Accepted Manuscript

Synthesis and *In vitro* α-Chymotrypsin Inhibitory Activity of 6-Chlorobenzimidazole Derivatives

Hina Siddiqui, Rabia Farooq, Bishnu P. Marasini, Rizwana Malik, Naima Syed, Syed Tarique Moin, Atta-ur-Rahman, M. Iqbal Choudhary

PII: S0968-0896(16)30342-X
DOI: http://dx.doi.org/10.1016/j.bmc.2016.05.018
Reference: BMC 13002

To appear in: *Bioorganic & Medicinal Chemistry*

Received Date: 23 February 2016
Revised Date: 4 May 2016
Accepted Date: 12 May 2016

Please cite this article as: Siddiqui, H., Farooq, R., Marasini, B.P., Malik, R., Syed, N., Moin, S.T., Atta-ur-Rahman, Iqbal Choudhary, M., Synthesis and *In vitro* α-Chymotrypsin Inhibitory Activity of 6-Chlorobenzimidazole Derivatives, *Bioorganic & Medicinal Chemistry* (2016), doi: http://dx.doi.org/10.1016/j.bmc.2016.05.018

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

ACCEPTED MANUSCRIPT

Synthesis and *In vitro* α-Chymotrypsin Inhibitory Activity of 6-Chlorobenzimidazole Derivatives

Hina Siddiqui[a*], Rabia Farooq[a], Bishnu P. Marasini[a], Rizwana Malik[b], Naima Syed[a],

Syed Tarique Moin[a], Atta-ur-Rahman[a], and M. Iqbal Choudhary [a,b,c*]

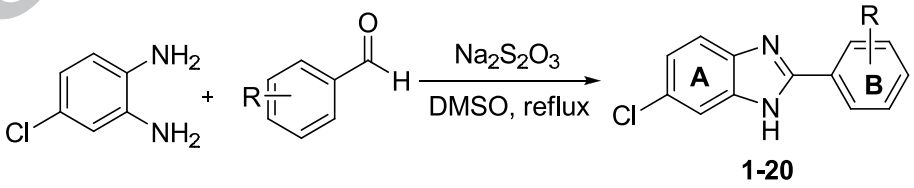
[a]H. E. J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, Karachi-75270, Pakistan

[b]Dr. Panjwani Center for Molecular Medicine and Drug Research, International Center for Chemical and Biological Sciences, University of Karachi, Karachi-75270, Pakistan

[c]Department of Biochemistry, Faculty of Science, King Abdul Aziz University, Jeddah-21452, Saudi Arabia

ABSTRACT

A library of benzimidazole derivatives **1-20** were synthesized, and studied for their α-chymotrypsin (α-CT) inhibitory activity *in vitro*. Kinetics studies and molecular docking studies were performed to identify the type of inhibition. Compound **1** was found to be a good inhibitor of α-chymotrypsin enzyme (IC_{50} = 14.8 µM, Ki = 16.4 µM), when compared with standard chymostatin (IC_{50} = 5.7±0.13 µM). Compounds **2-8**, **15**, **17**, and **18** showed significant inhibitory activities. All the inhibitors were found to be competitive inhibitors, except compound **17**, which was a mixed type inhibitor. The substituents (R) in *para* and *ortho* positions of phenyl ring B, apparently played a key role in the inhibitory potential of the series. Compounds **1-20** were also studied for their cytotoxicity profile by using 3T3 mouse fibroblast cells and compounds **3**, **5**, **6**, **8**, **12-14**, **16**, **17**, **19**, and **20** were found to be cytotoxic. Molecular docking was performed on the most active member of the series in comparison to the standard compound, chymostatin, to show the most likely binding modes. The compounds reported here can serve as templates for further studies of new inhibitors of α- chymotrypsin and other chymotrypsin-like serine proteases enzymes.



1-20

***Keywords*:** Benzimidazole; α-Chymotrypsin inhibition; Serine protease, docking

ACCEPTED MANUSCRIPT

1.0 Introduction:

Serine proteases are important targets for the development of drugs against several diseases[1]. The enzyme α-chymotrypsin (EC 3.4.21.1) belongs to the serine protease family, which plays a vital role in the digestion of dietary proteins after food intake[2]. Premature activation of chymotrypsin leads to chronic pancreatitis[3]. Chymotrypsin and cathepsin also catalyzes the cleavage of interleukin 1-*β* (IL-1*β*) precursor into active IL-1*β,* which causes arthritis[4]. Chymotrypsin also activates epithelial sodium channel (Enac) by proteolysis, and may cause cystic fibrosis[5].

Benzimidazole is an important scaffold, known to possess various biological activities, such as antimicrobial[6], angiotensin II receptor antagonist[7], phosphodiesterase inhibition, anti-HIV[8], anti-influenza[9], antiparasitic[10], anti-inflammatory[11], topoisomerase I inhibition, inhibition of the hepatitis C virus RNA polymerase, anti-fertility, anti-helmintic[12], antihistaminic[13] properties. Therefore, the synthesis of new benzimidazole derivatives has been vigorously pursued in medicinal chemistry. Several benzimidazole-based drugs, such as omeprazole, rabeprazole, and timoprazole, are widely used as proton pump inhibitors in the treatment of ulcer, and gastritis. Moreover, albendazole, and mebendazole are used against a variety of worm infestations (**Figure-1**).

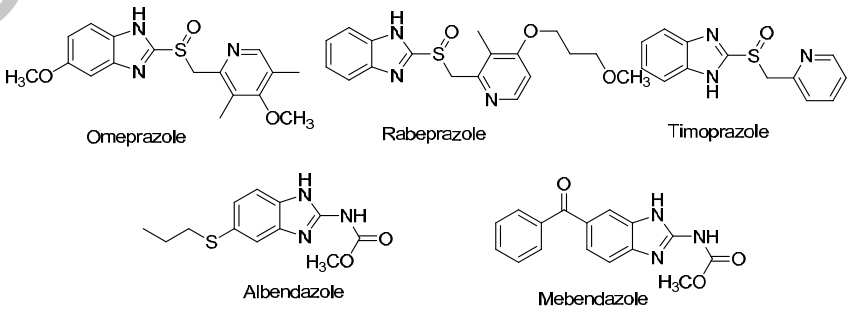


Figure-1: Structures of Some Common Drugs Based on Benzimidazole Pharmacophore.

ACCEPTED MANUSCRIPT

Current study describes the synthesis of 6-chlorobenzimidazole derivatives **1-20**, and evaluation of their α-chymotrypsin inhibitory, and cytotoxic activities using biochemical and cellular *in vitro* assays, respectively. All the compounds were found to be known except compound **10**. These compounds have been reported for antibacterial[14], antiproliferative[15], antimicrobial[16], modulators of ion channels[17], analgesic[18], antiinflammatory[19], cyclooxygenase inhibitory[19], and antioxidant[19] activities. However to the best of our knowledge compounds **1-20** have not been reported for the α-chymotrypsin, and related enzyme inhibition activity. Modern spectroscopic techniques were used to characterize the synthesized compounds.

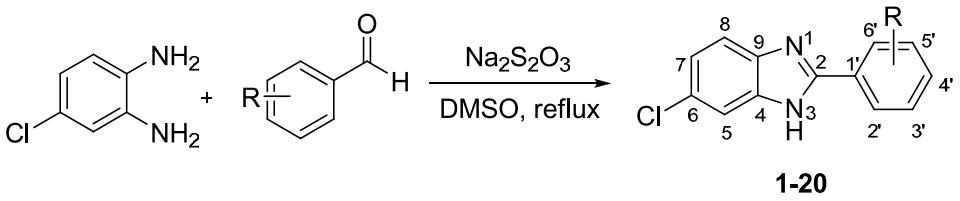
Molecular level characterization of binding pattern of these compounds into αchymotrypsin active site has provided insights into mechanism of action of these benzimidazole derivatives. Molecular docking was performed on the most active compound **1**, in comparison to the standard compound, chymostatin, to identify the most likely binding modes of benzimidazole derivatives which could contribute for the inhibitory activity of these compounds.

2.0 Results and Discussion:

2.1 Chemistry:

6-Chlorobenzimidazoles derivatives **1–20** were synthesized by condensation reaction between 4-chlorophenylenediamine and different aldehydes, by using a methodology described by Ridley *et al*[20] (**Scheme-1**). Resulting compounds were purified by solvent-solvent extraction, and obtained in good to excellent yields. In few cases compounds were purified by using normal phase column chromatography.

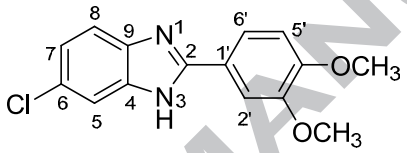
ACCEPTED MANUSCRIPT



Scheme-1: Synthesis of Benzimidazole Derivatives **1-20**.

Structure Elucidation of Representative Compound 1:

The structures of compounds **1-20** were deduced by using MS and NMR spectroscopic techniques. Structure elucidation of a representative member (compound **1**) is presented below;



6-Chloro-2-(3,4-dimethoxyphenyl)-1*H*-benzimidazole (**1**)

In the ^{1}H-NMR, H-6' was found coupled with H-5' and H-2', and appeared as a doublet of a doublet at δ_H 7.67 ($J_{6',5'}$ = 6.3 Hz, $J_{6',2'}$ = 1.5 Hz). H-5' was coupled with H-6' at δ_H 7.12 (d, 1H, $J_{5',6'}$ = 6.3 Hz). H-5 resonated as a doublet at δ_H 7.66 (d, 1H, $J_{5,7}$ = 1.5 Hz). In addition, H-8/ H-2' appeared as a broad singlet at δ_H 7.53. H-7 showed coupling with H-8 and H-5 at δ_H 7.23 (dd, 1H, $J_{7,8}$ = 6.3 Hz, $J_{7,5}$ = 1.2 Hz).

^{13}C-NMR spectrum showed characteristic C-2 carbon was resonated at δ 152.8. Other prominent peaks in the ^{13}C-NMR were δ 151.0 (C-3'and C-4'), 124.0 (C-4 and C-9), 123.2 (C-6 and C-1'), 121.1 (C-7 and C-6'), 112.9 (C-8 and C-5), 111.2 (C-2' and C-5'), and 56.5 (2OCH_3 at C-3' and C-4'). The HREI-MS was found to be *m/z* 288.0664 supporting the molecular formula $C_{15}H_{13}ClN_2O_2$ (Exact Mass: 288.0666).

ACCEPTED MANUSCRIPT

2.2 In Vitro αChymotrypsin Activity:

Compounds **1-20** were evaluated for their α-chymotrypsin inhibitory activity. Compounds which showed more than 50% inhibition of the enzyme at 500 µM concentration were selected for IC_{50} value measurement, and kinetics studies. The IC_{50} values were found to be in the range of 15 to 97 µM.

Table-1 presents the α-chymotrypsin inhibitory activity of the compounds **1-20**. Compound **1** was found to be the most active member of the series with an IC_{50} = 14.8 ± 0.1 µM, in comparison to standard drug chymostatin (IC_{50} = 5.7±0.13 µM). The activity of compound **1** may be attributed to the presence of two electron donating methoxy groups at C-3' and C-4' of ring B. Compounds **2** and **3** were also found to be active members of the series with IC_{50} values 15.9 ± 0.5, and 20.9 ± 0.1 µM, respectively. The activity was reduced with the removal of one of methoxy group from C-4', as observed in compound **17** (IC_{50}= 38.1± 0.6 µM). Activity also decreased two folds when dimethylamino group was present at the C-4' position, as in compound **4**. Compound **5** showed good activity with IC_{50} value 34.6 ± 0.5 µM, which may be because of two electron donating hydroxyl groups at C-2', and C-4'. The removal of hydroxyl from C-4' resulted in a complete loss of activity, as observed in compound **14**. Substitution of chloro group at C-2', and C-4' positions also resulted in a significant activity (compound **6)**, but changing the sequence of substitution resulted in a loss of activity many folds, as in compound **8** (IC_{50}= 96.4 ± 2.6 µM). Introduction of chloro and nitro group at C-2' and C-5' respectively, resulted in complete loss of activity as observed in the compound **10**. Use of pyridine as substituent resulted in reduced activity as observed in compound **7** IC_{50}= 48.9 ± 0.5 µM). In comparison replacement of pyridine with furan substituent resulted in reduced activity in compound **15** (IC_{50}= 93.1 ± 3.2 µM). Similarly use of styrene group resulted in loss of activity by many folds in compound **13**

ACCEPTED MANUSCRIPT

(IC_{50}= 486.1 ± 9.8 µM).It is proposed that benzimidazole moiety apparently interfere with the catalytic action of histidine-57 of α-chymotrypsin enzyme *via* accepting or donating hydrogen by N-1' or N-3', and thus contributing towards the affinity of the compounds with the enzyme.

2.3 Kinetic studies

Kinetics studies were performed on the most active members of the series, i.e. compounds **1-8**, **15**, **17** and **18**, to study the mode of inhibition. Table-1 shows the Ki values, and type of inhibitions of these compounds. The pattern of inhibition at variable concentrations of compound **1**, and substrate is graphically presented in Figures-2- 4.

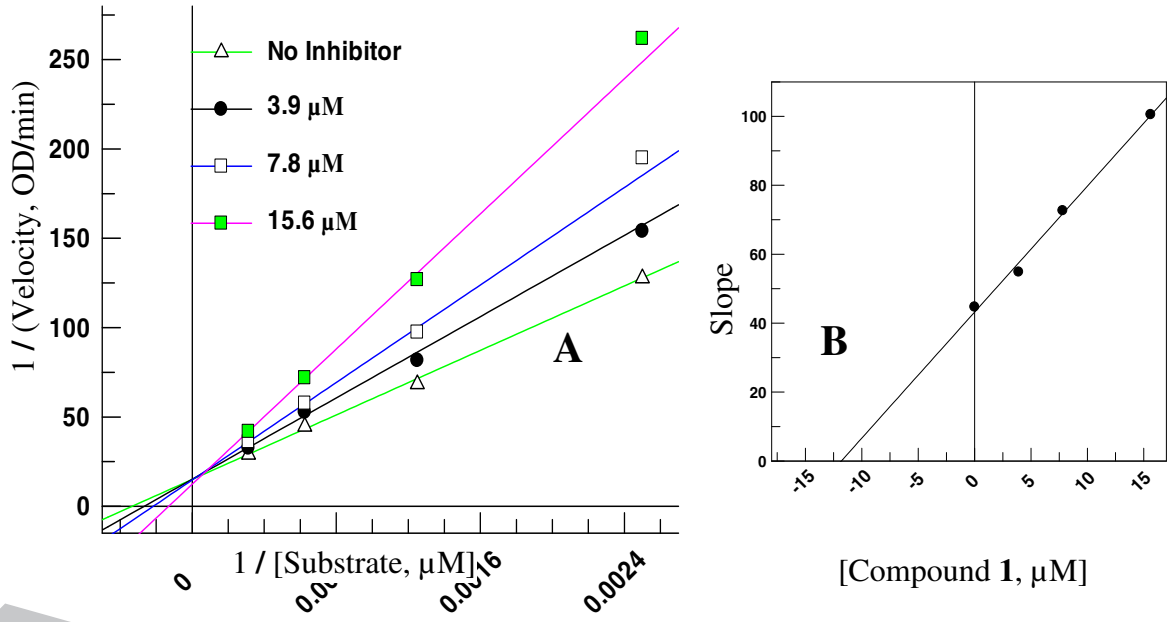


Figure-2: (**A**) Line-weaver Burk plot of various concentrations of compound **1**. In this steady-state kinetics, all the lines intersect each other at y-axis indicating a competitive-type of inhibition. (**B**) Secondary plot generated from Line-weaver Burk plot. *Ki* value is equivalent to the meeting point of the straight line at x-axis.

ACCEPTED MANUSCRIPT

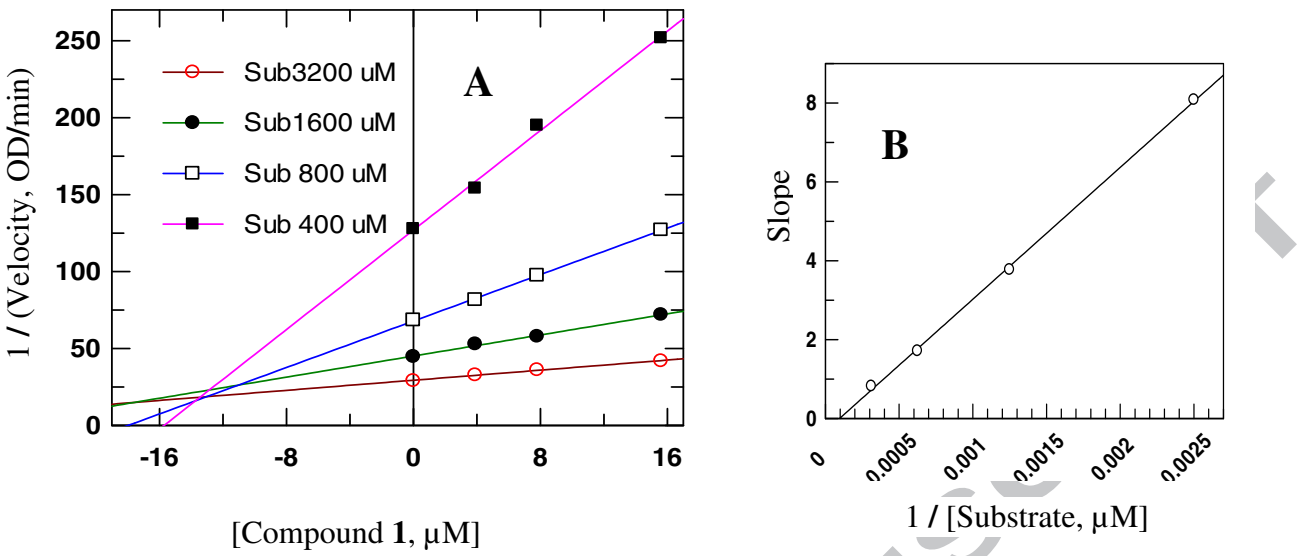


Figure-3: (**A**) shows the Dixon plot of compound **1** with various concentrations of substrate. In this graph, these lines meet at 2nd quadrant indicating a competitive type of inhibition. *Ki* value is equivalent to the meeting point of the straight line at x-axis **(B)** Secondary plot produced from Dixon plot for compound **1.** The line passes through origin clarifies confusion with mixed-type of inhibition.

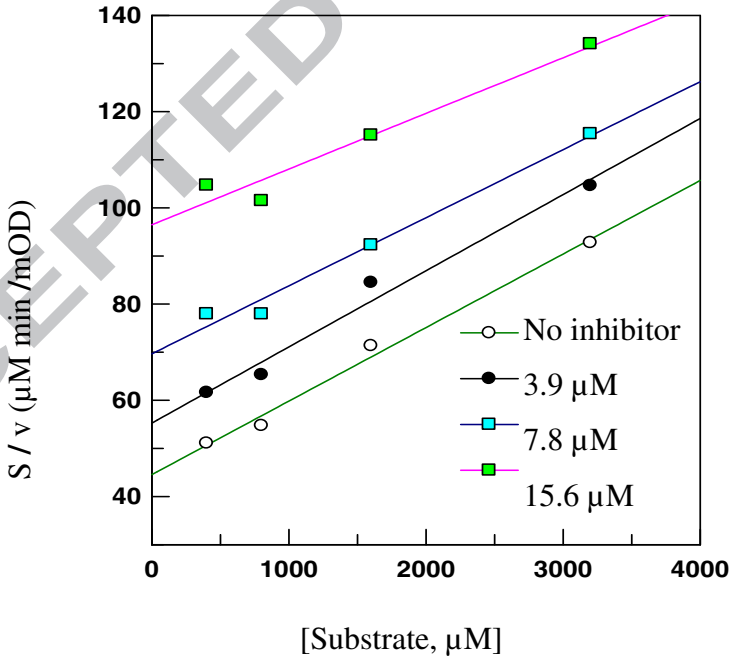


Figure-4: Hanes-Woolf plot of various concentrations of compound **1**. These parallel lines indicate that the compound is a competitive-type of inhibitor.

ACCEPTED MANUSCRIPT

2.4 Molecular Docking Studies

Active site of chymostrypsin is burried in solvent-inaccessible pocket. The active site of chymotrypsin contains Asp-102, His-57, and Ser-195, referred as catalytic triad which *via* a series of reaction sequence produces nucleophilic Ser-195[21]. Inhibition of chymotrypsin activity is linked with the blockage of nucleophilic catalytic reaction, carried out by Ser-195. Chymostatin[22], a standard compound was found to bind with Ser-195 and other residues, including hydrophobic residues in its close proximity. These residues involve Asp-194, Cys-191, Gly193, Met-192, Ser-217, and Tyr-228. Among these residues, backbone amide hydrogens of Ser-195 and Gly-193 pointing into the active site cavity form a region in space which is named as "oxyanion hole". This space plays an important role to enhance the rate of the catalytic reaction by several factors.

Chymostatin (CHY) is a large complex organic molecule, which is stabilized in the active site of α-chymotrypsin by polar interactions between aromatic rings of CHY, and Ser-195 and Tyr-228, as illustrated in **Figure-5**. In addition to these, His-57 and Ser-217 dynamically participate in hydrogen bonding with amide hydrogens of CHY. Met-192 and Asp-194 also provide extra stability to CHY and contribute in the inhibitory activity of the molecule. Nitrogen containing aliphatic ring was found to partially occupy the space of the oxyanion hole region that may causes influence on the rate of enzymatic activity. **Figure-6** depicts the binding of compound **1** in the enzyme's active site. Compared to CHY, compound **1** occupies less space of the active site but it has a major interaction between methoxy substituted aromatic ring and Ser-195, whereas His-57 was found to contribute in the stabilization effect *via* possible interaction with imidazole ring and methoxy aromatic ring. Apart from these catalytic residues, amide hydrogen of Gly-193 forms H-bonding with imidazole nitrogen, whereas chlorine atom of the benimidazole ring was

ACCEPTED MANUSCRIPT

partially exposed to solvent accessible region and thus could not find suitable residues to form favourable polar interactions. This may lead to lesser inhibitory activity of the compound **1** as compared to chymostatin. However, description of the binding pattern of compound **1** to the enzyme's active site may serve to rationalize its inhibitory potency in comparison to the standard compound, chymostatin which has higher inhibition potential as deduced from a lower IC_{50} value, yet the newly synthesized benzimidazole derivatives may therefore, be considered as new leads for further optimization.

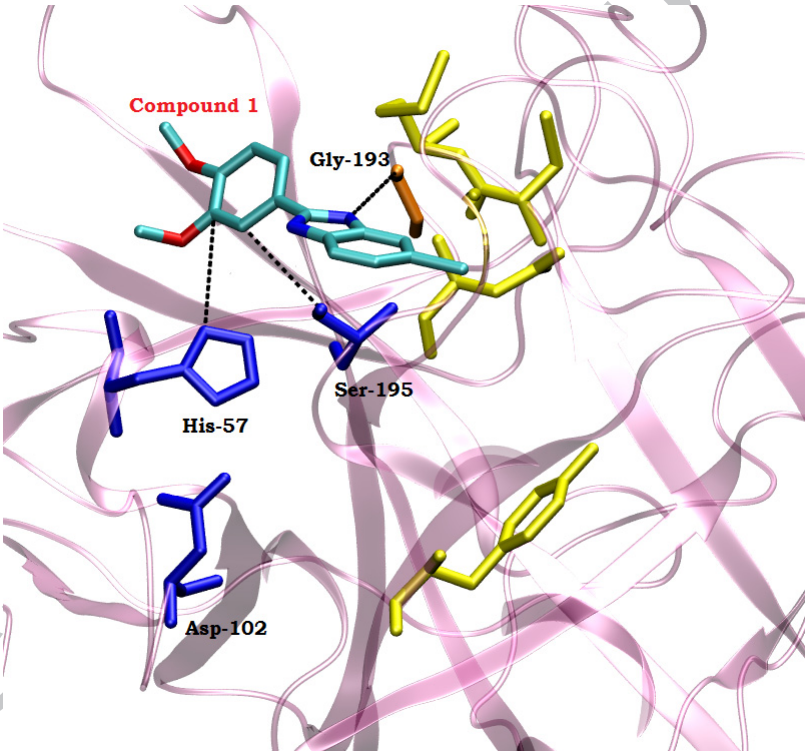


Figure-5: Binding of compound **1** with active site residues of chymotrypsin enzyme (hydrogens were removed for clarity)

ACCEPTED MANUSCRIPT

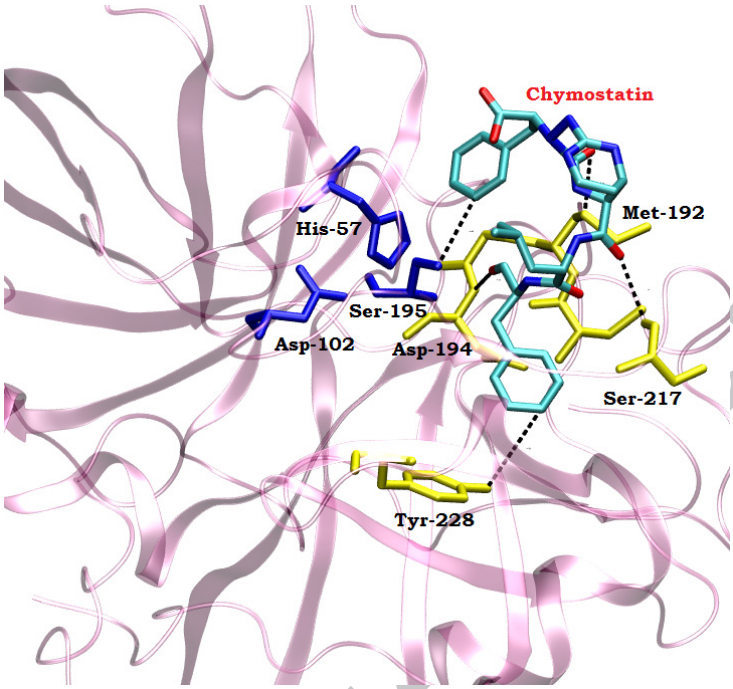


Figure-6: Binding of chymostatin (standard) with active site residues of chymotrypsin enzyme (hydrogens were removed for clarity)

3.0 Conclusion:

We have identified a series of chloro-benzimidazole derivatives as new inhibitors of α chymotrypsin inhibitors. This may serve as templates for the designing, and development of new inhibitors of α- chymotrypsin and chymotrypsin-like serine proteases enzymes and associated diseases.

4.0 Experimental

4.1 General Experimental Conditions:

All the chemicals were purchased from commercial suppliers and used for reaction without

ACCEPTED MANUSCRIPT

purification. Reaction was carried out in flame dried flasks, and with chemically dried solvents. EI-MS (Electron Impact Mass Spectra) were recorded on a JEOL JMS-600H mass spectrometer. The data was analyzed by using MASPEC data system. The NMR spectra were recorded in deuterated solvents on Avance Bruker 300, 400, 500, and 600 MHz NMR spectrometers. The chemical shifts (δ) and coupling constants (J) were measured in ppm and Hz, respectively, $SiMe_4$ was used as an internal standard. TLC analyses were performed on pre-coated ALUGRAM, SIL G/UV_{254} aluminum plates (Kieselgel 60, 20×20, 0.5 mm thick, E. Merk, Germany). Chromatograms developed on TLC plates were visualized under ultraviolet light at 254 nm for fluorescence quenching spots, and 365 nm for florescent spots.

4.2 General Procedure for Synthesis of Compounds 1-20:

The synthesis of 6-chlorobenzimidazole derivatives **1-20** was carried out by reaction of 4-chlorophenylenediamine with various aromatic aldehydes in DMF in very good yields. In a general reaction, 4-chlorophenylenediamine (1.00 eq), and aromatic aldehydes (1.00 eq) were dissolved in 10 mL DMF, followed by addition of sodium metabisulfite ($Na_2S_2O_5$) (0.29 eq), and refluxed till the reaction was completed. After the completion, the reaction mixture was cooled to room temperature, followed by the addition of ice cold water. Resulting precipitates were filtered, and purified through column chromatography. The structures of the products were deduced by ^{1}H-NMR spectroscopy. and EI-MS analysis.

4.2.1 6-Chloro-2-(3,4-dimethoxyphenyl)-1*H*-benzimidazole (1)

Yield: 0.29 gm (58 %): ^{1}H-NMR: (300 MHz, CD_3OD): δ_H 7.71 (d, 1H, $J_{5,7}$ = 1.5 Hz, H-5) 7.67 (dd, 1H, $J_{6',5'}$ = 6.3 Hz, $J_{6',2'}$ = 1.5 Hz, H-6'), 7.53 (bs, 2H, H-8/ H-2'), 7.23 (dd, 1H, $J_{5,4}$ = 6.3 Hz, $J_{7,5}$ = 1.2 Hz, H-7), 7.12 (d, 1H, $J_{5',6'}$= 6.3 Hz, H-5'), 3.95 (s, 3H, OCH_3-3'), 3.90 (s, 3H, OCH_3-4')

ACCEPTED MANUSCRIPT

^{13}C-NMR: (125 MHz, CD_3OD): δ 152.8 (C-2), 150.96 (C-3' and C-4'), 124.0 (C-4 and C-9), 123.2 (C-6 and C-1'), 121.1 (C-7 and C-6'), 112.9 (C-8 and C-5), 111.2 (C-2' and C-5'), 56.5 (OCH_3-3', and C-4'); EI-MS: *m/z* (rel. abund %): 290 ([M+2]$^+$, 95), 288 (M$^+$, 100), 230 (15), 192 (19); HREI-MS: Calculated for $C_{15}H_{13}ClN_2O_2$ Exact Mass: 288.0666 found 288.0664.

4.2.2 4-(6-Chloro-1*H*-benzimidazol-2-yl)-2-ethoxyphenol (2)

Yield: 0.4 gm (80%): ^{1}H-NMR: (300 MHz, CD_3OD): δ_H 7.67 (d,1H, $J_{5,7}$ = 2.1 Hz, H-5), 7.54 (m, 2H, H-8/H-6'), 7.50 (d, 1H, $J_{2',6'}$ = 1.8 Hz, H-2'), 7.21 (dd, 1H, $J_{7,8}$ = 8.7 Hz, $J_{7,5}$ = 1.8 Hz, H-7), 6.95 (d, 1H, $J_{5',6'}$ = 8.4 Hz, H-5'), 4.23 (q, 2H, $J_{2'',3''}$ = 6.9 Hz, H-2″), 1.50 (t, 3H, $J_{3'',2''}$ = 6.9 Hz, H-3″); EI-MS: *m/z* (rel. abund %): 290 ([M+2]$^+$, 36), 288 (M$^+$, 100), 260 (75); ^{13}C-NMR: (125 MHz, CD_3OD): δ 150.7 (C-2), 148.6 (C-3' and C-4'), 123.8 (C-7 and C-6'), 122.0 (C-4 and C-9), 121.3 (C-5 and C-8), 116.7 (C-5'), 112.5 (C-2'), 112.4 (C-6 and C-1'), 65.7 (C-2″), 15.0 (C-3″); HREI-MS: Calculated for $C_{15}H_{13}ClN_2O_2$ Exact Mass: 288.0666 found 288.0671.

4.2.3 6-Chloro-2-(1-naphthyl)-1*H*-benzimidazole (3)

Yield: 0.28 gm (58%): ^{1}H-NMR: (300 MHz, CD_3OD): δ_H 8.47 (m, 1H, H-8'), 8.08 (d, 1H, $J_{3'/2'/4'}$ = 8.4 Hz, H-3'), 8.00 (m, 2H, H-7'/ H-4'), 7.88 (dd, 1H, $J_{2',3'}$ = 7.2 Hz, $J_{2',4'}$= 0.9 Hz, H-2'), 7.65 (m, 4H, H-7'/ H-6'/ H-5'/ H-8), 7.32 (dd, 1H, $J_{7,8}$ = 8.7 Hz, $J_{7,5}$ = 1.8 Hz, H-7); EI-MS: *m/z* (rel. abund %): 279 ([M+2]$^+$, 44), 277 (M$^+$, 100), 278 (71), 242 (29), 141 (25); HREI-MS: Calculated for $C_{17}H_{11}ClN_2$ Exact Mass: 278.0611 found 278.0601.

4.2.4 4-(6-Chloro-1*H*-benzimidazol-2-yl)-*N, N*-dimethylaniline (4)

Yield: 0.25 gm (53%): ^{1}H-NMR: (300 MHz, CD_3OD): δ_H 7.92 (d, 2H, $J_{2',3'/6',5'}$ = 9.0 Hz, H-2'/H-6'), 7.52 (d, 1H, $J_{5,7}$ = 1.8 Hz, H-5), 7.50 (d, 1H, $J_{8,7}$ = 8.7 Hz, H-8), 7.22 (dd, 1H, $J_{7,8}$ = 8.7 Hz,

ACCEPTED MANUSCRIPT

$J_{7,5}$ = 1.8 Hz, H-7), 6.86 (d, 2H, $J_{3',2'/5',6'}$= 9.0 Hz, H-3'/ H-5'), 3.05 (s, 6H, $N(CH3)_2$); EI-MS: *m/z* (rel. abund %): 273 ($[M+2]^+$, 36), 271 (M^+, 100), 255 (44), 221 (18); HREI-MS: Calculated for $C_{15}H_{14}ClN_3$ Exact Mass: 271.0876 found 271.0891.

4.2.5 4-(6-Chloro-1*H*-benzimidazol-2-yl)-1, 3-benzenediol (5)

Yield: 0.20 gm (44%): ^{1}H-NMR: (300 MHz, CD_3OD): δ_H 7.72 (d, 1H, $J_{8,7}$ = 6.0 Hz, H-8), 7.53 (s, 1H, H-5), 7.49 (d, 1H, $J_{6',5'}$ = 6.3 Hz, H-6'), 7.19 (dd, 1H, $J_{7,8}$ = 8.7 Hz, $J_{7,5}$ = 1.8 Hz, H-7), 6.44 (d, 1H, $J_{3',5'}$ = 1.5 Hz, H-3'), 6.42 (overlapped, 1H, H-5'); EI-MS: *m/z* (rel. abund %): 262 ($[M+2]^+$, 47), 260 (M^+, 100), 203 (23); HREI-MS: Calculated for $C_{13}H_9ClN_2O_2$ Exact Mass: 260.0353 found 260.0339.

4.2.6 6-Chloro-2-(2, 4-dichlorophenyl)-1*H*-benzimidazole (6)

Yield: 0.35gm (68%): ^{1}H-NMR: (300 MHz, CD_3OD): δ_H 7.84 (d, 1H, $J_{6',5'}$ = 8.4 Hz, H-6'), 7.70 (d, 1H, $J_{3',5'}$ = 2.1 Hz, H-3'), 7.63 (bs, 2H, H-5/ H-8), 7.53 (dd, 1H, $J_{5',6'}$ =8.4 Hz, $J_{5',3'}$ = 2.1 Hz, H-5'), 7.29 (dd, 1H, $J_{7,8}$ = 8.7 Hz, $J_{7,5}$ = 1.8 Hz, H-7); EI-MS: *m/z* (rel. abund %): 298 ($[M+2]^+$, 100), 296 (M^+, 100); HREI-MS: Calculated for $C_{13}H_7Cl_3N_2$ Exact Mass: 295.9675 found 295.9674.

4.2.7 6-Chloro-2-(3-pyridinyl)-1*H*-benzimidazole (7)

Yield: 0.34 gm (65%): ^{1}H-NMR: (300 MHz, CD_3OD): δ_H 9.24 (d, 1H, $J_{2',6'}$ = 1.5 Hz), 8.68 (dd, 1H, $J_{4',5'}$ = 4.8 Hz, $J_{4',6'}$ =1.5 Hz, H-4'), 8.50 (dt, 1H, $J_{6',5'}$= 8.1 Hz, $J_{6'/2',4'}$=1.8 Hz, H-6'), 7.63 (m, 3H, H-5'/ H-8/ H-5), 7.30 (dd, 1H, $J_{7,8}$ = 8.7 Hz, $J_{7,5}$ = 2.1 Hz, H-7); EI-MS: *m/z* (rel. abund %): 231 ($[M+2]^+$, 94), 229 (M^+, 100), 203 (60), 226 (45); HREI-MS: Calculated for $C_{12}H_8ClN_3$ Exact Mass: 229.0407 found 229. 0404.

ACCEPTED MANUSCRIPT

4.2.8 6-Chloro-2-(2, 6-dichlorophenyl)-1*H*-benzimidazole (8)

Yield: 0.38 gm (74%): ^{1}H-NMR: (300 MHz, CD_3OD): δ_H 7.60 (bs, 1H, H-5), 7.60 (overlapped, 1H, H-8), 7.56 (m, 2H, H-3'/ H-5'), 7.52 (d, 1H, $J_{4'/3',5'}$ = 4.8 Hz, H-4'), 7.32 (dd, 1H, $J_{7,8}$ = 8.7 Hz, $J_{7,5}$ =1.8 Hz, H-7); EI-MS: *m/z* (rel. abund %): 298 ([M+2]$^+$, 76), 296 (M$^+$, 100), 271 (20), 226 (45); HREI-MS: Calculated for $C_{13}H_7Cl_3N_2$ Exact Mass: 295.9675 found 298.9680.

4.2.9 6-Chloro-2-phenyl-1*H*-benzimidazole (9)

Yield: 0.25 gm (64%): ^{1}H-NMR: (300 MHz, CD_3OD): δ_H 8.08 (m, 2H, H-5/8), 7.58 (m, 3H, H-3'/4'/5'), 7.53 (m, 2H, H-2'/ H-6'), 7.25 (dd, 1H, $J_{7,8}$ = 8.4 Hz, $J_{7,5}$ =1.8 Hz, H-7); EI-MS: *m/z* (rel. abund %): 230 ([M+2]$^+$, 61), 228 (M$^+$, 100), 229 (31); HREI-MS: Calculated for $C_{13}H_9ClN_2$ Exact Mass: 228.0454 found 228.0452.

4.2.10 6-Chloro-2-(2-chloro-5-nitrophenyl)-1*H*-benzimidazole (10)

Yield: 0.35 gm (66%): ^{1}H-NMR: (300 MHz, $CDCl_3$): δ_H 10.9 (s, 1H, H-3), 8.75 (s. 1H, H-6'), 7.99 (d, 1H, $J_{4',3'}$ = 6.3 Hz, H-4'), 7.72 (s. 1H, H-5), 7.68 (d, 1H, $J_{3',4'}$ = 6.3 Hz, H-3'), 7.29 (d, 1H, $J_{8,7}$ = 6.3 Hz, H-8), 7.02 (d, 1H, $J_{7,8}$ = 6.6 Hz, H-7); EI-MS: *m/z* (rel. abund %): 309 ([M+2]$^+$, 100), 307 (M$^+$, 100), 226 (85).

4.2.11 4-(6-Chloro-1*H*-benzimidazol-2-yl)benzonitrile (11)

Yield: 0.31 gm (70%): ^{1}H-NMR: (300 MHz, CD_3OD): δ_H 8.23 (d, 2H, $J_{3',2'/5',6'}$= 8.4 Hz, H-3'/H-5'), 7.91 (d, 2H, $J_{2',3'/6',5'}$ = 8.4 Hz, H-2'/H-6'), 7.62 (overlapped, 1H, H-8), 7.58 (s, 1H, H-5), 7.29 (dd, 1H, $J_{7,8}$ = 8.7 Hz, $J_{7,5}$ =1.8 Hz, H-7); EI-MS: *m/z* (rel. abund %): 255 ([M+2]$^+$, 97), 253 (M$^+$, 100), 217 (15); HREI-MS: Calculated for $C_{14}H_8ClN_3$ Exact Mass: 253.0407 found 253.0406.

ACCEPTED MANUSCRIPT

4.2.12 6-Chloro-2-(4-methylphenyl)-1*H*-benzimidazole (12)

Yield: 0.28 gm (66%): ^{1}H-NMR: (300 MHz, CD_3OD): δ_H 7.96 (d, 2H, $J_{2',3'/6',5'}$ = 8.4 Hz, H - 2'/ H-6'), 7.56 (Overlaped, 2H, H-5/ H-8), 7.36 (d, 2H, $J_{3',2'/5',6'}$ = 8.1 Hz, H-3'/ H-5'), 7.23 (dd, 1H, $J_{7,8}$ = 8.4 Hz, $J_{7,5}$ = 1.8 Hz, H-7), 2.413(s, 3H, CH_3); EI-MS: *m/z* (rel. abund %): 244 ([M+2]$^+$, 87), 242 (M$^+$, 100), 207 (20); HREI-MS: Calculated for $C_{14}H_{11}ClN_2$ Exact Mass: 242.0611 found 242.0602.

4.2.13 6-Chloro-2-[2-phenylethenyl]-1*H*-benzimidazole (13)

Yield: 0.24 gm (54%): ^{1}H-NMR: (300 MHz, CD_3OD): δ_H 7.66 (d, 1H, $J_{1',2'}$ = 16.5 Hz, H-1'), 7.63 (d, 2H, $J_{2'',3''/6'',5''}$ = 7.2 Hz, H-2''/ H-6''), 7.53 (bs, 2H, H-5/ H-8), 7.39 (m, 3H, H-3''/ H-4''/ H-5''), 7.22 (dd, 1H, $J_{7,8}$ = 8.4 Hz, $J_{7,5}$ = 1.8 Hz, H-7), 7.15 (d, 1H, $J_{2',1'}$ = 16.5 Hz, H-2'); EI-MS: *m/z* (rel. abund %): 255 ([M+2]$^+$, 39), 253 (M$^+$, 100), 218 (22); HREI-MS: Calculated for $C_{15}H_{11}ClN_2$ Exact Mass: 254.0611 found 254.0587.

4.2.14 2-(6-Chloro-1*H*-benzimidazol-2-yl)phenol (14)

Yield: 0.3 gm (71%): ^{1}H-NMR: (300 MHz, CD_3OD): δ_H 7.92 (bs, 1H, H-5), 7.60 (m, 2H, H-8/H-6'), 7.35 (t, 1H, $J_{4',5'}$ = 7.2 Hz, H-4'), 7.25 (dd, 1H, $J_{7,8}$ = 8.7 Hz, $J_{7,5}$ = 1.8 Hz, H-7), 7.02 (m, 2H, H-3'/H-5'); EI-MS: *m/z* (rel. abund %): 264 ([M+2]$^+$, 34), 244 (M$^+$, 100), 216(35); HREI-MS: Calculated for $C_{13}H_9ClN_2O$ Exact Mass: 244.0403 found 244.0408.

4.2.15 6-chloro-2-(furan-2-yl)-1-methyl-1H-benzimidazole (15)

Yield: 0.31 gm (77%): ^{1}H-NMR: (300 MHz, CD_3OD): δ_H 7.52 (bs, 2H, H-5/H-3'), 7.23 (dd, 1H, $J_{7,8}$ = 8.7 Hz, $J_{7,5}$ = 1.8 Hz, H-7), 7.09 (d, 1H, $J_{8,7}$ = 3.3 Hz, H-8), 6.28 (d, 1H, $J_{5',4'}$ = 3.3 Hz, H-5'), 6.0 (m, 1H, H-4'), 2.43 (s, 3H, N-Me); EI-MS: m/z (rel. abund %): 234 ([M+2]$^+$, 29),232

ACCEPTED MANUSCRIPT

(M^+, 100), 217(22), 202(25); HREI-MS: Calculated for $C_{12}H_9ClN_2O$ Exact Mass: 232.0403 found 232.0405.

4.2.16 6-Chloro-2-(4-isopropylphenyl)-1*H*-benzimidazole (16)

Yield: 0.32 gm (68%): ^{1}H-NMR: (300 MHz, CD_3OD): δ_H 8.00 (d, 2H, $J_{2',3'/6',5'}$ = 8.4 Hz, H-2'/H-6'), 7.56 (bs, 2H, H-5/H-8), 7.43 (d, 2H, $J_{3'2',/5',6'}$ = 8.4 Hz, H-3'/H-5'), 7.23 (dd, 1H, $J_{7,8}$ = 8.4 Hz, $J_{7,5}$ = 1.5 Hz, H-7), 7.29 (m, 1H, $CH(CH_3)_2$), 1.31 (d, 6H, J = 6.9 Hz); EI-MS: *m/z* (rel. abund %): 272 ([M+2]$^+$, 17), 270 (M^+, 50), 255(100); HREI-MS: Calculated for $C_{16}H_{15}ClN_2$ Exact Mass: 270.0924 found 270.0913.

4.2.17 3-(6-Chloro-1*H*-benzimidazol-2-yl)phenyl methyl ether (17)

Yield: 0.34 gm (76%): ^{1}H-NMR: (300 MHz, CD_3OD): δ_H 7.66 (d, 1H, $J_{8,7}$ = 2.4 Hz, H-8), 7.62 (s, 1H, H-5), 7.58 (bs, 2H, H-2′/ H-6′), 7.44 (t, 1H, $J_{5'/4',6'}$ = 8.1, H-5′), 7.26 (dd, 1H, $J_{7,8}$ = 8.4, $J_{7,5}$ = 3 Hz , H-7), 7.10 (dd, 1H, $J_{4',5'}$ = 8.4 Hz, $J_{4',2'}$ = 2.4 Hz, H-4′), 3.89 (s, 3H, OCH_3-3′); EI-MS: *m/z* (rel. abund %): 260 ([M+2]$^+$, 36), 258 (M^+, 100), 228 (40); HREI-MS: Calculated for $C_{14}H_{11}ClN_2O$ Exact Mass: 258.0560 found 258. 0553.

4.2.18 6-Chloro-2-(2-fluorophenyl)-1*H*-benzimidazole (18)

Yield: 0.36 gm (83%): ^{1}H-NMR: (300 MHz, CD_3OD): δ_H 8.19 （td, 1H, $J_{4',6'}$ = 1.8 Hz, $J_{4'/3',5'}$ = 9.3 Hz, H-4′), 7.632 (m, 1H, H-3′), 7.53 (m, 2H, H-5′/ H-6′), 7.40 (overlapped, 2H, H-5/H-8), 7.27 (dd, 1H, $J_{7,8}$ = 8.7, $J_{7,5}$ = 1.8 Hz, H-7); EI-MS: *m/z* (rel. abund %): 248 ([M+2]$^+$, 39), 246 (M^+, 100); HREI-MS: Calculated for $C_{13}H_8ClFN_2$ Exact Mass: 246.0360 found 246.0357.

ACCEPTED MANUSCRIPT

4.2.19 2-(3-bromophenyl)-6-chloro-1H-benzimidazole (19)

Yield: 0.38 gm (71%): ^{1}H-NMR: (300 MHz, CD_3OD): δ_H 8.27 (s, 1H, H-5), 8.04 (d, 1H, $J_{6',5'}$ = 7.8 Hz, H-6'), 7.68 (d, 1H, $J_{8,7}$ = 7.8 Hz, H-8), 7.60 (m, 1H, H-4'), 7.55 (s, 1H, H-2'), 7.47 (t, 1H, $J_{5'/4',6'}$ = 7.8 Hz, H-5'), 7.28 (dd, 1H, $J_{7,8}$ = 8.7 Hz, $J_{7,5}$ = 2.1 Hz, H-7); EI-MS: *m/z* (rel. abund %): 309 ($[M+2]^+$, 26), 307 (M^+, 100), 227 (72), 192 (82); HREI-MS: Calculated for $C_{13}H_8BrClN_2$ Exact Mass: 307.9716 found 307.9712.

4.2.20 6-Chloro-2-(4-nitrophenyl)-1*H*-benzimidazole (20)

Yield: 0.42 gm (79%): ^{1}H-NMR: (300 MHz, CD_3OD): δ_H 8.42 (d, 2H, $J_{3',2'/5',6'}$ = 9 Hz, H - 3'/ H-5'), 8.31 (d, 2H, $J_{2',3'/6',5'}$ = 9 Hz, H - 2'/ H-6'), 7.64 (d, 1H, $J_{8,7}$ = 3.0 Hz, H-8), 7.60 (s, 1H, H-5), 7.30 (dd, 1H, $J_{7,8}$ = 8.7 Hz, $J_{7,5}$ = 1.8 Hz, H-7); EI-MS: *m/z* (rel. abund %): 275 ($[M+2]^+$, 83), 273 (M^+, 100), 227(90), 192(75); HREI-MS: Calculated for $C_{13}H_8ClN_3O_2$ Exact Mass: 273.0305 found 273.0303.

4.3 α-Chymotrypsin Inhibitory Assay

α-Chymotrypsin inhibitory activity was determined in 50 mM *tris*-HCl buffer pH 7.6 with 10 mM $CaCl_2$. α-Chymotrypsin (12 units/mL, in buffer) with the various concentrations of test compounds in DMSO was incubated at 30 °C for 25 min. The reaction began with the addition of the substrate, *N*-succinyl-*L*-phenylalanine-*p*-nitroanilide (SPpNA; 0.4 mM final, in the buffer). The variations in the absorbance with the released *p*-nitroaniline was monitored at 410 nm[23]. All the experiments were performed in triplicate in a final volume of 200 µL, using a micro plate reader (SpectraMax M2, Molecular Devices, CA, USA).The % of inhibition was calculated as:

$$\% \text{ Inhibition} = 100 - \left(\frac{\text{OD/min of test compound}}{\text{OD/min of positive control}}\right) \times 100$$

ACCEPTED MANUSCRIPT

IC_{50} (Inhibition of enzymatic hydrolysis of the substrate SPpNA by 50%) values were calculated using EZ-Fit enzyme kinetics program (Perellela Scientific, Inc., Amherst, USA). α-Chymostatin (Standard) was used as the positive control, whereas DMSO was added in the negative control instead of compounds

4.4 α-Chymotrypsin Inhibition Kinetic Study

Per minute change in optical density (OD/min) was measured by incorporating various concentrations of test compound with various concentrations (between 0.4 and 3.2 mM) of substrate (SPpNA). Reciprocal of the rate of the reactions against the reciprocal of the substrate concentration as Lineweaver–Burk plot (and its secondary plot; slope *vs* compound concentration); the Dixon plot (and its secondary plot; slope *vs* reciprocal of compound concentration), and then Hanes-Woolf plot were plotted[24]. Graphs were plotted using GraFit 4 (Erithacus Software Limited, Surrey, UK), and Graph Pad Prism5 (Graph Pad Software, California, USA). Graphical views of Dixon plots, Lineweaver-Burk plots, and their secondary plots, as well as Hanes-Woolf plot were used to determine the types of inhibition. The Ki values, obtained directly from the software, were cross-checked also in these graphs. Final concentration of DMSO was maintained at 5.5 %.

4.5 Cytotoxicity Assay:

The cytotoxicity of all compounds (**1-20**) was evaluated by using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay against 3T3 (Mouse fibroblast) cells[25]. Briefly 3T3

ACCEPTED MANUSCRIPT

cells were grown in DMEM (containing 100 µg/mL streptomycin, 5% of FBS and 100 IU/mL penicillin), and incubated at 37 ^{o}C in 5% CO_2. Cells were maintained at 5x10^4 cells/ mL concentration, and introduced into 96-well plates and allowed to stand for overnight period. Medium was removed, and 200 µL of fresh medium was added, various concentrations of the test compounds were also added and allowed to react for 48 hrs. 0.5 mg/mL of MTT was added to each well and incubated for 4 more hours. DMSO was added and reduction of MTT to formazan dye was calculated by recording the absorbance at 540 nm using a micro plate reader (Spectra Max plus, Molecular Devices, CA, USA). Cycloheximide was used as positive control whereas DMSO was added in the negative control instead of compounds. The percent inhibition was calculated for each cell by using the following formula:

% Inhibition = 100-{(mean of O.D. of test compound – mean of O.D. of negative control)/ (mean of O.D. of positive control – mean of O.D. of negative control) x 100}

4.6 Molecular Docking Studies:

Crystal structure of chymotrypsin complexed with benzohydroxamic acid/vanadate (BVA) (PDB ID: 2P8O)[26] was used for the docking studies using AutoDock 4.2, executed with the help of graphical user interface, AutoDockTools 1.4.5 (ADT)[27]. Docking studies were performed using the Lamarckian Genetic Algorithm (LGA) as a search engine. The GUI (Graphical User Interface) helped to prepare the protein and ligands structure files for the grid and docking calculations. The free active site was obtained after removing coordinates of co-crystallized ligand as well as water molecules were also removed. Coordinates of ligands *i.e.* compound **1**,

ACCEPTED MANUSCRIPT

chymostatin (standard compound), and BVA were built and quantum mechanically optimized using Gaussian 09[28] at Density Functional Theory level with B3LYP functional and 6-31G (d, p) basis sets were utilized for all atoms. Point atomic charges were applied to ligands which were adopted from the Mulliken Population Analysis. The docking protocol was validated by reproducing binding pose of the BVA inhibitor into the active site on the basis of root mean square deviation, and that came out to be around 1.00 Å, as shown in Figure-7. The resolution and dimensions of three-dimensional energy scoring grids were set to 0.375 Å and 40 × 40 × 40 Å dimensions to carry out docking of BVA ligand, whereas the dimensions of other ligands were increased to 44× 44 × 44 Å in order to keep the ligand inside the grid. Other parameters for LGA consist of default setting for each docking calculation with a total of 30 runs with a maximum of 2,500,000 energy evaluations, and cluster analysis using a root–mean–square (rms) tolerance of 1.5 Å.

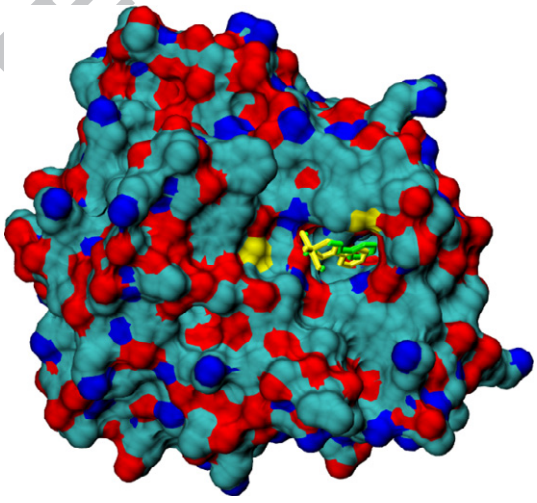
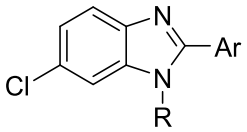


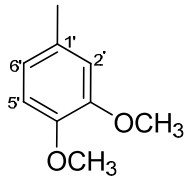
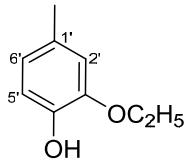
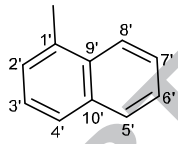
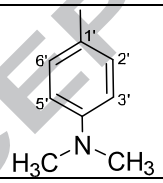
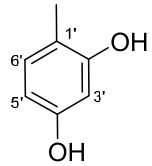
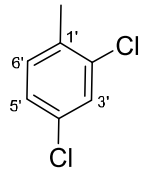
Figure-7 Superposition of co-crystallized (yellow) and re-docked (green) conformation into the protein active site

ACCEPTED MANUSCRIPT

Table-1: Inhibition of α-chymotrypsin by 6-chlorobenzimidazole derivatives **1-20,** and their cytotoxicity.

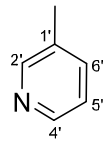
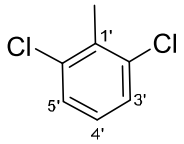
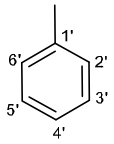
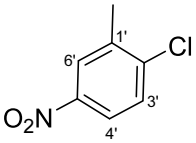
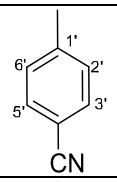
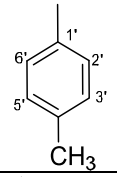
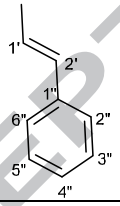
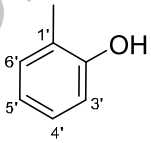
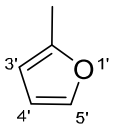


1-20

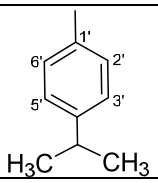
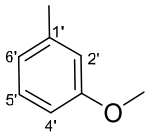
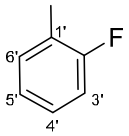
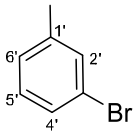
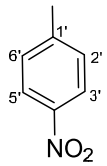
Compound	-Ar	-R	IC_{50} ± SEM (µM)	Ki ± SEM (µM)	Type of inhibition	Cytotoxicity IC_{50} ± SD (µM)
1		H	14.8 ± 0.1	16.4 ± 0.9	Competitive	>30
2		H	15.9 ± 0.5	17.3 ± 0.8	Competitive	>30
3		H	20.9± 0.1	25.31 ± 3.1	Competitive	11.577±0.467
4		H	24.2 ± 2.1	17.5 ± 0.8	Competitive	>30
5		H	34.6 ± 0.5	33.3 ± 1.1	Competitive	28.146±1.731
6		H	35.6 ± 2.2	39.6 ± 2.7	Competitive	13.794±0.970

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

7		H	48.9 ± 0.5	50.1 ± 2.9	Competitive	>30
8		H	96.4 ± 2.6	99.8 ± 7.4	Competitive	25.180±0.384
9		H	>500	NC	NC	>30
10		H	>500	NC	NC	>30
11		H	>500	NC	NC	>30
12		H	>500	NC	NC	25.99±0.573
13		H	486.1±9.8	NC	NC	12.460±0.240
14		H	>500	NC	NC	3.792±0.200
15		CH_3	93.1±3.2	71.4±5.9	Competitive	>30

ACCEPTED MANUSCRIPT

16		H	427.6±10.2	NC	NC	7.913±0.242
17		H	38.1±0.6	36.5±5.8	Mixed	18.371±0.668
18		H	118.2±6.1	79.3±8.7	Competitive	>30
19		H	457.5±8.8	NC	NC	6.903±0.084
20		H	470.6±8.2	NC	NC	7.218±0.199
21	Chymostatin (Standard)	-	5.7±0.13	-	-	-
22	Cycloheximide (Standard)	-	-	-	-	0.26±0.1

S.E.M. = Standard Error of Mean at n=3
SD = Standard Deviation
** = Anomalous Type of Inhibition
NC = Not calculated (because of % of inhibition shown was less than 50% at 500 µM.

5.0. Conflict of Interest:

The authors declare no conflict of interest.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

6.0 References:

1. Pochet, L.; Doucet, C.; Dive, G.; Wouters, J.; Masereel, B.; Reboud-Ravaux, M.; Pirotte, B., Coumarinic derivatives as mechanism-based inhibitors of α-chymotrypsin and human leukocyte elastase. *Bioorganic & Medicinal Chemistry* **2000,** *8* (6), 1489-1501.

2. Wilcox, P., [5] Chymotrypsinogens—chymotrypsins. *Methods in Enzymology* **1970,** *19*, 64-108.

3. Zhou, J.; Sahin‐Tóth, M., Chymotrypsin C mutations in chronic pancreatitis. *Journal of Gastroenterology and Hepatology* **2011,** *26* (8), 1238-1246.

4. Stehlik, C., Multiple interleukin‐1β–converting enzymes contribute to inflammatory arthritis. *Arthritis & Rheumatism* **2009,** *60* (12), 3524-3530.

5. Rauh, R.; Diakov, A.; Tzschoppe, A.; Korbmacher, J.; Azad, A. K.; Cuppens, H.; Cassiman, J. J.; Dötsch, J.; Sticht, H.; Korbmacher, C., A mutation of the epithelial sodium channel associated with atypical cystic fibrosis increases channel open probability and reduces Na+ self inhibition. *The Journal of Physiology* **2010,** *588* (8), 1211-1225.

6. (a) Ganesh, R.; Mohammad, U.; Rajesh, P.; Deepak, R., Synthesis of fluorobenzimidazoles as antimicrobial agents. *Indian Journal of Heterocyclic Chemistry* **2009,** *18*, 419-420;

   (b) Singh, N.; Pandurangan, A.; Rana, K.; Anand, P.; Ahamad, A.; Tiwari, A. K., Benzimidazole: A short review of their antimicrobial activities. *International Current Pharmaceutical Journal* **2012,** *1* (5), 119-127.

7. Ries, U. J.; Mihm, G.; Narr, B.; Hasselbach, K. M.; Wittneben, H.; Entzeroth, M.; Van Meel, J. C.; Wienen, W.; Hauel, N. H., 6-Substituted benzimidazoles as new nonpeptide angiotensin II receptor antagonists: synthesis, biological activity, and structure-activity relationships. *Journal of Medicinal Chemistry* **1993,** *36* (25), 4040-4051.

8. Demirayak, Ş.; Mohsen, U. A.; Karaburun, A. Ç., Synthesis and anticancer and anti-HIV testing of some pyrazino [1, 2-a] benzimidazole derivatives. *European Journal of Medicinal Chemistry* **2002,** *37* (3), 255-260.

9. Khan, K.; Shah, Z.; Ahmad, V.; Ambreen, N.; Khan, M.; Taha, M.; Rahim, F.; Noreen, S.; Perveen, S.; Choudhary, M., 6-Nitrobenzimidazole derivatives: Potential phosphodiesterase inhibitors: Synthesis and structure–activity relationship. *Bioorganic & Medicinal Chemistry* **2012,** *20* (4), 1521-1526.

10. Valdez, J.; Cedillo, R.; Hernández-Campos, A.; Yepez, L.; Hernández-Luis, F.; Navarrete-Vazquez, G.; Tapia, A.; Cortés, R.; Hernández, M.; Castillo, R., Synthesis and antiparasitic activity of 1H-benzimidazole derivatives. *Bioorganic & Medicinal*

ACCEPTED MANUSCRIPT

Chemistry Letters **2002,** *12* (16), 2221-2224.

11. Lazer, E. S.; Matteo, M. R.; Possanza, G. J., Benzimidazole derivatives with atypical antiinflammatory activity. *Journal of Medicinal Chemistry* **1987,** *30* (4), 726-729.

12. Márquez-Navarro, A.; Nogueda-Torres, B.; Hernández-Campos, A.; Soria-Arteche, O.; Castillo, R.; Rodríguez-Morales, S.; Yépez-Mulia, L.; Hernández-Luis, F., Anthelmintic activity of benzimidazole derivatives against Toxocara canis second-stage larvae and Hymenolepis nana adults. *Acta Tropica* **2009,** *109* (3), 232-235.

13. Iemura, R.; Kawashima, T.; Fukuda, T.; Ito, K.; Tsukamoto, G., Synthesis of benzimidazole derivatives as potential H1-antihistaminic aggents. *Journal of Heterocyclic Chemistry* **1987,** *24* (1), 31-37.

14. Chase, B.; Weller, W., The synthesis of some potential antibacterial agents. J*ournal of Pharmacy and Pharmacology* **1964**, 16 (3), 163-173.

15. Błaszczak-Świątkiewicz, K.; Olszewska, P.; Mikiciuk-Olasik, E., Antiproliferative activity of new benzimidazole derivatives. *Acta Biochimica Polonica* **2013**, 60 (3), 427-433.

16. Tavman, A.; Birteksoz, A. S., Spectral characterization and antimicrobial activity of 4-(5-H/Me/Cl/NO_2-1H-benzimidazol-2-yl)-benzene-1, 3-diols and some metal complexes. *Reviews in Inorganic Chemistry* **2009**, 29 (4), 255-272.

17. Wilson, D. M.; Termin, A. P.; Gonzalez III, J. E.; Zimmermann, N.; Zhang, Y.; Fanning, L. T., Benzimidazoles useful as modulators of ion channels. Google Patents: 2010.

18. (a) Özden, S.; Ertepinar, H.; Geban, Ö., QSAR study on antibacterial effects of benzimidazole and imidazopyridine derivatives. *Collection of Czechoslovak Chemical Communications* **1995**, 60 (12), 2178-2188;

(b) Paglietti, G.; Pirisi, M.; Loriga, M.; Grella, G.; Sparatore, F.; Satta, M.; Manca, P.; Peana, A., [Preparation and pharmacologic activity of 2-(4'R') benzyl-5R-benzimidazole. Analgesic activity and effect on conditioned avoidance response]. *Il Farmaco; Edizione Scientifica* **1988**, 43 (3), 203-214.

19. Sahoo, U.; Seth, A.; Balaraman, R.; Velmurugan, R., Design, Synthesis of Some Novel Thiazolidin-4-one Derivatives Bearing Benzimidazole Nucleus and Biological Evaluation of their Possible in vitro Antiinflammatory as Cyclooxygenase Inhibitors and Antioxidant Activity. *Asian Journal of Chemistry* **2015**, 27 (3), 961.

20. Ridley, H. F.; Spickett, R. G. W.; Timmis, G. M., A new synthesis of benzimidazoles and aza-analogs. Journal of Heterocyclic Chemistry 1965, 2 (4), 453-456.

21. Polgar, L., The catalytic triad of serine peptidases. *Cellular and Molecular Life Sciences*

ACCEPTED MANUSCRIPT

CMLS 2005, 62 (19-20), 2161-2172.

22. Tatsuta, K.; Mikami, N.; Fujimoto, K.; UMEZAW, S.; UMEZAWA, H.; AOYAGI, T., The structure of chymostatin, a chymotrypsin inhibitor. *The Journal of Antibiotics* 1973, 26 (11), 625-646.

23. Choudhary, M. I.; Adhikari, A.; Rasheed, S.; Marasini, B. P.; Hussain, N.; Kaleem, W. A., Cyclopeptide alkaloids of Ziziphus oxyphylla Edgw as novel inhibitors of α-glucosidase enzyme and protein glycation. *Phytochemistry Letters* 2011, 4 (4), 404-406.

24. (a) Lineweaver, H.; Burk, D., The determination of enzyme dissociation constants. *Journal of the American Chemical Society* 1934, 56 (3), 658-666;

(b) Dixon, M., The determination of enzyme inhibitor constants. Biochemical Journal 1953, 55 (1), 170; (c) Irwin, H., Enzyme Kinetics: Behavior and analysis of rapid equilibrium and steady-state enzyme systems. New York: Wiley Classics Library Edition, John Wiley & Sons, Inc: 1993.

25. (a) Tsvetkova, D. D.; Obreshkova, D. P.; Petkova, V. B.; Atanasov, P. Y.; Malik, R.; Siddiq, S.; Hadjieva, B.; Dimitrov, M. V., Investigation of antiproliferative activity of Galantamine peptide GAL-VAL against 3T3 cell lines. *World Journal of Pharmacy and Pharmaceutical Sciences* **2014,** *3* (3), 10-19;

(b) Mosmann, T., Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods* **1983,** *65* (1), 55-63.

26. Moulin, A.; Bell, J. H.; Pratt, R.; Ringe, D., Inhibition of chymotrypsin by a complex of ortho-vanadate and benzohydroxamic acid: Structure of the inert complex and its mechanistic interpretation. *Biochemistry* **2007,** *46* (20), 5982-5990.

27. Morris, G. M.; Huey, R.; Lindstrom, W.; Sanner, M. F.; Belew, R. K.; Goodsell, D. S.; Olson, A. J., AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. *Journal of Computational Chemistry* **2009,** *30* (16), 2785-2791.

28. Frisch, M.; Trucks, G.; Schlegel, H. B.; Scuseria, G.; Robb, M.; Cheeseman, J.; Scalmani, G.; Barone, V.; Mennucci, B.; Petersson, G. e., Gaussian 09. Gaussian, Inc. Wallingford, CT: 2009.

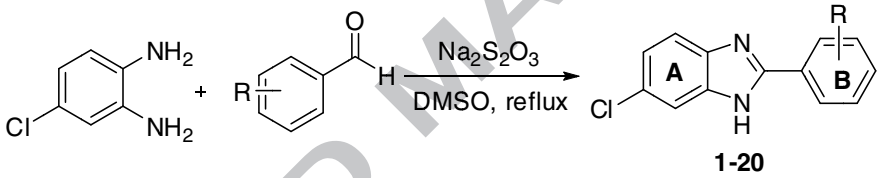
ACCEPTED MANUSCRIPT

Graphical Abstract

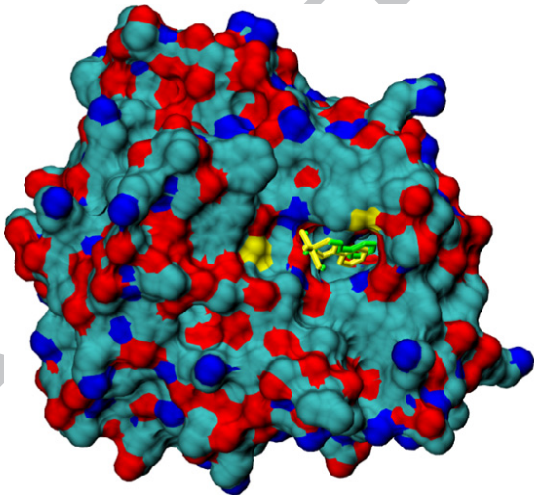
Synthesis and *In vitro* α-Chymotrypsin Inhibitory Activity of 6-Chlorobenzimidazole Derivatives

Hina Siddiqui[a*], Rabia Farooq[a], Bishnu P. Marasini[a], Rizwana Malik[b], Naima Syed[a], Syed Tarique Moin[a], Atta-ur-Rahman[a], and M. Iqbal Choudhary [a,b,c*]

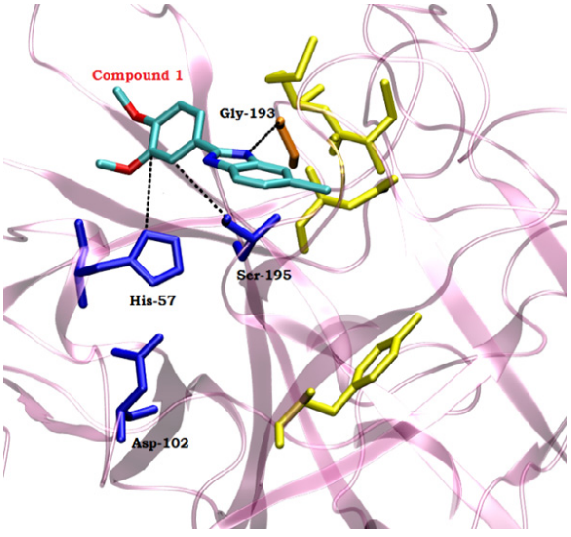
[a]H. E. J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, Karachi-75270, Pakistan
[b]Dr. Panjwani Center for Molecular Medicine and Drug Research, International Center for Chemical and Biological Sciences, University of Karachi, Karachi-75270, Pakistan
[c]Department of Biochemistry, Faculty of Science, King Abdul Aziz University, Jeddah-21452, Saudi Arabia



1-20



Binding of chymostatin (standard), a competitive inhibitor, at the active site residues of chymotrypsin enzyme
IC_{50} =5.7±0.13 µM



*Binding of compound **1**, a competitive inhibitor at the active site residues of chymotrypsin enzyme*
IC_{50} =14.8 ± 0.1 µM
Ki ± 16.4 ± 0.9 µM