyde, urea, ethyl acetoacetate. Yield 97%. m.p. 202 °C [64]. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.09 (t, 3H, J = 7.2 Hz, OCH_2CH_3), 2.21 (s, 3H, CH_3), 4.01 (q, 2H, J = 7.2 Hz, OCH_2), 5.14 (d, 1H, J = 3.3 Hz, *CH*), 7.22–7.33 (m, 5H, *Ar-H*), 7.76 (br s, 1H, *NH*), 9.22 (br s, 1H, *NH*) ^{13}C NMR (75 MHz, DMSO-d_6): δ 14.5 (OCH_2CH_3), 18.2 (CH_3), 54.4 (*C*H-Ar), 59.6 (OCH_2), 98.1 (*C*-COOEt), 126.7–129.0 (C-aromatic), 148.8 (CH_3C=C), 152.6 (C=O), 165.6 (*C*OOEt): EIMS calculated for $C_{14}H_{16}N_2O_3$ ($M^{+\bullet}$) 260.

4.3.1.14. 5-Aceto-6-methyl-4-phenyl-3,4-dihydropyrimidin-2(1H)-one (14). **14** was prepared according to the general procedure by using benzaldehyde, urea, acetylacetone. Yield 91%. m.p. 241–242 °C [69,70]. ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.07 (s, 3H, CH_3), 2.32 (s, 3H, CH_3CO), 5.10 (d, 1H, J = 3.3 Hz, *CH*), 7.69 (br s, 1H, *NH*), 9.20 (br s, 1H, *NH*), 7.0–7.6 (m, 5H, *Ar-H*). ^{13}C NMR (75 MHz, DMSO-d_6): δ 17.9 (CH_3), 29.4 (CH_3CO), 53.3 (*C*H-Ar), 101.1 (*C*-COMe), 120.2–127.0 (C-aromatic), 145.9 (CH_3C=C), 152.9 (C=O), 194.7 (*C*OMe); EIMS calculated for $C_{13}H_{14}N_2O_2$ ($M^{+\bullet}$) 230.

4.3.1.15. 5-Benzoyl-4,6-diphenyl-3,4-dihydropyrimidin-2(1H)-one (15). **15** was prepared according to the general procedure by using benzaldehyde, urea, dibenzoylmethane. Yield 67%. m.p. 213 °C [71]. ^{1}H NMR (300MHz, DMSO-d_6): δ 5.10 (d, 1H, J = 3.3 Hz, *CH*), 7.22–7.35 (m, 15H, *Ar-H*), 7.78 (br s, 1H, *NH*), 9.20 (br s, 1H, *NH*). ^{13}C NMR (75 MHz, DMSO-d_6): δ 53.6 (*C*H-Ar), 101.4 (*C*-COPh), 120.2–142.0 (C-aromatic), 145.9 (CH_3C=C), 152.9 (C=O), 192.4 (*C*OPh); EIMS calculated for $C_{23}H_{18}N_2O_2$ ($M^{+\bullet}$) 354.

4.3.1.16. 5-(Ethoxycarbonyl)-4-(3-hydroxyphenyl)-6-methyl-3,4-dihydropyrimidin-2(1H)-one (16). **16** was prepared according to the general procedure by using 3-hydroxybenzaldehyde, urea, ethyl acetoacetate. Yield 89%. m.p. 160–162 °C [72]. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.14 (t, 3H, J = 7.1 Hz, OCH_2CH_3), 2.18 (s, 3H, CH_3), 4.03 (q, 2H, J = 7.3 Hz, OCH_2), 5.07 (d, 1H, J = 3.3 Hz, *CH*), 6.61– 6.67 (m, 3H, *Ar-H*), 7.12 (t, 1H, 8.1 Hz, *Ar-H*), 7.72 (br s, 1H, *NH*), 9.16 (br s, 1H, *NH*), 9.3 (s, 1H, OH). ^{13}C NMR (75 MHz, DMSO-d_6): δ 14.1 (OCH_2CH_3), 18.0 (CH_3), 54.9 (*C*H-Ar), 59.2 (OCH_2), 98.7 (*C*-COOEt), 126.7–129.0 (C-aromatic), 147.6 (CH_3C=C), 152.1 (C=O), 157.3 (*C*-OH), 164.5 (*C*OOEt); EIMS calculated for $C_{14}H_{16}N_2O_4$ ($M^{+\bullet}$) 276.

4.3.1.17. 5-(Ethoxycarbonyl)-4-(4-hydroxy-3-methoxyphenyl)-6-methyl-3,4-dihydropyrimidin-2(1H)-one (17). **17** was prepared according to the general procedure by using vanillin, urea, ethyl acetoacetate. Yield 87%. m.p. 232–233 °C [68]. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.14 (t, 3H, J = 7.1 Hz, CH_3), 2.24 (s, 3H, CH_3), 3.73 (s, 3H, OCH_3), 4.10 (q, 2H, J = 7.1 Hz, OCH_2CH_3), 5.16 (d, 1H, J = 3.3 Hz, *CH*), 6.40 (s, 1H, *Ar-H*), 6.45 (d, 1H, *H*-aromatic), 6.50 (d, 1H, *Ar-H*), 7.79 (br s, 1H, *NH*), 8.97 (br s,1H, *NH*), 10.67 (s, 1H *OH*). ^{13}C NMR (75 MHz, DMSO-d_6): δ 14.3 (OCH_2CH_3), 18.2 (CH_3), 53.6 (*C*H-Ar), 56.4 (OCH_3), 60.1 (OCH_2), 101.4 (*C*-COOEt), 119.7–144. (C-aromatic), 144.1 (C-aromatic-OH), 147.9, (CH_3C=C), 151.3 (C=O), 151.2 (C-aromatic-OCH_3), 164.1 (*C*OOEt); EIMS calculated for $C_{15}H_{18}N_2O_5$ ($M^{+\bullet}$) 306.

4.3.1.18. 5-Benzoyl-4-(4-hydroxy-3-methoxyphenyl)-6-phenyl-3,4-dihydropyrimidin-2(1H)-one (18). **18** was prepared according to the general procedure by using vanillin, urea, dibenzoylmethane. Yield 66% 74%. m.p. 222 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 3.73 (s, 3H, OCH_3), δ 5.10 (d, 1H, J = 3.3 Hz, *CH*), 6.40 (s, 1H, *Ar-H*), 6.45 (d, 1H, *H*-aromatic), 6.50 (d, 1H, *Ar-H*), 7.22–7.35 (m, 10H, *Ar-H*), 7.63 (br s, 1H, *NH*), 9.10 (br s, 1H, *NH*), 10.12 (s, 1H, OH). ^{13}C NMR (75 MHz, DMSO-d_6): δ 54.1 (*C*H-Ar), 101.2 (*C*-COPh), 144.1 (C-aromatic-OH), 146.5 (CH_3C=C), 151.2 (C-aromatic-OCH_3), 152.4 (C=O), 193.1 (*C*OPh); EIMS calculated for $C_{24}H_{20}N_2O_4$ ($M^{+\bullet}$) 400.

4.3.1.19. 5-(Ethoxycarbonyl)-6-methyl-4-(4-N,N-dimethylphenyl)-3,4-dihydropyrimidin-2(1H)-one (19). **19** was prepared according to the general procedure by using 4-(N,N-dimethyl)benzaldehyde, urea, ethyl acetoacetate. Yield 89%. m.p. 259–260 °C [73]. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.12 (t, 3H, J = 6.9 Hz, OCH_2CH_3), 2.30 (s, 3H, CH_3), 3.37 (s, 6H, $N(CH_3)_2$), 4.09 (q, 2H, J = 7.2 Hz, OCH_2), 5.04 (d, 1H, J = 3.3 Hz, *CH*), 6.62 (d, 2H, J = 8.5 Hz, *Ar-H*), 7.05 (d, 2H, J = 8.5 Hz, *Ar-H*), 7.81 (br s, 1H, *NH*), 9.11 (br s, 1H, *NH*). ^{13}C NMR (75 MHz, DMSO-d_6): δ 14.1 (OCH_2CH_3), 17.9 (CH_3), 44.3 ($N(CH_3)_2$), 54.4 (*C*H-Ar), 59.2 (OCH_2), 100.6 (*C*-COOEt), 126.7–129.0 (C-aromatic), 147.6 (CH_3C=C), 149.6 (*C*-$N(CH_3)_2$), 151.8 (C=O), 164.9 (*C*OOEt); EIMS calculated for $C_{16}H_{21}N_3O_3$ ($M^{+\bullet}$) 303.

4.3.1.20. 5-(Ethoxycarbonyl)-6-methyl-4-(4-N,N-dimethylphenyl)-3,4-dihydropyrimidin-2(1H)-one (20). **20** was prepared according to the general procedure by using 4-(N,N-dimethyl)benzaldehyde, urea, diethylmalonate. Yield 67%. m.p. 202–205 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.14 (t, 6H, J = 7.1 Hz, OCH_2CH_3), 3.37 (s, 6H, $N(CH_3)_2$), 4.10 (q, 4H, J = 7.1 Hz, OCH_2), 5.10 (d, 1H, J = 3.3 Hz, *CH*), 6.62 (d, 2H, J = 8.5 Hz, *Ar-H*), 7.05 (d, 2H, J = 8.5 Hz, *Ar-H*), 7.61 (br s, 1H, *NH*), 9.11 (br s, 1H, *NH*). ^{13}C NMR (75 MHz, DMSO-d_6): δ 12.4, 14.7 (OCH_2CH_3), 44.4 ($N(CH_3)_2$), 54.4 (*C*H-Ar), 59.4, 60,8 (OCH_2), 101.2 (*C*-COOEt), 125.0–141.0 (C-aromatic), 145.9 (CH_3C=C), 149.3 (*C*-$N(CH_3)_2$), 152.5 (C=O), 164.2 (*C*OOEt); EIMS calculated for $C_{17}H_{23}N_3O_4$ ($M^{+\bullet}$) 333.

4.3.1.21. 5-(Ethoxycarbonyl)-6-ethoxy-4-(4-hydroxyphenyl)-3,4-dihydropyrimidin-2(1H)-one (21). **21** was prepared according to the general procedure by using 4-hydroxybenzaldehyde, urea, diethylmalonate. Yield 71%. m.p. 188 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.18 (t, 6H, J = 7.1 Hz, OCH_2CH_3), 3.90 (q, 4H, J = 7.2 Hz, OCH_2), 5.10 (d, 1H, J = 3.3 Hz, *CH*), 6.62 (d, 2H, J = 8.5 Hz, *Ar-H*), 7.05 (d, 2H, J = 8.5 Hz, *Ar-H*), 7.77 (br s, 1H, *NH*), 9.11 (br s, 1H, *NH*), 9.96 (br s, 1H, OH). ^{13}C NMR (75 MHz, DMSO-d_6): δ 12.7, 14.1 (OCH_2CH_3), 17.8 (CH_3), 54.2 (*C*H-Ar), 59.5, 60.6 (OCH_2), 98.8 (*C*-COOEt), 126.7–138.0

(C-aromatic), 147.6 (CH_3C=C), 152.1 (C=O), 157.9 (*C*-OH), 165.1 (*C*OOEt); EIMS calculated for $C_{15}H_{18}N_2O_5$ ($M^{+\bullet}$) 306.

4.3.1.22. 4-(4-Chlorophenyl)-5-(ethoxycarbonyl)-6-methyl-3,4-dihydropyrimidin-2(1H)-one (22). **22** was prepared according to the general procedure by using 4-chlorobenzaldehyde, urea, ethyl acetoacetate. Yield 70%. m.p. 218–219 °C [73]. ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.97 (t, 3H, J = 7.1 Hz, OCH_2CH_3), 4.10 (q, 4H, J = 7.2 Hz, OCH_2), 5.04 (d, 1H, J = 3.3 Hz, *CH*), 7.14–7.24 (m, 4H, *Ar-H*). 7.63 (br s, 1H, *NH*), 9.15 (br s, 1H, *NH*). ^{13}C NMR (75 MHz, DMSO-d_6): δ 14.9 (OCH_2CH_3), 17.9 (CH_3), 54.9 (*C*H-Ar), 60.3 (OCH_2), 101.4 (*C*-COOEt), 126.7–137.0 (C-aromatic), 148.9 (CH_3C=C), 151.7 (C=O), 164.1 (*C*OOEt); EIMS calculated for $C_{14}H_{15}N_2O_3Cl$ ($M^{+\bullet}$) 294.

4.3.1.23. 5-(Ethoxycarbonyl)-6-methyl-4-(3-pyridyl)-3,4-dihydropyrimidin-2(1H)-one (23). **23** was prepared according to the general procedure by using pyridine-3-carboxaldehyde, urea, ethyl acetoacetate. Yield 79%. m.p. 213–214 °C [74]. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.08 (t, J = 7.5Hz), 2.28 (s, 3H, CH_3), 4.01 (q, 2H, J = 7.1 Hz, OCH_2CH_3), 5.14 (d, 1H, J = 3.3 Hz, *CH*), 7.35–7.62 (m, 2H, *Ar-H*), 7.76 (br s,1H, *NH*), 9.22 (br s,1H, *NH*). 8.5 (s, 2H, *Ar-H*), ^{13}C NMR (75 MHz, DMSO-d_6): δ 14.1 (OCH_2CH_3), 17.9 (CH_3), 54.1 (*C*H-Ar), 59.4 (OCH_2), 98.1 (*C*-COOEt), 126.7–129.0 (C-aromatic), 145.9 (CH_3C=C), 151.2 (C=O), 165.1 (*C*OOEt); EIMS calculated for $C_{13}H_{15}N_3O_3$ ($M^{+\bullet}$) 261.

4.3.1.24. 5-Aceto-1,6-dimethyl-4-phenyl-3,4-dihydropyrimidin-2(1H)-one (24). **24** was prepared according to the general procedure by using benzaldehyde, N-methylurea, acetylacetone. Yield 73%. m.p. 154–155 °C [66]. ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.10 (s, 3H, CH_3), 2.29 (s, 3H, CH_3), 2.93 (s, 3H, N-CH_3), 5.14 (d, 1H, J = 3.3 Hz, *CH*), 7.69 (br s, 1H, *NH*), 7.0–7.6 (m, 5H, *Ar-H*). ^{13}C NMR (75 MHz, DMSO-d_6): δ 18.1 (CH_3), 28.7 (CH_3CO), 30.3 (N-CH_3), 54.1 (*C*H-Ar), 99.7 (*C*-COMe), 127.0–129.4 (C-aromatic), 148.2 (CH_3C=C), 152.2 (C=O), 195.7(*C*OMe); EIMS calculated for $C_{13}H_{14}N_2O_2$ ($M^{+\bullet}$) 244.

4.3.1.25. 1-Benzyl-5-(ethoxycarbonyl)-6-methyl-4-phenyl-3,4-dihydropyrimidin-2(1H)-one (25). **25** was prepared according to the general procedure by using benzaldehyde, N-benzylurea, ethyl acetoacetate. Yield 71%. m.p. 88–89 °C [75]. ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.97 (t, 3H, CH_3), 2.35 (s, 3H, CH_3), 4.03 (q, 2H, J = 7.1 Hz, OCH_2CH_3), 4.59 (d, 1H, J = 16.8 Hz, *CH*), 4.93 (d, 1H, J = 16.8 Hz, *CH*), 5.24 (d, 1H, J = 3.3 Hz, *CH*), 7.93 (br s, 1H, *NH*), 7.06–7.35 (m, 10H, *Ar-H*). ^{13}C NMR (75 MHz, DMSO-d_6): δ 14.7 (OCH_2CH_3), 17.9 (CH_3), 47.2 (N-CH_2), 54.4 (*C*H-Ar), 60.4 (OCH_2), 100.0 (*C*-COOEt), 127.3–139.8 (C-aromatic), 147.6 (CH_3C=C), 152.1 (C=O), 165.6 (*C*OOEt); EIMS calculated for $C_{21}H_{22}N_2O_3$ ($M^{+\bullet}$) 350.

4.3.2. General procedure for the synthesis of 2-amino-1,4-dihydropyrimidines

A mixture of an aldehyde (10 mmol), guanidine hydrochloride (10 mmol), 1,3-dicarbonyl compound (10 mmol) and sodium bicarbonate (3.35 g, 40 mmol), in DMF (20 mL) was taken in a Pyrex tube and irradiated in an ultrasonic bath at 70–75 °C. The reaction was monitored by TLC. After the completion of the reaction, the resulting precipitate was filtered and crude product was recrystallized from an appropriate solvent or purified with silica gel column chromatography. The purity of compounds was checked by TLC in different solvent systems. All the products were confirmed by MS ^{1}H NMR and ^{13}C NMR data.

4.3.2.1. Ethyl 2-amino-6-ethoxy-4-(4-mothoxyphenyl)-1,4-dihydropyrimidine-5-carboxylate (26). **26** was prepared according to the general procedure by using anisaldehyde, guanidine HCl, diethylmalonate. Yield 71%. m.p. 217 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.18 (t, 6H, J = 7.1 Hz, OCH_2CH_3), 3.90 (q, 4H, J = 7.1 Hz, OCH_2), 3.72 (s, 3H, OCH_3), 3.73 (s, 3H, OCH_3), 4.01 (q, 4H, OCH_2), 4.98 (s, 1H, *CH*), 5.87 (s, 2H, NH_2), 6.66 (d, 1H, *Ar-H*), 7.00 (d, 1H, *Ar-H*), 7.1 (s, 1H, *NH*). ^{13}C NMR (75 MHz, DMSO-d_6): δ 12.4, 14.7 (OCH_2CH_3), 17.9 (CH_3), 54.8 (*C*H-Ar), 56.7 (OCH_3), 59.4, 60.1 (OCH_2), 100.2 (*C*-COOEt), 126.7–139.0 (C-aromatic), 147.9 (CH_3C=C), 150.3 (C-aromatic-OCH_3), 160.5 (*C*-NH_2), 165.1 (*C*OOEt); EIMS calculated for $C_{16}H_{21}N_3O_4$ ($M^{+\bullet}$) 319.

4.3.2.2. Ethyl 2-amino-4-(4-hydroxy-3-methoxyphenyl)-6-methyl-1,4-dihydropyrimidine-5-carboxylate (27). **27** was prepared according to the general procedure by using vanillin, guanidine HCl, ethyl acetoacetate. Yield 65%. m.p. 196 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.11 (t, 3H, J = 7.1 Hz, CH_3), 2.25 (s, 3H, CH_3), 3.71 (s, 3H, OCH_3), 4.01 (q, 2H, J = 7.1 Hz, OCH_2CH_3), 5.24 (s, 1H, J = 2.24 Hz, *CH*), 5.78 (s, 2H, NH_2), 6.40 (s, 1H, *Ar-H*), 6.45 (d, 1H, *H*- aromatic), 6.50 (d, 1H, *Ar-H*), 7.79 (br s, 1H, *NH*), 8.97 (br s,1H, *NH*), 10.51 (s, 1H *OH*). ^{13}C NMR (75 MHz, DMSO-d_6): δ 14.7 (OCH_2CH_3), 18.5 (CH_3), 54.6 (*C*H-Ar), 56.2 (OCH_3), 60.4 (OCH_2), 100.8 (*C*-COOEt), 119.7–144 (C-aromatic), 144.3 (C-aromatic-OH), 147.2 (CH_3C=C), 151.2 (C-aromatic-OCH_3), 161.5 (*C*-NH_2), 164.1 (*C*OOEt); EIMS calculated for $C_{15}H_{19}N_3O_4$ ($M^{+\bullet}$) 305.

4.4. In vitro urease inhibition studies

The shortlisted compounds in initial hits and other synthesized compounds were screened according to literature procedure [55]. The reaction mixtures comprising 25 μL of enzyme (jack bean urease) solution and 55 μL of buffer containing 100 mM urea were incubated with 5 μL of test compounds (0.5 mM concentration) at 30 °C for 15 min in 96-well plates. Urease activity was determined by measuring ammonia production using the indophenol method in which 45 μL each phenol reagent (1% (w/v) phenol and 0.005% (w/v) sodium nitroprusside) and 70 μL of alkali reagent (0.5% (w/v) NaOH and 0.1% active chloride NaOCl) were added to each well. The increasing absorbance at 630 nm was measured after 50 min, using a microplate reader (Molecular Device, USA). All reactions were performed in triplicate in a final volume of 200 μL. The results (change in absorbance per min) were processed by using soft Max Pro software (molecular Device, USA). The entire assay was performed at pH 6.8. Percentage inhibition was calculated using the following formula:

$$100 - \left(\frac{\text{OD test well}}{\text{OD control}}\right) \times 100$$

Thiourea was used as the standard urease inhibitor.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jmgm.2013.04.006.

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