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Original Article

Design and synthesis of potent and selective β- glucuronidase inhibitor by virtual and in vitro screening

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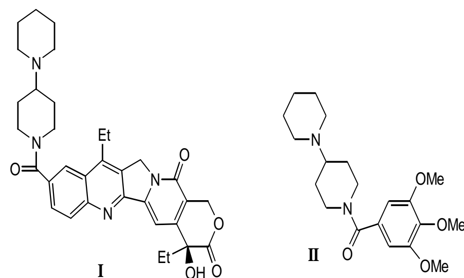
ABSTRACT

Piperidine and pyrrolidine constitute an important fragment of biomolecules present in the naturally occurring compounds and they have shown potential biological activity. We have designed 4-(1-pyrrolidinyl) piperidine derivatives and virtually screened for β-glucuronidase by computational docking using Argus Lab followed by in vitro screening. Compounds were also screened for urease, phosphodiesterase and α- chymotrypsin inhibition to find selective inhibitor. Compound 2, 3, 4 have shown beta glucuronidase inhibition greater than standard D-Sacchric acid 1, 4 lactone (IC_{50} =48.4±1.25µm). All the compounds were inactive for the other tested enzymes except compound 3 which exhibited weak urease inhibition. Compound 2, 4 and 6 can be used as selective β - glucuronidase inhibitor. Compound 2 {1-[2-(4″-chloro-phenyl)-2-oxo-ethyl]- 4-pyrrolidin-1′-yl- piperidinium bromide} showed remarkable inhibition against β-glucuronidase, with an IC_{50} value of 17.10 ± 0.61 µm, this is about three times more active than the standard drug.

KEY WORDS: 4-(1- Pyrrolidinyl) Piperidine, β-Glucuronidase, urease

INTRODUCTION

Piperidine and pyrrolidine containing compounds possess diverse biological activities.[1-8] Piperidine containing Irinotecan (I) (FDA approved drug) is a semi-synthetic analogue of camptothecine exhibit significant antineoplastic activity.[9] Due to piperidino-piperidine component Irinotecan exhibited better profile of activity than camptothecine. Compound II is also piperidine containing compound with β-glucuronidase inhibition (IC_{50} = 0.306 µM) .[10]



In current study we designed different derivatives of 4-(1-pyrrolidinyl) piperidine in which we replaced one piperidine with pyrrolidine and studied β-glucuronidase inhibition as well as other enzyme inhibition including urease, phosphodiesterase and α-chymotrypsin. Pyrrolidine containing compounds have been reported to be β-glucosidase, β-galactosidase and β-mannosidase inhibitor, anti-acetylcholinesterase and histamine H_1 and H_3 receptor antagonist.[11-13]
β-Glucuronidase is an exoglycosidase enzyme that catalyzes the cleavage of glucuronosly-O-bonds. The enzyme is present in many organs, body fluids, blood cells, liver, spleen, kidney, gastric juice, lung, muscle, bile, urine and serum. In certain disease such as cancer, inflammatory joint disease, some hepatic diseases and AIDS the activity of β-glucuronidase is increased. Human β-glucuronidase also has a role in the deconjugation of glycosaminoglycans. Endogenous biliary β-glucuronidase deconjugates the glucuronides of

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bilirubin and causes the development of cholelithiasis in human bile. Liver damage cause an increase in this enzyme level in blood, liver cancer is also suspected to be related to the over expression of this enzyme. Many β-glucuronidase inhibitors such as 8-hydroxytriceine glucuronide, isovitexin trihydroxy pipecolic acid, scoparic acid A and C have already been isolated from different plants and some are used clinically.[13-14]

Urease (EC 3.5.1.5; urea amidohydrolase) is a metal containing enzyme that catalyzes the hydrolysis of urea into ammonia and carbon dioxide.[15] Urease-producing bacteria have a negative effect on human health. Urease is known to be a major cause of pathologies induced by Helicobacter pylori, as it allows the bacteria to survive at the low pH of stomach during colonization, which leads to gastric and peptic ulcers and, in some cases, may lead to cancer.[16] They are directly involved in urinary tract infections[17]

MATERIAL AND METHODS

General Experimental: Melting points were recorded on Gallenkamp melting point apparatus and are uncorrected. Ultraviolet (UV) spectra were recorded on a Hitachi U-3200 spectrophotometer. Infra Red (IR) spectra were measured on a Shimadzu IR 460 spectrophotometer using KBR disc. Mass (EIMS) were determined on Massen spectrometer MAT 311A Varian Bermen spectrometer. EIMS-HR were determined by MAT95XP Thermo Finnigan. ^{1}H Nuclear magnetic resonance (^{1}HNMR) spectra were recorded on AVANCE AV 300 spectrometer. Reactions were monitored by using TLC pre-coated silica gel, GF-254.

Chemistry: 4-(1-pyrrolidinyl) piperidine derivatives were synthesized by quaternization of piperidine with different aromatic groups. 15-20ml (0.5mmole) acetone solution of 4-(1-Pyrrolidinyl) piperidine added to15-20ml (0.5 mmole) acetone solution of acetonaphthone and various substituted phenacyl halides. The reaction mixture stirred in round bottom flask at 52-54°C for 4-8 hours showed in table 1. The reaction was monitored by thin layer chromatography. The crude solid product was purified through recrystallization by using warm ethanol and ether.

General proedure for the synthesis of compounds 2-6: Equimolar quantities of 4-(1-Pyrrolidinyl) piperidine and other reactant were dissolved separately in 15-20 ml of acetone and after mixing, stirred the solution at 52-54°C for 4-8 hours, precipitate appeared spontaneously. The reaction was monitored by TLC. After the completion of the reaction the resulting product was filtered and thoroughly washed with acetone to remove the traces of reactants. The product thus obtained was purified through recrystallization by using warm ethanol and ether. Pure compounds were dried in desiccators over anhydrous calcium sulphate. Melting point was recorded and spectral data were obtained to confirm the structure of compounds **2-6.**

Molecular docking studies: Molecular structures were drawn by using the Marvin Sketch software[19]. Partial atomic charges were calculated by the mulikan method in Argus lab software. [20-21] The crystal structure of the β-Glucuronidase enzyme (PDB ID: 3HS5) was obtained from the protein data bank.[22] All hydrogen atoms in the protein were allowed to optimize. Minimization was performed by Argus lab4.0.1 software using Hartree-Fock method.

β-Glucuronidase Enzyme Inhibition: β-Glucuronidase (E.C. 3.2.1.31) from *E. coli*, (*p*-nitrophenyl-β-D-glucuronide) was obtained from Sigma Chemical Co. Other reagents were also purchased from commercial sources. β-Glucuronidase activity was determined by measuring the absorbance at 405 nm of *p*-nitrophenol formed from the substrate by the spectrophotometric method. The total reaction volume is 250 µl. The reaction mixture contained 185 µl of 0.1 M acetate buffer, 5 µl of test compound solution, 10µl of enzyme solution incubated at 37 °C for 30 min. The plates were read on a multiplate reader (SpectraMax plus 384) at 405 nm after the addition of 50 µl of 0.4mM *p*-nitrophenyl- b-D-glucuronide. All assays were run in triplicate[18].The IC_{50} values were calculated using the EZ-Fit Enzyme Kinetics program, Perrella Scientific Inc., Amherst, U.S.A.

Urease Inhibition: Reaction mixtures comprising 1 unit of urease enzyme (*Bacillus pasteurii or Jack bean*) solution and 55μl of buffers containing 100mM urea were incubated with 5μl of test compounds (1mM concentration) at 30°C for 15 min in 96-well plates. Urease activity was determined by measuring ammonia production using the indophenol method as described by Weatherburn.[23] Briefly, 45μl each of phenol reagent (1% w/v phenol and 0.005% w/v sodium nitroprusside) and 70μl 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 200μl. The results (change in absorbance

per min) were processed by using Soft- Max Pro software (Molecular Device, USA). All reactions were performed in triplicate. All the assays were performed at pH 8.2 (0.01 M $K_2HPO_4.3H_2O$, 1mM EDTA and 0.01 M $LiCl_2$). Percentage inhibitions were calculated from the formula 100-($OD_{testwell}$/ $OD_{control}$) x 100. Thiourea was used as the standard inhibitor of urease.

***α* -Chymotrypsin Inhibition:** The inhibitory activity of α-chymotrypsin is performed in 50 mM Tris-HCl buffer pH 7.6 with 10 mM CaCl2 as mentioned by Carnell et al, (1988)[24] with the slight modification. The enzyme; α-chymotrypsin (12 Units/mL prepared in buffer mentioned above) with the test compound (0.5 mM) prepared in DMSO (final concentration 7%) is incubated at 30°C for 25 min. The reaction is started by the addition of the substrate N-succinyl-L-phenylalanine-p-nitroanilide (SPpNA; 0.4 mM prepared in the buffer as above). The change in absorbance by released p-nitroanilide will be continuously monitored at 410 nm. The positive control without test compound and the negative control without enzyme or with standard inhibitor run in parallel. The % of inhibition is based upon initial velocity and calculated as:

% of Inhibition =100 – {OD/min of Test Compound / OD/min Positive Control x100}

Phosphodiesterase Inhibition: Activity against the snake venom phosphodiesterase I (Sigma P 4631) (EC 3.1.4.1) was assayed by using the reported method[25] with the following modifications. 33 mM Tris–HCl buffer, pH 8.8, 30 mM Mg-acetate with 0.000742 U/well final concentrations using microtiter plate assay and 0.33 mM bis-(p-nitro phenyl) phosphate (Sigma N-3002) as a substrate. Cysteine and EDTA12–14 (E. Merck) was used as positive controls (IC50 = 748 lM ± 0.15, 274 lM ± 0.07, respectively). After 30 min of incubation, the enzyme activity was monitored spectrophotometrically at 37°C on a microtiter plate spectrophotometer (Spectra Max, Molecular Devices) by following the release of p-nitrophenol from p-nitrophenyl phosphate at 410 nm. All the reactions were performed in triplicate and the initial rates were measured as the rate of change in OD/min (optical density/min) and used in subsequent calculations.

RESULT AND DISCUSSION

β- glucuronidase inhibition: Compounds **1-6** were screened for invitro β-glucuronidase urease, phosphodiesterase and α- chymotrypsin inhibition. The compounds which have shown activity greater than 50% were selected for IC_{50} and results are shown in Table 1. Parent compound 4-(1-pyrrolidinyl) piperidine **1** showed no activity. Among the synthesized analogues of compound **1**, compounds **2, 3, 4** and **6** showed reasonable inhibition, while compound **5** displayed no activity. Compound **2** showed remarkable inhibition with an IC_{50} value of 17.10±0.61 µm, which is about three times more than the standard drug 1,4 lactone D-saccharic acid (IC_{50} = 48.4± 1.25). Compounds **3** and **4,** with IC_{50} values of 45.5 µM and 34.3 µM are also more potent than the standard drug. SAR studies of all compounds demonstrate that the binding of different phenacyl halides and the naphthalene containing molecule, except compound **5** (a fluoro derivative) reveal β-glucuronidase inhibitory activity. Whereas naphthalene derivative **6** showed low activity with IC_{50}= 131. We conclude that single phenyl ring bearing chloro, methoxy and bromo substituent may have better affinity for the tested enzyme than the naphthyl group. Analogues **2-5** with different functional groups at the para position reveal that this position is responsible for the activity of the molecule. All compounds were inactive for urease, phosphodiesterase and α-chymotrypsin except compound 3 which showed urease inhibition as shown in figure -1

Molecular Docking Studies: Molecular docking studies of the compounds were carried out by using Argus Lab Software The compound with least docking energy exhibited highest activity. The Compound **2** showed interaction between oxygen of carbonyl and amino of Arg 21. Second interaction for this compound appeared between Ser 87 and nitrogen of piperidine ring. The activity of compound **4** is attributed to the interaction of ketonic oxygen with that of amino group of Arg 21 indicating a distance of 2.2799 Å. The second interaction is observed between nitrogen of pyrrolidinyl fragment with that of carboxylic group of Gly 20. The conformational attachment seems to be most suitable for the molecule with that of two different amino acids. Graphical representation is shown in Figure-2 and 3. The most active compound 2 binds the β- glucouronidase enzyme with GAScore of -9.54236. A better profile of activity is exhibited by the compound possessing chlorine atom at the para position of phenyl ring. This increase in activity may be attributed due to its docking confirmation with the respective enzyme.

1-[2-(4″-chloro-phenyl)-2-oxo-ethyl]-4-Pyrrolidin-1′-yl-Piperidinium bromide (2): From 4-(1-Pyrrolidinyl) piperidine and 2-bromo-4′-chloroacetophenone. White crystalline powder Yield: 80.5%, m.p: 237°C; UV λ_{max} (MeOH) nm: 239,205; IR ν_{max} (KBr) cm^{-1}: 3422, 2942, 1651, 1592; ^{1}H-NMR (D_20, 300 MHz) δ: 7.75-7.78 (d,2H, J=8.8Hz H-2″ H-6″), 7.44-7.41 (d, 2H,

J=8.29Hz, H-3″ H-5″), 4.60 (s, 2H, H-7), 3.5-3.3 (m, 1H, Hz, H-4), 2.97-2.88 (t, 4H, , J=13.3Hz, H-2′, H-5′), 2.30-2.26 (d, 4H, , J=13.5Hz , H-2, H-6), 1.9-1.7 (m, 8H, H-3′, H-4′, H-3, H-5); EIMS m/z: 306 (M^+-HBr, $C_{17}H_{23}N_2OCl$), 292, 251, 223, 208, 191,180,167,158,153,139, 124,110, 83,70, 55; HR-EIMS: 306.1499 (M^+ -HBr $C_{17}H_{23}N_2OCl$) Calculated 306.1527

1-[2-(4″-methoxy-phenyl)-2-oxo-ethyl]- 4-Pyrrolidin-1′-yl-piperidinium bromide (3): From 4-(1-Pyrrolidinyl) piperidine and 2-bromo-4′-methoxyacetophenone White crystalline Powder Yield: 81.5%, m. p: 258ºC; UV λ_{max} (MeOH) nm: 389,276,218,201; IR ν_{max} (KBr) cm^{-1}: 3506, 1676, 1510, 1226, 572. ^{1}H-NMR (D_2O, 300 MHz)) δ: 7.74-7.71(d, 2H, J=11.5Hz, H-2″ H-6″), 6.86-6.83 (d, 2H, J=8.96HZ, H-3″ H-5″), 4.60(s, 3H, OCH3-4″), 3.82 (s, 2H, H-7), 3.69 (s, 1H, H-4), 3.19-2.90 (m, 8H, H-2′, H-5, H-2, H-6), 2.15-1.86(m, 4H, H-3′, H-4′), 1.63-1.55 (m, 4H, H-3, H-5). EIMS m/z: 302.4 (M^+-HBr, $C_{18}H_{26}N_2O$), 167.2, 153, 135, 124, 121, 110, 98, 96, 55HR-EIMS: 302.1986 (M^+ -HBr $C_{18}H_{26}N_2O$) Calculated 302.1994.

1-[2-(4″-bromo-phenyl)-2-oxo-ethyl]- 4-Pyrrolidin-1′-yl-piperidinium; bromide (4): From 4-(1-Pyrrolidinyl) piperidine and 2, 4′-dibromoacetophenone White crystalline powder.Yield: 71.6%; m p: decomposed at 259ºC; UV λ_{max} (MeOH) nm: 389,256,280. IR ν_{max} (KBr) cm^{-1} 3456, 576,472. ^{1}H-NMR D_2O, 300 MHz) δ: 7.67-7.64(d, 2H, J=8.58Hz, H-2″, H-6″), 7.56-7.53 (d, 2H, J=0.02HZ, H-3″, H-5″), 4.60 (s, 2H, H-7), 4.19 (d, 1H, J=4. 2HZ, H-4), 3.44-2.91 (m, 8H, H-2′, H-5′, H-2, H-6), 1.88-1.71(m, 8H, H-3′, H-4′, H-3, H-5). EIMS m/z: 352 (M^+-Br, $C_{17}H_{24}N_2OBr$), 281,254,226,198,183,167, 153, 139, 124, 11098, 85, 80, 70, 55; HR-EIMS: 352.2523 (M^+-Br, $C_{17}H_{24}N_2OBr$), Calculated 352.2893.

1-[2-(4″-floro-phenyl)-2-oxo-ethyl]- 4-Pyrrolidin-1′-yl -piperidinium; bromide (5): From 4-(1-Pyrrolidinyl) piperidine and 2-bromo-4′-flouroacetophenone. White crystalline powder Yield: 77.2%; m p: 145±3ºC UV λ_{max} (MeOH) nm: 227,202; IR ν_{max} (KBr) cm^{-1}:3425,2798, 1651, 1508.^{1}H-NMR (MeOD, 300 MHz) δ:7.85-7.80(t, 2H, J=5.4Hz, H″-2″,H-6″),7.10-7.04 (t, 2H, J=8.8Hz, H-3″, H-5″), 4.60 (s, 2H, H-7), 3.91-3.88 (d, 1H, J=8.4Hz, H-4), 3.31-2.94(m, 8H, H-2′, H-5′ H-2, H-6), 2.18-1.83 (m, 4H, H-3′, H-4′), 1.65-1.57 (m, 4H, H-3, H-5), EIMS m/z: 290 (M^+-HBr, $C_{17}H_{23}N_2OF$),221,192,167,153,124,110,98,83,70,5 5, HR-EIMS: 290.1794 (M^+-HBr, $C_{17}H_{23}N_2OF$) Calculated 290.1794.

1-[2-Naphthalen-2-yl-2-oxo-ethyl]- 4-Pyrrolidin-1′-yl-piperidinium; bromide (6): From 4-(1-Pyrrolidinyl) piperidine and 2-bromo-2′acetonaphthone. White crystalline powder Yield: 73.2%, m p: 288ºC; UV λ_{max} (MeOH) nm: 333,294,248,208; IR ν_{max} (KBr) cm^{-1}: 3423, 2611, 1626, 1068, 933, 474. ^{1}HNMR (MeOD, 300 MHz) δ: 8.66 (s, 1H, H-10″), 8.08-7.94 (m, 4H, H-2″, H-8″, H-3″, H-5″), 7.69-7.66(t,1H, J=3.7Hz, H-6″),7.63-7.60(t,1H, J=4.4Hz, H-7″)4.7(s 2H, *H*-7), 3.63-3.4(m, 1H, *H*-4), 3.34-3.05(m, 8H, *H*-2′, *H*-5, *H*-2, *H*-6), 2.40-2.38 (d, 4H, *J*=12.8Hz, *H*-3′, *H*-4′), 2.16-2.12 (d, 4H, J=17.6Hz, *H*-3, *H*-5); EIMS m/z: 323(M^+-Br, $C_{21}H_{27}N_2O$), 322,293,253,224,167,153,141,127,110,96,80,70, 55; HR-EIMS: 322.2045 (M^+-HBr, $C_{21}H_{26}N_2O$) Calculated 322.2015

CONCLUSION

The studied showed that compounds 2 and 4 can be considered as selective inhibitor for β-glucuronidase, while 3 is a nonselective inhibitor and can inhibit both β-glucuronidase as well as urease. Furthermore, none of the compounds 1-6 showed inhibition against α-chymotrypsin and phosphodiesterase.

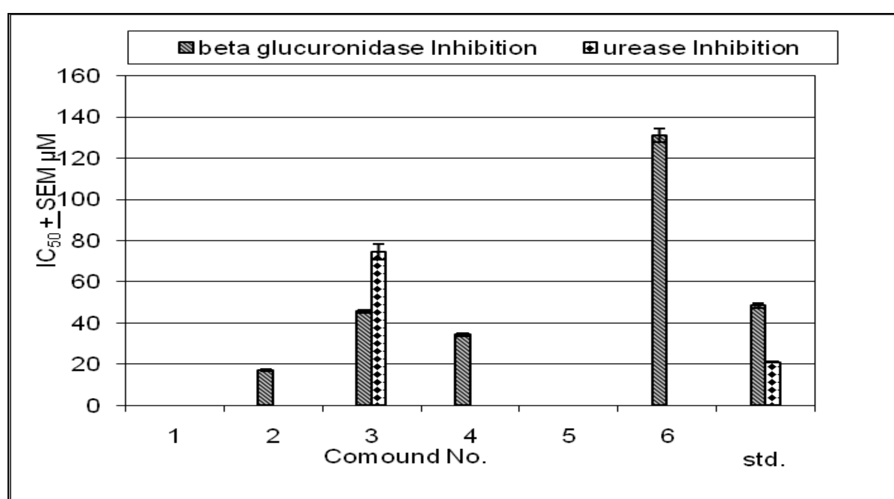


Figure 1: IC50 for β-Glucuronidase and Urease Inhibition

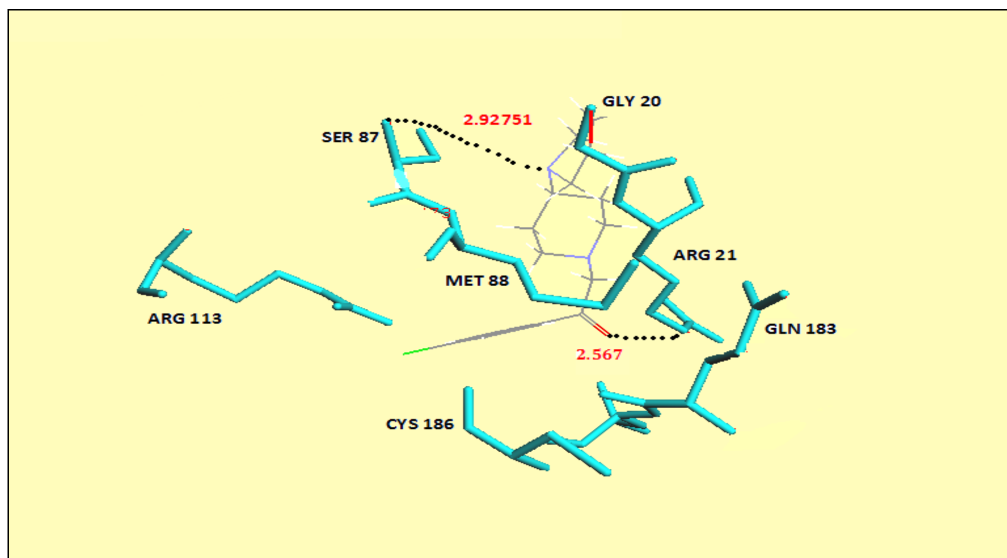


Figure-2. Docked structures of compound 2 into the active site of β-Glucuronidase enzyme

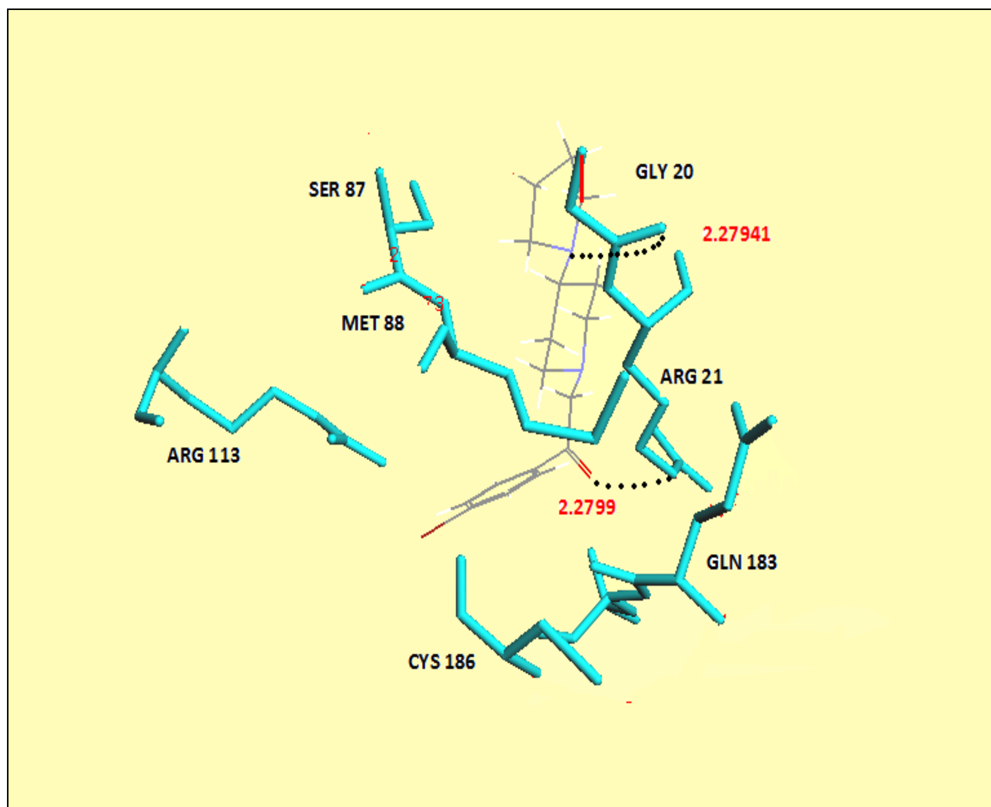
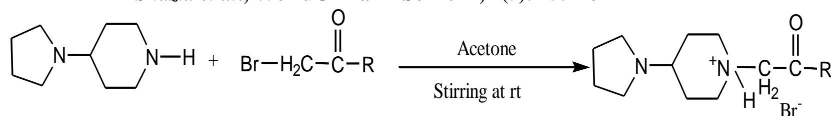


Figure-3. Docked structures of compound 4 into the active site of β-Glucuronidase enzyme



Compound no.	R	β- Glucuronidase Inhibition		Urease Inhibion
		IC50+SEM; µM	Docking Score (k.cal/mol)	IC50+SEM; µM
Parent **1**	-	ND	-6.92208	ND
2	4-chlorophenyl (Cl)	17.1 ±0.61	-9.54236	ND
3	4-methoxyphenyl (OCH_3)	45.5 ±0.89	-6.63307	74.35±3.81
4	4-bromophenyl (Br)	34.33 ± 0.61	-8.25481	ND
5	4-fluorophenyl (F)	ND	-6.92208	ND
6	naphthyl	131 ±3.26	-8.10354	ND
1,4 lactone D-saccharic acid		48.4±1.25 µm	-8.15911	-
Thiourea	H_2N–C(=S)–NH_2	-	-	21± 0.11

Table 1: Docking scores (kcal/mol) and IC50 values of 4-(1-Pyrrolidinyl) piperidine derivatives for β-Glucuronidase and urease inhibition (ND= Not Determine).

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