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INHIBITORY POTENTIAL OF SOME CHALCONES ON CATHEPSINS B, H AND L

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Abstract

Cathepsins, intracellular proteases, are known to be involved in a number of physiological processes ranging from degradation of extracellular proteins, prohormone processing, progressions of atherosclerosis etc. High levels of cathepsins have also been indicated in various pathological conditions like arthritis, cancer etc. One of the reasons for these elevated levels is attributed to decrease in inhibitor concentration. Therefore, the work on the identification of small molecular weight compounds as inhibitors of cysteine proteases is of great therapeutic significance. In the present work, we report synthesis of a small library of chalcones and their study as inhibitors to cysteine proteases. After a preliminary screening of the compounds as inhibitors to cysteine proteases in general, studies were carried out to evaluate their inhibitory effects on cathepsin B, H and L. The most potent inhibitors among all the compounds were nitro substituted compounds for cathepsin B and cathepsin L and chloro substituted compounds for cathepsin H, respectively.

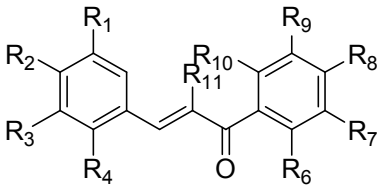
Keywords: Chalcones, Cathepsin B, Cathepsin H, Cathepsin L, inhibitors

Introduction

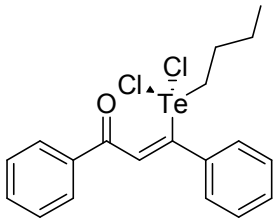
Recent studies have shown that tumour growth, invasion and metastasis are strongly correlated to the behaviour of cellular proteolytic enzymes [1]. Cellular proteases are generally subdivided into four groups: serine, cysteine, aspartic and metalloproteinases. Cathepsins B, H and L (CB,

CH and CL) lysosomal proteolytic enzymes, belonging to the cysteine protease family, widely distributed in almost all mammalian cells are responsible for intracellular protein turnover [2]. It has been demonstrated that cathepsins cannot only degrade components of the extracellular matrix, such as laminin, collagen and elastin structures of basement membranes, but also activate other proteolytic enzyme systems [3]. In vivo, the activity of cysteine proteases is controlled by specific endogenous inhibitors, e.g. sterns and cystatins [4]. The catalytic cathepsin activity is regulated by the balance between the amount of active enzyme and the amount of the inhibitor. Imbalance between proteases and their inhibitors is believed to promote tumour progression [5]. Recent studies have shown that cytosols of tumours tissues contain higher levels of CB, CH, CL than normal adjacent tissue. Higher enzyme activity correlates with tumour progression and shortened patient survival [6, 7]. This has been demonstrated in lung, head and neck, colon and other cancers [7- 9].

Literature survey suggests that a large work has been done on peptidyl or peptidyl analogues as inhibitors to cysteine proteases [10, 11]. However, these inhibitors are not considered to be viable drug candidates for treating diseases like cancer, apoptosis etc. because of gastric instability or the possibility of immunogenic reactions. Therefore, research on non-peptidyl drugs has become an important aspect in drug research and development [12, 13]. Chalcone derivatives (I and II) [14, 15] have been reported as inhibitors to cysteine proteases.



Chalcone derivative (I)



4,4-dichloro-1,3-diphenyl-4-telluraoct-2-en-1-one(II)

Toward this endeavor we have also studied the effect of some semicarbazones, thiosemicarbazones, pyrazoles and pyrazolines as inhibitors of endogenous proteolytic activities [16-21]. Some of the bischalcones and their derivatives [22], Acyl Hydrazides and Triazoles [23], chalcone hydrazones [24] have been reported as effective non-peptidyl inhibitors for cathepsin B & H from our laboratory. Chalcones constitute an important group of natural products and have been reported to exhibit a wide variety of pharmacological effects including antitumor activity [25], antimalarial [26], antiplatelet [27], antiviral [28], immunomodulatory [29], anti-inflammatory [28 and anticancer agents [30]. These molecules are known to inhibit various enzymes found responsible in the diseased conditions.

Previously we have reported that 2'-hydroxychalcones are effective inhibitors of cathepsin B & H [31]. In another study 4'-phenylchalcones are also established as effective inhibitors of cathepsins B, H and L [32]. To explore the work further and to establish a structure- activity relationship, a small library of chalcones with varying substituent having potential role as antitumor and anti-inflammatory agents has ignited a thought to evaluate the designed molecules as inhibitors to cysteine proteases, cathepsin B, H and L which may provide new therapeutic opportunities in cancer treatment.

RESULT AND DISCUSSION

Synthesis of substituted 1, 3-diarylpropenones in quantitative yields by a quick solvent free condensation method motivated us to synthesize a library of chalcones with varying substituent (Scheme 1). During the process we observed that the yield was more and the process was fast, the product was obtained in just 15 to 30 min. The resulting chalcones were identified with the help of spectral studies. In the IR spectra of chalcones 1-110, the identification peak at 1651 - 1645 cm^{-1} represented conjugated >C=O stretching vibrations, another characteristic peak at

1605 - 1585 cm^{-1} was attributed to C=C stretching vibrations. The results show the presence of double bond in conjugation with carbonyl group in the synthesized compounds. ^{1}HNMR ($CDCl_3$) spectrum, the C-2 and C-3 protons are observed as doublets with coupling constant ~ 16 Hz. which shows that stereochemistry across C-2, C-3 double bond is Trans. The other protons were revealed at their respective position as detailed in supplementary material.

Effect of synthesized compounds on *in-vitro* endogenous proteolysis in Liver homogenate

The effect of synthesized chalcones on proteolysis of endogenous protein substrates was observed at pH 5.0. The proteolytic studies on endogenous protein substrates were conducted for 3h and 24h. Table S1(supplementary data) presents the inhibition of endogenous proteolytic study in presence of different compounds at pH 5.0 where most of the proteolytic activity is attributed to cysteine proteases [33]. It can be observed that proteolytic activity is inhibited appreciably in presence of these compounds. In some cases ~100% inhibition is achieved at 1×10^{-4}M concentration. The results show that enzymatic activities were influenced by the substituents on the ring and play a crucial role in inhibiting the proteolytic activities. The enzyme activities vary from compound to compound depending on the substituent present in all the synthesized compounds.

It may be mentioned here that the enzyme preparation contain many proteases. To establish a general line of structure activity relationship is very difficult in this experiment but certainly some trends can be visualized. One observation is that chalcones synthesized by the reaction of various arylmethylketone with nitro substituted benzaldehyde from all the series show maximum inhibition. The behavior might be attributed to its strong electron withdrawing nature. In most of the cases; inhibition was more at 3h and less at 24h. The reason may be that binding of proteases with synthesized compounds might have been reversed with time. In the initial stages, binding is more and results in high inhibition and activity is regenerated with time resulting in lesser or no inhibition after 24h reaction time. It may also be interpreted that with time some enzymes might have been activated which are less

sensitive towards these synthesized compounds.

It can be observed from Table S1(supplementary data) that among various chalcones tested maximum inhibition was observed in the case of 3-(3-nitrophenyl)-1-(furan-2-yl)prop-2-en-1-one **(1i)** followed by 3-(3-nitrophenyl)-1-(4-hydroxyphenyl)prop-2-en-1-one **(5i)**, 3-(4-nitrophenyl)-1-(4-(N'-4-bromophenyl)methylene)aminophenyl)prop-2-en-1-one **(11h)**, 3-(2-nitrophenyl)-1-(4-aminophenyl)prop-2-en-1-one **(2h)**, 3-(3-nitrophenyl)-1-(4-fluorophenyl)prop-2-en-1-one **(6i)**, 3-(3-nitrophenyl)-1-(4-bromophenyl)prop-2-en-1-one **(10i)**, 3-(4-nitrophenyl)-1-(4-(N'methylphenyl)prop-2-en-1-one**(7j)**, 3-(2-nitrophenyl)-1-(pyridin-2-yl)-2-en-1-one **(9h)**, 3-(3-nitrophenyl)-1-(4-nitrophenyl)prop-2-en-1-one **(3i)**, 3-(2-nitrophenyl)-1-(napthalen-3-yl)-2-en-1-one **(8h)**, 3-(4-nitrophenyl)-1-(thien-2-yl)prop-2-en-1-one **(4j)** at 3h reaction. The same trend was observed at 24h reaction but the extent of inhibition was decreased (Figure 2).

The data reported here indicate that these compounds act as inhibitors to proteases active at pH 5.0, and inhibit endogenous protein hydrolysis significantly. In some cases ~100% inhibition is achieved at 1×10^{-4}M concentration. Once the inhibitory effects of compounds, series **1-11,** was established on cysteine proteases in general, their effect was observed on cathepsin B, H and L, keeping in view the broad utility of these class of compounds as anticancer drugs as well as cysteine protease inhibitor potentiality.

Effect of synthesized compounds on the activity of Cathepsin B

The activities of cathepsin B were estimated at varying concentrations of different categories of synthesized compounds. Figure 3i show the relationship between the enzyme activity and concentration of most inhibitory chalcone of each series **(1-11),** respectively. Among the various compounds tested, it has been found that cathepsin B activity is inhibited and is affected by the substituent present in compounds.

It was found that in chalcones from each series (1-11), 4-nitrochalcone phenyl 3-(2-nitrophenyl)-1-

(furan-2-yl)prop-2-en-1-one **1h** showed maximum inhibition. Similarly, nitro substituted chalcone in each series e.g. 3-(3-nitrophenyl)-1-(4-aminophenyl)prop-2-en-1-one **2i,** 3-(4-nitrophenyl)-1-(4-nitrophenyl)prop-2-en-1-one **3j,** 3-(3-nitrophenyl)-1-(thien-2-yl)prop-2-en-1-one **4i,** 3-(3-nitrophenyl)-1-(4-hydroxyphenyl)prop-2-en-1-one **5i,** 3-(3-nitrophenyl)-1-(4-fluorophenyl)prop-2-en-1-one **6i,** 3-(4-nitrophenyl)-1-(4-(methylphenyl)prop-2-en-1-one **7i,** 3-(3-nitrophenyl)-1-(napthalen-3-yl)-2-en-1-one **8h,** 3-(3-nitrophenyl)-1-(pyridin-2-yl)-2-en-1-one **9i,** 3-(2-nitrophenyl)-1-(4-bromophenyl)prop-2-en-1-one **10h** and 3-(4-nitrophenyl)-1-(4-(N'-4-methyl)methylene)aminophenyl)prop-2-en-1-one **11j** have been found to inhibit cathepsin B appreciably..

Effect of synthesized compounds on the activity of Cathepsin H

The activities of cathepsin H were estimated at varying concentrations of different categories of synthesized compounds. Figure 3ii show the relationship between the enzyme activity and concentration of most inhibitory substituted chalcone of each series **(1-11),** respectively. Among the various compounds tested, it has been found that cathepsin H activity is inhibited and is also affected by the substituent present in compounds.

It was found that in chalcones from each series (1-11), 3-(3-chlorophenyl)-1-(furan-2-yl)prop-2-en-1-one **1c** showed maximum inhibition. The compounds 3-(3-chlorophenyl)-1-(4-aminophenyl)prop-2-en-1-one **2c,** 3-(4-chlorophenyl)-1-(4-nitrophenyl)prop-2-en-1-one **3d,** 3-(2-chlorophenyl)-1-(thien-2-yl)prop-2-en-1-one **4b,** 3-(3-chlorophenyl)-1-(4-hydroxyphenyl)prop-2-en-1-one **5c,** 3-(3-chlorophenyl)-1-(4-fluorophenyl)prop-2-en-1-one **6c,** 3-(4-chlorophenyl)-1-(4-(N'methylphenyl)prop-2-en-1-one **7d,** 3-(2-chlorophenyl)-1-(napthalen-3-yl)-2-en-1-one **8b,** 3-(2-chlorophenyl)-1-(pyridin-2-yl)-2-en-1-one **9b,** 3-(2-chlorophenyl)-1-(4-bromophenyl)prop-2-en-1-one **10b,** 3-(4-chlorophenyl)-1-(4-(N'-4-nitrophenyl)methylene)aminophenyl)prop-2-en-1-one **11c** also inhibited cathepsin H to a

greater extent when compared with other substituent in the series.

Effect of synthesized compounds on the activity of Cathepsin L

The activities of cathepsin L were estimated at varying concentrations of different categories of synthesized compounds. Figure 3iii show the relationship between the enzyme activity and concentration of most inhibitory substituted chalcone of each series **(1-11)**, respectively. Among the various compounds tested, it has been found that cathepsin L activity is inhibited and is also affected by the substituent present in compounds.

It was found that in chalcones from each series (1-11), 3-(2-nitrophenyl)-1-(furan-2-yl)prop-2-en-1-one **1h** showed maximum inhibition, followed by 3-(3-nitrophenyl)-1-(4-aminophenyl)prop-2-en-1-one **2i,** 3-(2-nitrophenyl)-1-(4-nitrophenyl)prop-2-en-1-one **3h,** 3-(3-nitrophenyl)-1-(thien-2-yl)prop-2-en-1-one **4i,** 3-(4-nitrophenyl)-1-(4-hydroxyphenyl)prop-2-en-1-one **5j,** 3-(3-nitrophenyl)-1-(4-fluorophenyl)prop-2-en-1-one **6i,** 3-(2-nitrophenyl)-1-(4-(methylphenyl)prop-2-en-1-one **7h,** 3-(4-methoxyphenyl)-1-(napthalen-3-yl)-2-en-1-one **8g,** 3-(3-nitrophenyl)-1-(pyridin-2-yl)-2-en-1-one **9i,** 3-(3-nitrophenyl)-1-(4-bromophenyl)prop-2-en-1-one **10i,** 3-(4-nitrophenyl)-1-(4-(N'-4-methylphenyl)methylene)aminophenyl)prop-2-en-1-one **11j** in that order.

Enzyme kinetic studies

After establishing the inhibitory action of synthesized compounds on cathepsin B, cathepsin H and cathepsin L, experiments were designed to evaluate the type of inhibition and to determine the K_i value of these compounds on cathepsins B, H and L. For that, enzyme activities were evaluated at different substrate concentrations in presence and absence of a fixed concentration of different compounds. The enzyme concentration was kept constant in all the experiments. Line-weaver Burk plots drawn between 1/S and 1/V in presence and absence of most inhibitory compound for cathepsin B, H and L are shown in figure 3iv, 3v and 3vi, respectively. It was

found that the plots of 1/V and 1/S were straight lines intersecting at the Y-axis and showed that value of V_{max} remained constant in all the compounds whereas the value of K_m changes in presence of each compound. These studies suggested a competitive type of inhibition exhibited by these compounds for cathepsin B, cathepsin H and Cathepsin L. Using the Line-weaver Burk equation for competitive inhibition the K_i values were calculated and the K_i values for most inhibitory compound of each series has been presented in table 1. The Ki value of most inhibiting compound for cathepsin B in the each series has been evaluated as ~6.40x10^{-6}M, ~6.20x10^{-6}M, ~5.98x10^{-6}M, ~6.57x10^{-6}M, ~5.84x10^{-6}M, ~5.94x10^{-6}M, ~6.05x10^{-6}M, ~6.12x10^{-6}M, ~5.91x10^{-6}M, ~5.871x10^{-6}M and ~5.81x10^{-6}M for compounds 1h, 2i, 3j, 4i, 5i, 6i, 7i, 8h, 9i, 10h and 11j, respectively; Similarly for cathepsin H, the K_i value of most inhibiting compound in the each series has been evaluated as ~297.8x10^{-6}M, ~128.0x10^{-6}M, ~405.4x10^{-6}M, ~216.94x10^{-6}M, ~163.3x10^{-6}M, ~31.69x10^{-6}M, ~41.6x10^{-6}M, ~98.9x10^{-6}M, ~47.24x10^{-6}M, ~21.42x10^{-6}M and ~13.3x10^{-6}M for compounds 1c, 2c, 3d, 4b, 5c, 6c, 7d, 8b, 9b, 10b and 11c, respectively. For cathepsin L, the K_i value of most inhibiting compound in the each series has been evaluated as ~34.9x10^{-9}M, ~19.4x10^{-9}M, ~7.79x10^{-9}M, ~72.9x10^{-9}M, ~5.32x10^{-9}M, ~6.41x10^{-9}M, ~9.21x10^{-9}M, ~10.66 x10^{-9}M, ~14.48x10^{-9}M, ~5.73x10^{-9}M and ~4.4x10^{-9}M for compounds 1h, 2i, 3h, 4i, 5j, 6i, 7h, 8g, 9i, 10i and 11j, respectively.

Table-1: K_i values exerted by most inhibitor compounds of each series on Cathepsin B, Cathepsin H and Cathepsin L

S.No.	Cathepsin B		Cathepsin H		Cathepsin L	
	Code of compound	K_i (10^{-6}M)	Code of compound	K_i (10^{-6}M)	Code of compound	K_i (10^{-9}M)
1.	1h	6.40	1c	297.8	1h	34.9
2.	2i	6.20	2c	128.0	2i	19.4
3.	3j	5.98	3d	405.4	3h	7.79
4.	4i	6.57	4b	216.94	4i	72.9
5.	5i	5.84	5c	163.3	5j	5.32
6.	6i	5.94	6c	31.69	6i	6.41
7.	7i	6.05	7d	41.6	7h	9.21
8.	8h	6.12	8b	98.9	8g	10.66
9.	9i	5.91	9b	47.24	9i	14.48
10.	10h	5.871	10b	21.42	10i	5.73
11.	11j	5.81	11c	13.3	11j	4.4

Lineweaver – Burk plots were plotted between 1/V and 1/S to find the type of inhibition. It was found that all the compounds showed competitive reversible inhibition on cathepsin B having specific activity 11.15 nmoles/min/mg, cathepsin H having specific activity 22.91nmoles/min/mg and cathepsin L having specific activity16.78 nmoles/min/mg (Fig 3iv-vi). The K_i values were calculated using Lineweaver – Burk equations for competitive reversible inhibitions.

Table-2: Docking studies showing decrease in different energies of cathepsin B in presence of different chalcones (most inhibitor chalcones from different series)

Compound	Total Energy	VDW	H Bond	Electronic
BANA	-129.75	-92.20	-34.78	-2.77
Leupeptin	-106.89	-84.65	-22.23	0
1h	-91.63	-53.33	-38.54	0.23
2i	-92.81	-71.54	-21.5	0.23
3j	-84.86	-66.05	-19.14	0.32

4i	**-87.33**	**-67.22**	**-21.23**	**1.12**
5i	**-96.49**	**-74.39**	**-22.33**	**0.23**
6i	**-86.57**	**-67.67**	**-19.55**	**0.65**
7i	**-83.26**	**-71.71**	**-11.79**	**0.24**
8h	**-93.58**	**-71.56**	**-22.85**	**0.82**
9i	**-88.07**	**-65.65**	**-23.05**	**0.63**
10h	**-97.79**	**-66.89**	**-31.13**	**0.23**
11j	**-100.66**	**-88.54**	**-12.12**	**0**

The results are one of the docking experiments run using iGemdock under drug screening settings. The ligands were loaded as MDL mol file. The active site was extracted from the structure of cathepsin B retrieved from Protein Data Bank (http://www.rcsb.org/) as cav2IPP B_PYS.pdb[34].

Table-3: Docking studies showing decrease in different energies of cathepsin H in presence of different chalcones (most inhibitor chalcones from different series)

Compound	Total Energy	VDW	H Bond	Electronic
Leu- βNA	**-75.71**	**-72.21**	**-3.5**	**0**
LeuCH_2Cl	**-61.28**	**-44.86**	**-16.41**	**0**
1c	**-67.49**	**-61.92**	**-5.57**	**0**
2c	**-72.52**	**-62.05**	**-10.47**	**0**
3d	**-86.01**	**-63.85**	**-22.16**	**0**
4b	**-64.34**	**-64.34**	**0**	**0**
5c	**-70.42**	**-60.33**	**-10.08**	**0**

6c	**-69.77**	**-62.89**	**-6.87**	**0**
7d	**-68.67**	**-68.67**	**0**	**0**
8b	**-71.76**	**-65.76**	**-5.99**	**0**
9b	**-66.84**	**-56.34**	**-10.5**	**0**
10b	**-67.72**	**-58.88**	**-8.83**	**0**
11c	**-98.57**	**-89.07**	**-9.5**	**0**

The results are one of the docking experiments run using iGemdock under drug screening settings. The ligands were loaded as MDL mol file. The active site was extracted from the structure of cathepsin H retrieved from Protein Data Bank (http://www.rcsb.org/) as cav8PCH H_NAG.pdb[35].

Table-4: Docking studies showing decrease in different energies of cathepsin L in presence of different chalcones (most inhibitor chalscones from different series)

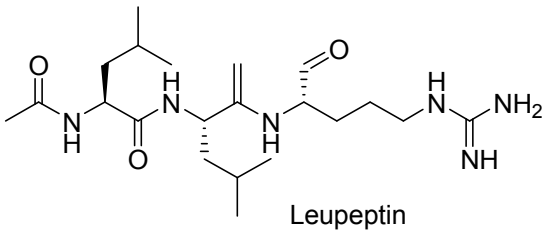
Compound	Total Energy	VDW	H Bond	Electronic
Leupeptin	**120.59**	**87.627**	**-31.93**	**-1.724**
Z-Phe-Arg-4mβNA	**-136.04**	**-107.26**	**-31.06**	**2.290**
1h	**-92.022**	**-68.150**	**-23.87**	**00**
2i	**-91.008**	**-75.3242**	**-16.5**	**0.816**
3h	**-87.8985**	**-81.7823**	**-6.11**	**0**
4i	**-88.216**	**-71.0995**	**-18.34**	**1.225**
5j	**-87.806**	**-82.1074**	**-5.69**	**0**
6i	**-93.6011**	**-74.4905**	**-19.91**	**0.799**
7h	**-93.4324**	**-71.183**	**-23.27**	**1.029**

8g	**-88.1839**	**-81.2786**	**-6.90**	**0**
9i	**-94.4156**	**-79.1661**	**-15.25**	**0**
10i	**-90.341**	**-70.9597**	**-20**	**0.618**
11j	**-102.184**	**-95.229**	**-6.95**	**0**

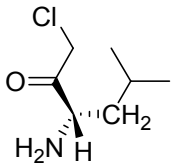
The results are one of the docking experiments run using iGemdock under drug screening settings. The ligands were loaded as MDL mol file. The active site was extracted from the structure of cathepsin L retrieved from Protein Data Bank (http://www.rcsb.org/) as cav3BC3L_CSW[36].

Structure Activity Relationship Studies

In vitro inhibition studies of differently functionalized chalcones on cathepsins B, H and L clearly demonstrated that the designed compounds inhibit these enzymes effectively. We have demonstrated here that in general, these compounds exhibited an inhibition order cathepsin L> > cathepsin B > cathepsin H. Establishing these compounds as potential inhibitors to cathepsins B, H and L with target-directed chemical specificity suggests an optimistic future for their use as cysteine protease inhibitors. These chalcones also provide a backbone skeleton for a number of compounds to be used as therapeutic agents in a number of disease processes and are important intermediates for various biologically active compounds. In order to ascertain inhibition ability of the studied compounds, results were compared with Leupeptin, a known inhibitor of cathepsins B and L and Leu-CH_2-Cl for cathepsin H. It is reported that, Leupeptin is a potential peptide inhibitor of cathepsin B [37], inhibited the goat brain cathepsin B [38] competitively with K_i value of $12{\cdot}5 \times 10^{-9}$M whereas K_i value for human liver cathepsin B [39] was reported to be $7{\cdot}0 \times 10^{-9}$ M. In contrast, K_i value for human liver cathepsin H [39] was reported to be 9.2×10^{-6} M. It has been reported that cathepsin L is inhibited by leupeptin with a K_i value of 1.45×10^{-9} M [40]



Leupeptin



Leu-CH_2-Cl

It can be observed that leupeptin showed ~ 98.5% inhibition at 10^{-6} M concentration for cathepsin B. It showed ~ 51.89% inhibition at 10^{-5} M concentration for cathepsin H and ~ 9.02% inhibition is observed for cathepsin L at 10^{-9} M concentration. Similarly, Leu-CH_2-Cl

showed ~ 10% inhibition at 10^{-5} M concentration for cathepsin B and 93.99% inhibition for cathepsin L at 10^{-5} M concentration whereas it showed ~ 91.89% inhibition at 10^{-5}M concentration for cathepsin H. The results obtained are comparable with earlier results reported for brain cathepsin H, cathepsin B and cathepsin L [38, 41,42].

The inhibitory potential of chalcones can be attributed to the presence of α-ß unsaturated carbonyl moiety in the molecule. Chalcones have previously been reported as inhibitors of cathepsin B [43, 44]. The $–CH_2SH$ moiety present at the active site of enzymes can interact with the electron deficient centre present in the chalcones inhibiting the enzymes activity. In the present study, substitution pattern in each series was carefully altered to acquire a comprehensible correlation between the electronic environment of ligands and the enzyme active site or binding site. Among the similarly substituted designed compounds nitro substituent has been evaluated as most inhibitory to enzymes cathepsin B and L. In a similar study on bischalcones and quinazoline-2(1H)-one and quinazoline-2(1H)-thione derivatives [22], we have evaluated that nitro substituted quinazoline-2(1H)-one and quinazoline-2(1H)-thione derivatives are potential inhibitors to cathepsins B and H. In another study, carried out in order to identify non peptidyl inhibitors to cathepsins B and H, we have reported that 3-phenyl-5-(4-nitrophenyl)-4-amino-1,2,4-triazole and 3-phenyl-5-(3'-nirophenyl)-4-amino-1,2,4-triazole [23] inhibited cathepsins B and H effectively. In the present study, it can be observed that nitro-substitution provided an effective interaction for cathepsins B and L. All the synthesized compounds were evaluated as better inhibitors to cathepsins B and L as compared to cathepsin H. It may be an indication that active sites of cathepsins B and L are more susceptible to these compounds as compared to cathepsin H. the results when compare with previously reported inhibitory potential of 2'-hydroxychalcones and their cyclized derivatives also suggest that –

NO_2 group plays an important role in effective binding of ligand with the enzyme active site. The study reports that cathepsin B is strongly inhibited by nitro substituted chalcone with the K_i value of $6.18x10^{-8}$ M [31]. In addition, it is further observed that 2'-hydroxy group plays an important role in the binding of chalcone with enzyme, as suggested by the K_i values reported for 2'-hydroxychalcones [31]. In case of Cathepsin H, we evaluated that halogen substituted chalcones were most inhibitory. The results are similar to those reported previously [23] where halogen substituted pyrazolines inhibited Cathepsin H appreciably. In the present study, we have discussed that backbone structure as well as substituent effects the enzyme activities. And found that in chalcones, Ar-CO-CH=CH-Ar', substituents in the Ar' affected the enzyme inhibition to a significant extent. However, distinctive difference in inhibition pattern is not observed when we changed the Ar group. The results are further discussed with this background. The results were found consistent when compared with *in- silico* docking studies.

3.4. Molecular docking experiment

On the basis of the interaction data of docking experiments that include total energy and individual energy terms, an indicative of the fitness of a predicted pose in the binding site, it is suggested that the level of interaction is highest for 3-(4-nitrophenyl)-1-(4-(N'-4-methyl)methylene)aminophenyl)prop-2-en-1-one **11j** with in the active site of cathepsin B (table 2) Fig 4 (i-xi) shows the result of the best docking pose of most inhibitory compounds of each series 1h, 2i, 3j, 4i, 5i, 6i, 7i, 8h, 9i, 10h and 11j, respectively into the active site of Cathepsin B. In cathepsin B, all the compounds showed a lesser interaction than the peptidyl inhibitor, leupeptin. Decrease in total energy for leupeptin-cathepsin B has come out be -106.89 whereas with substrate BANA it has been computed as, -129.76 of which the contribution of van der Waal interactions are more with a score of -84.66 as compared to H-bonds with a score

of -22.23. This is due to peptide-protein interaction. Leupeptin is peptidyl in nature and therefore binds effectively with the enzyme active site resulting in higher binding energy. As compared to this the binding energy of title compounds are less (table 2). The *in-silico* predictable behavior of enzyme-ligand interaction can give an idea about the interaction. Docking methods have been used to provide valuable insight into the binding mode between the ligand and the enzyme active site thereby have an important role in the understanding of ligand-enzyme interactions. When compared with in the designed series to evaluate the interaction with cathepsin B, the results clearly support the *in vitro* inhibition studies. *In-vitro* inhibition studies (table S1) (supplementary data) also show that 3-(4-nitrophenyl)-1-(4-(N'-4-methyl)methylene)aminophenyl) prop-2-en-1-one **11j** is the most potent inhibitor of cathepsin B. It can be observed that Gln-23, Glu-28, Cys-29, Trp-30, Glu-74 and Gly-198 have been found to interact with the substrate through H-bonds. These amino acids interact with the compounds under consideration; thereby suggest a competitive type of inhibition (Fig 3iv). The proposed mechanism is shown in scheme 2. The results clearly indicate the significance of the in-vitro inhibition studies.

In cathepsin H, the decrease in total energy with the reference inhibitor Leu-CH_2Cl was less as compared to all the designed compounds (Table 3). Here, it can be seen that though Leu-CH_2Cl is specific inhibitor for cathepsin H [42, 45], but possess only one amino acid residue as compared to leupeptin–cathepsin B. therefore, the leu-CH_2Cl-cathepsin H interaction cause a decrease in energy of –61.282 of which -44.868 is the interaction and -16.414 is due to hydrogen bonds. As listed in table 1, all the designed compounds have been found to show higher decrease in ligand cathepsin H interaction energy than leu-CH_2Cl- cathepsin H. In case of cathepsin H, the level of interaction is highest for 3-(4-chlorophenyl)-1-(4-(N'-4-nitrophenyl)

methylene) aminophenyl) prop-2-en-1-one **11c** with the active site of cathepsin H (table 3)**.** Figure 5 (i-xi) show the docked poses of the most inhibitory compounds of each series 1c, 2c, 3d, 4b, 5c, 6c, 7d, 8b, 9b, 10b and 11c into the binding site of Cathepsin H, respectively. As we can see that the designed compound and the substrate leu-βNA binds at same site i.e. Ser-69, Gln-78 and Asn-112 and this supports the results obtained during the in-vitro studies; the compounds are evaluated as competitive inhibitors (Fig. 3v).

In case of cathepsin L, on the basis of the interaction data of docking experiments that include total energy and individual energy terms, an indicative of the fitness of a predicted pose in the binding site, it is suggested that the level of interaction is highest for 3-(4-nitrophenyl)-1-(4-(N'-4-methyl)methylene)aminophenyl)prop-2-en-1-one **11j** with the active site of cathepsin L (table 4). Fig 6 (i-xi) shows the result of the best docking pose of most inhibitory compounds of each series i.e., 1h, 2i, 3h, 4i, 5j, 6i, 7h, 8g, 9i, 10i and 11j, respectively into the active site of Cathepsin L. In cathepsin L, all the compounds showed a lesser interaction than the peptidyl inhibitor, leupeptin. Decrease in total energy for leupeptin-cathepsin L has come out be -120.59 which is equivalent to substrate Z-Phe-Arg-4mβNA, -136.05 of which the contribution of van der Waal interactions are more with a score of -87.67 as compared to H-bonds with a score of -31.19. This difference can be explained on the basis of peptidyl nature of leupeptin as has been explained for cathepsin L. As compared to this the binding energy of title compounds are less (table 4). The amino acids Gln-19, Trp -24 and Gly-164 have been found to interact with the compounds as well as with substrate Z-Phe-Arg-4mβNA thus indicating a competitive type of inhibition (Fig. 3vi) and the results clearly support the *in vitro* inhibition studies.. 3-(4-nitrophenyl)-1-(4-(N'-4-methyl)methylene)aminophenyl) prop-2-en-1-one **11j** show maximum inhibition (table 1) and therefore can account for maximum inhibition.

In the present work decrease in total energy of enzyme –legand complex is presented as binding affinity of ligand with in the active site of enzyme. However, Zhang et. al. have used free energy based approach in understanding such interactions [46].

Experimental Section

All the chemicals (analytical grade) and biochemicals Fast Garnet GBC (*o*-aminoazotoluene diazonium salt, substrate a-N-benzoyl-D, L-arginine-2-naphthylamide (BANA), Leu-ßNA and Z-Phe-Arg-4mβNA were purchased either from Sigma Chemical Co., USA or from Bachem Feinchemikalien AG, Switzerland. All the solutions used were prepared fresh in glass-distilled conductivity water. The protein sample was concentrated using Amicon stirred cells with YM 10 membrane under nitrogen pressure of 4–5 psi. The source of enzyme, fresh goat liver, was obtained from local slaughter house.

General Procedure

Melting points were determined in open capillary tubes and are thus uncorrected. All the chemicals and solvents used were of laboratory grade. IR spectra (KBr, cm^{-1}) were recorded on a PerkinElmer spectrometer. ^{1}H NMR spectra was recorded on Bruker 300 MHz NMR spectrometer (chemical shifts in d ppm) using TMS as an internal standard. Thin layer chromatography on aluminium plates percoated with silica gel G (Merck) in various solvent systems using iodine vapors as detecting agent or by irradiation with ultraviolet lights (254 nm) were used to monitor progress of reaction. ELISA plate reader was used for measuring absorbance in the visible range. Refrigerated ultracentrifuge Remi C-24BL was used for centrifugation purpose.

General method for the synthesis of 1, 3-diarylprop-2-en-1-ones

1, 3-diarylprop-2-en-1-ones were prepared under solvent free condensation of arylmethylketone and arylaldehydes by grinding them with a mortar and pestle in the presence of solid sodium

hydroxide. For this, equimolar ratio of arylmethylketone and potassium hydroxide pellets were grinded in mortar with the help of pestle after making a paste, added equimolar ratio of aryl aldehydes. The reaction was worked up in ice cold water. It was then filtered, washed with ice cold water, dried and recrystallised from ethanol. The purity of compounds was checked with the help of melting points, IR, ^{1}H NMR and ^{13}C NMR (supplementary material).

PROTEOLYTIC STUDIES

Preparation of liver homogenate: Goat liver, purchased fresh from the local slaughter house was washed with cold isotonic saline solution. The tissue was then homogenized in 0.1M acetate buffer pH 5.5 containing 0.2M NaCl in a mixer-cum-blender to obtain 10% (w/v) homogenate and was stored at 4^{o}C.

Assay for proteolytic activity: The proteolytic activity was estimated at pH 5.0, 37^{o}C using 0.1 M acetate buffer as the incubation medium. The homogenate prepared above was incubated with the buffer at 37^{o}C for 3 h and 24 h., separately. The reaction was stopped by the addition of TCA and the resulting solution was centrifuged to precipitate proteins. The acid soluble proteins were quantitated in the supernatant using Bradford method [47]. The experiments were conducted in triplicate and the results are presented in table S1(supplementary data) (figure 1 and figure 2).

Purification of goat brain cathepsin B, cathepsin H and cathepsin L

All the purification steps were carried out at 4°C. Cathepsin B, H and L were isolated, separated and purified from goat liver using the following procedure [48]. Goat liver acetone powder homogenisation in cold 0.1 M sodium acetate buffer pH 5.5 containing 0.2 M NaCl and 1mM EDTA, Acid-autolysis by lowering pH from 5·5 to 4·0 by gradual addition of cold 1 N HCl, 30-80% ammonium sulphate fractionation. Fractionation of proteases based on molecular weight

on Sephadex G-100 column chromatography and finally cation-exchange chromatography on CM-Sephadex C-50 and DEAE Sephadex A-50 column. The specific activities of the cathepsin B, cathepsin H and cathepsin L were ~11.15 nmoles/min/mg, ~22.91 nmoles/min/mg and ~16.78 nmoles/min/mg, respectively.

Effect of compounds on the activity of Cathepsin B

Cathepsin B was determined spectrophotometrically using BANA as a substrate at pH 6.0. The activities of cathepsin B was estimated at varying concentrations of synthesized compounds (table S1 (supplementary data) shows activities of cathepsin B at minimum inhibitory concentration of synthesized compounds). First of all, enzyme was equilibrated in phosphate buffer of pH 6.0 at 37^{o}C. The stock solutions of compounds were prepared in DMSO. Then appropriate amount of individual compounds were added in the reaction mixture separately to effect the final concentration of each compound as 0.1, 0.5 and 1.0 X 10^{-4} M final concentration. After an incubation time of 30 min. residual enzyme activity was estimated by the usual enzyme assay [22] at pH 6.0 using α-N-benzoyl-D, L-arginine-2-naphthylamide (BANA) as substrate. The experiments were performed in triplicate for each concentration and averaged before further calculations. The % activity in each case has been calculated with respect to the control where no compound was added but an equivalent amount of solvent was present. The activities of cathepsin B estimated at varying concentrations of most inhibitory compounds of each series have been shown in figure 3i.

Effect of compounds on the activity of Cathepsin H

Cathepsin H was determined spectrophotometrically using Leu-βNA as a substrate having specific activity 22.91 nmoles/min/mg. The activities of cathepsin H was estimated at varying concentrations of synthesized compounds (table S1 (supplementary data) shows activities of

cathepsin H at maximum inhibitory concentration of synthesized compounds). First of all, enzyme was equilibrated in phosphate buffer of pH 7.0 at 37^{o}C. The stock solutions of compounds were prepared in DMSO. Then appropriate amount of individual compounds were added in the reaction mixture separately to effect the final concentration of each compound as 0.1, 0.5 and 1.0 X 10^{-3} M final concentration. After an incubation time of 30 min. residual enzyme activity was estimated by the usual enzyme assay [22] at pH 7.0 using Leu-βNA as substrate. The experiments were performed in triplicate for each concentration and averaged before further calculations. The % activity in each case has been calculated with respect to the control where no compound was added but an equivalent amount of solvent was present. The activities of cathepsin H estimated at varying concentrations of most inhibitory compounds of all series have been shown in figure 3ii.

Effect of compounds on the activity of Cathepsin L

Cathepsin L was determined spectrophotometrically using Z-Phe-Arg-4-mβNA as a substrate having specific activity 16.78 nmoles/min/mg. The activities of cathepsin H was estimated at varying concentrations of synthesized compounds (table S1 (supplementary data) shows activities of cathepsin L at maximum inhibitory concentration of synthesized compounds). First of all, enzyme was equilibrated in phosphate buffer of pH 6.0 at 37^{o}C. The stock solutions of compounds were prepared in DMSO. Then appropriate amount of individual compounds were added in the reaction mixture separately to effect the final concentration of each compound as 0.1, 0.5 and 1.0 X 10^{-6} M final concentration. After an incubation time of 30 min. residual enzyme activity was estimated by the usual enzyme assay [38] at pH 6.0 using Z-Phe-Arg-4mβNA as substrate. The experiments were performed in triplicate for each concentration and averaged before further calculations. The % activity in each case has been calculated with

respect to the control where no compound was added but an equivalent amount of solvent was present. The activities of cathepsin L estimated at varying concentrations of most inhibitory compounds of all series have been shown in figure 3iii.

Kinetic studies of synthesized compounds on cathepsin B, cathepsin H and cathepsin L

After establishing the inhibitory action of synthesized compounds on cathepsin B, cathepsin H and cathepsin L; experiments were designed to evaluate the type of inhibition and to determine the K_i value of these compounds on respective enzymes. For that, enzyme activities were evaluated at different substrate concentrations in presence and absence of a fixed concentration of inhibitor. The enzyme concentration was kept constant in all the experiments. Line-weaver Burk plot were drawn between 1/S and 1/V in presence and absence of compounds on cathepsin B, cathepsin H and cathepsin L (Figure 3 iv, v, vi). The K_i values were calculated using the Line-weaver Burk equation for competitive inhibition $K_m' = K_m(1+I/ K_i)$.

DRUG MODELING STUDIES

All docking studies were performed using iGemdock. For these studies, small molecular weight ligands and enzyme active site structure is required. The structure of cathepsin B [34], cathepsin H and cathepsin L were retrieved from Protein Data Bank as (cav2IPP B_PYS.pdb), and (cav8PCH H_NAG.pdb) [35] and cav3BC3L_CSW [36], respectively. The structures were prepared in Marvin sketch, minimized and were saved as MDL Mol File. The prepared ligands and the binding site were loaded in the iGemdock software and docking was started by setting the GA- parameters at drug screening setting. The results are presented in table 2, 3 and 4 pertain to the interaction data. Fitness is the total energy of a predicted pose in the binding site. The empirical scoring function of iGemdock is the sum total of Van der Waal, H-bonding and electrostatic energy. The docked poses of the ligands in the active site of cathepsin B, H and L

along with their respective substrates and most inhibitory compounds of each series are shown in figure 4,5 and 6.

CONCLUSION

In summary, we have demonstrated that these compounds showed more inhibition on cathepsin L activity in comparison to cathepsin B and H. Analysis of the effect of these compounds on Cathepsin B, H and L by the combination of serendipitous biologic selectivity with target-directed chemical specificity suggests an optimistic future for their use as cysteine protease inhibitors as therapy in a number of disease processes.

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Figure Captions

Figure 1: Effect of chalcones on the endogenous proteolytic activity for 3 hr reaction. The data in each bar represents the % Residual Activity in presence of individual compound w.r.t. control taken as 100.

Figure-2: Effect of compounds on the endogenous proteolytic activity 24 hr reaction. The data in each bar represents the % Residual Activity in presence of individual compound w.r.t. control taken as 100.

Figure 3: Effect of varying concentration of most inhibitory chalcones from each series, 1h, 2i, 3j, 4i, 5i, 6i, 7i, 8h, 9i, 10h and 11j on cathepsin B figure 3(i) , 1c, 2c, 3d, 4b, 5c, 6c, 7d, 8b, 9b, 10b and 11c, on cathepsin H figure 3(ii) and 1h, 2i, 3h, 4i, 5j, 6i, 7h, 8g, 9i, 10i, 11j, on cathepsin L activity 3(iii), respectively. Results are mean of experiments conducted in triplicate. % Residual activities are presented w.r.t control which contain equivalent amount of solvent.

Line - weaver Burk plot for cathepsin B at varying concentrations of BANA in presence of $1x10^{-5}$M concentration of most inhibitory chalcone of each series (3iv), at pH 6.0. The K_m value for control have been found to be $3.64x10^{-4}$ M. Line - weaver burk plot for cathepsin H on varying concentrations of leu-ßNA in presence of $1x10^{-4}$M concentration of most inhibitory chalcone of each series (3v), at pH 7.0. The Km value for control has been

found to be $5.34x10^{-4}$M. Line-weaver Burk plot for cathepsin L at varying concentrations of Z-Phe-Arg-4mβNA in presence of $1x10^{-7}$M concentration of most inhibitory chalcone of each series (3vi), at pH 6.0. The K_m value for control has been found to be $6.024x10^{-5}$ M. The K_i values as calculated from this graph are presented in table 1.

Figure 4: Docking results showing the alignment of most inhibitory compounds along with the BANA in the active site of cathepsin B (cav2IPP B_PYS.pdb). Here fig. 4(i-xi) show alignment of 1h, 2i, 3j, 4i, 5i, 6i, 7i, 8h, 9i, 10h and 11j along with BANA in the active site of cathepsin B (cav2IPP B_PYS.pdb), respectively. Active site amino acid Cys-29 interacts with all the compounds as well as with BANA through H-bonds explaining the competitive type of inhibition (Fig 3iv).

Figure 5: Docking results showing the alignment of most inhibitory compounds along with the LeußNA in the active site of cathepsin H (cav8PCH H_NAG.pdb) Here fig. 5(i-xi) show alignment of 1c, 2c, 3d, 4b, 5c, 6c, 7d, 8b, 9b, 10b and 11c, along with LeußNA in the active site of cathepsin H (cav8PCH H_NAG.pdb), respectively. Ser-69, Gln-78 and Asn-112 interact with the designed compound and the substrate leu-βNA as well through H-bonds suggesting a competitive type of inhibition (Fig 3v).

Figure 6: Docking results showing the alignment of most inhibitory compounds along with the z-Phe-Arg-4mβNA in the active site of cathepsin L (cav3BC3L_CSW.pdb). Here fig. 6(i-xi) show alignment of 1h, 2i, 3h, 4i, 5j, 6i, 7h, 8g, 9i, 10i, 11j, along with Z-Phe-Arg-4mβNA in the active site of cathepsin L (cav3BC3L_CSW.pdb), respectively. The amino acids Gln-19, Trp -24 and Gly-164 have been found to interact with the compounds as well as with substrate Z-Phe-Arg-4mβNA through H-bonds thus indicating a competitive type of inhibition (Fig 3vi).

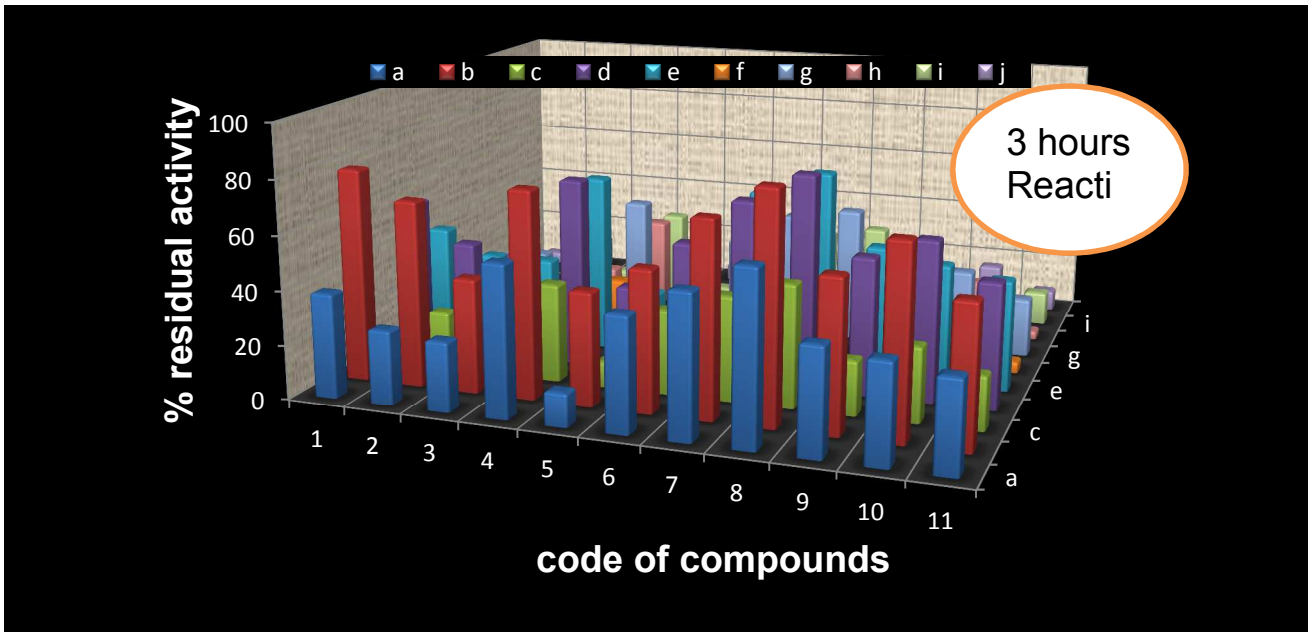


Figure 1

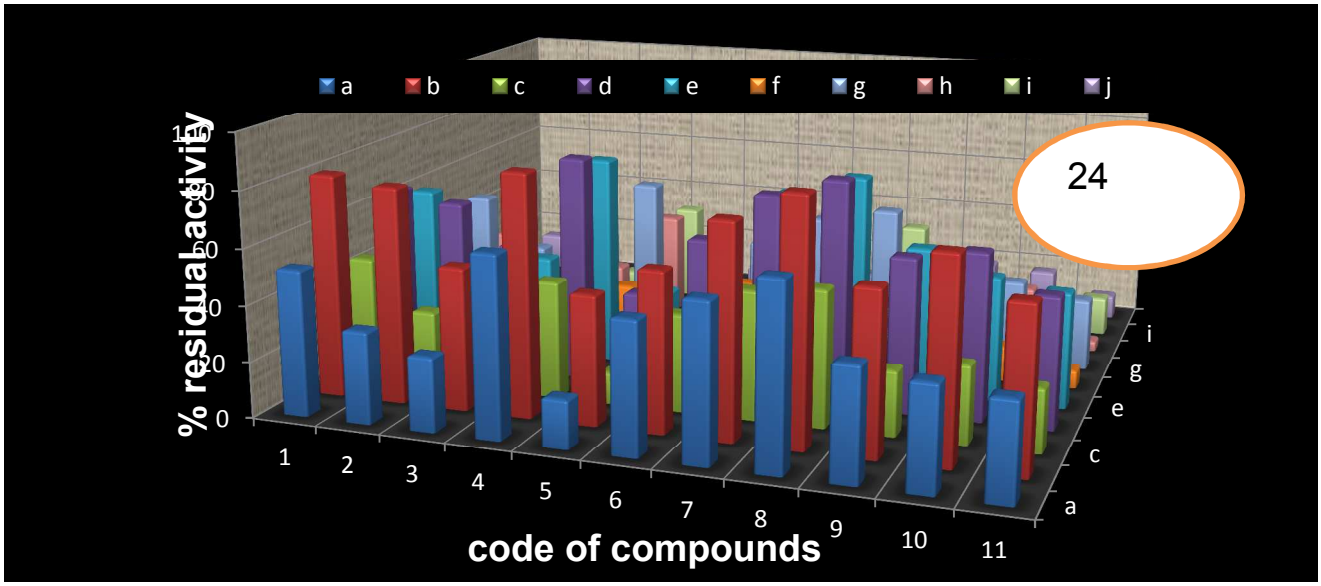


Figure-2

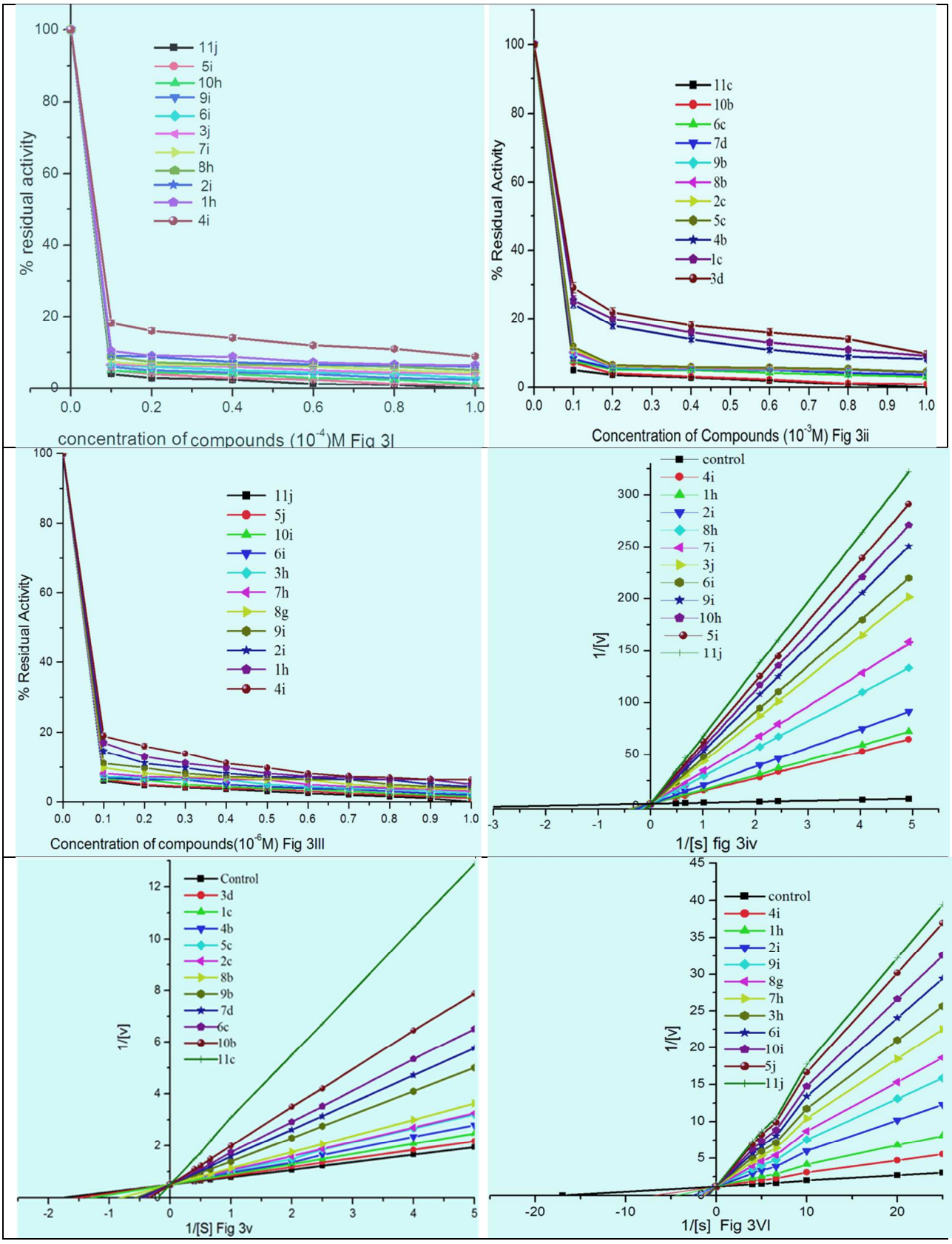
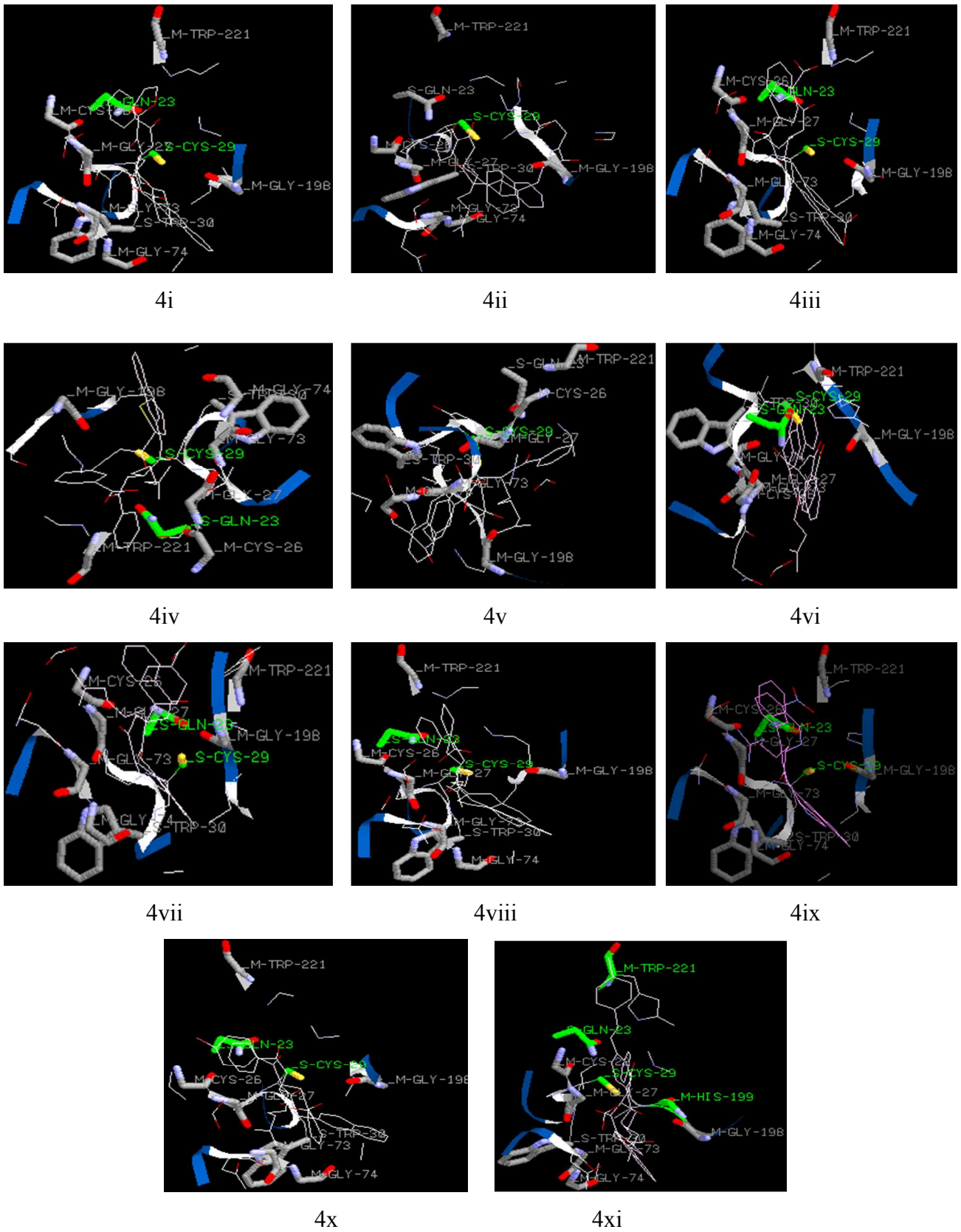


Figure 3



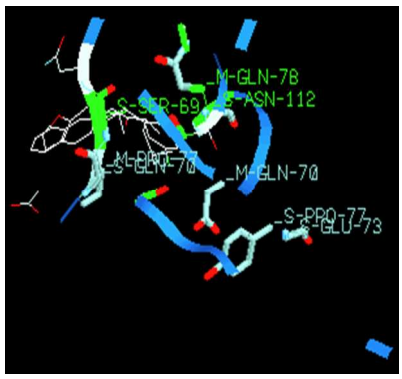
4i 4ii 4iii

4iv 4v 4vi

4vii 4viii 4ix

4x 4xi

Figure 4



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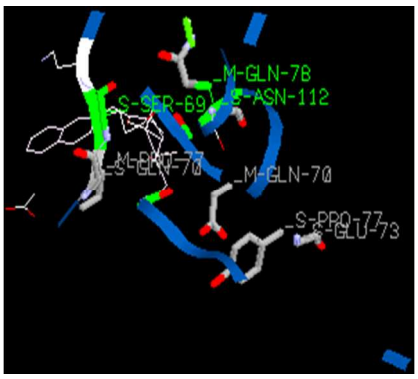

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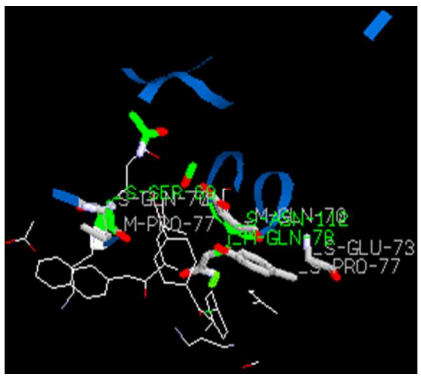

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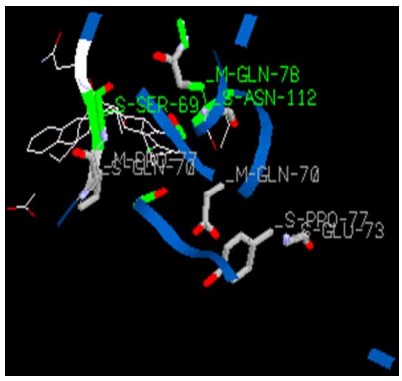

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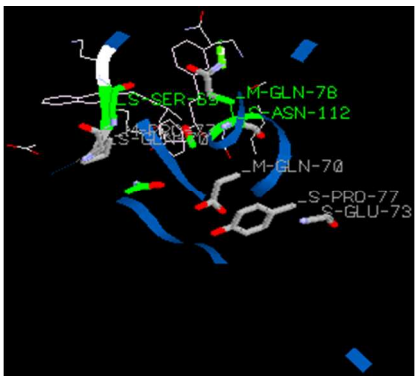

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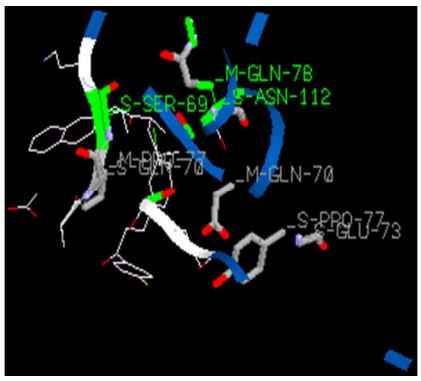

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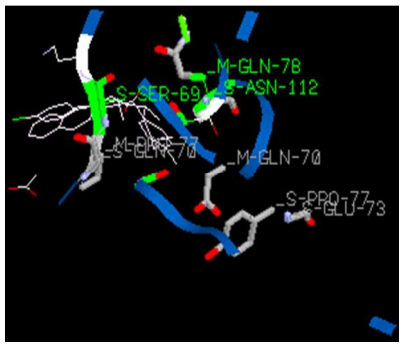

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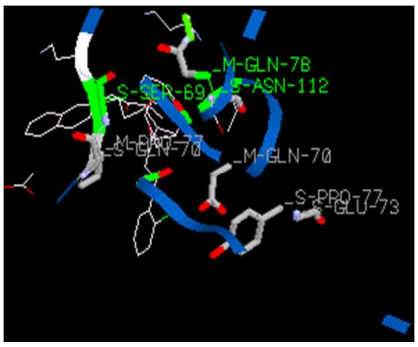

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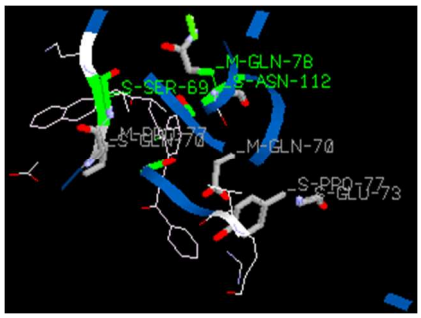

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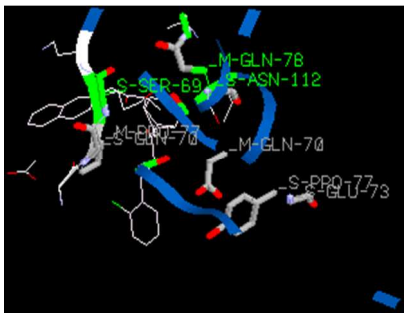

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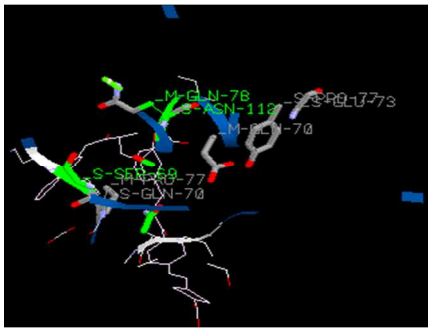

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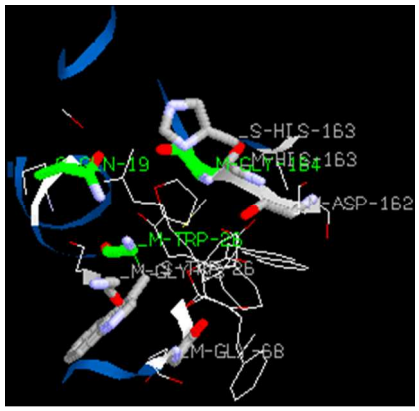

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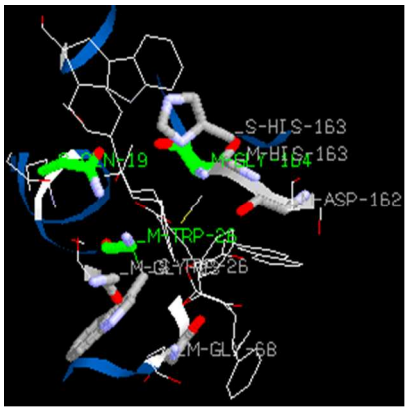

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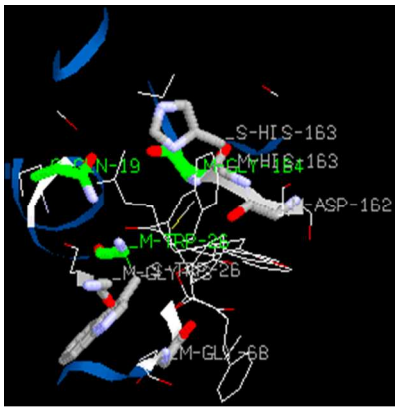

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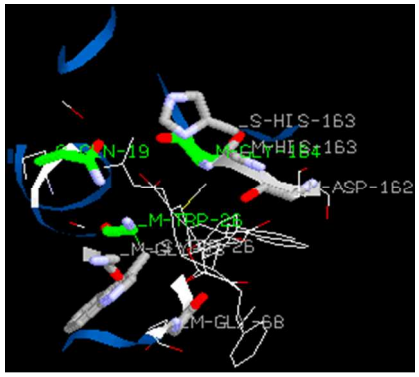

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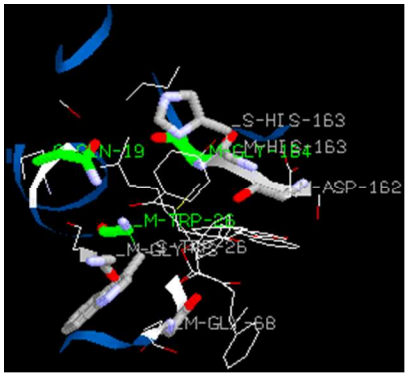

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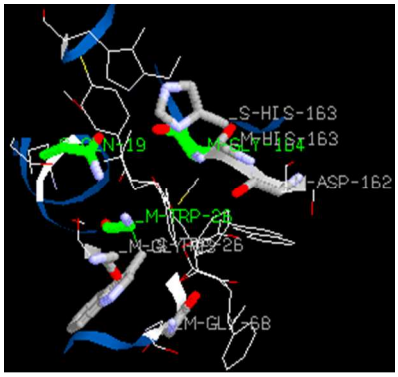

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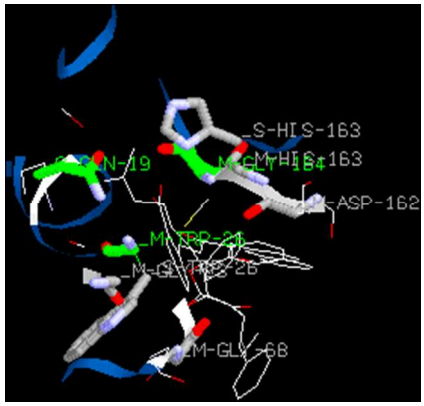

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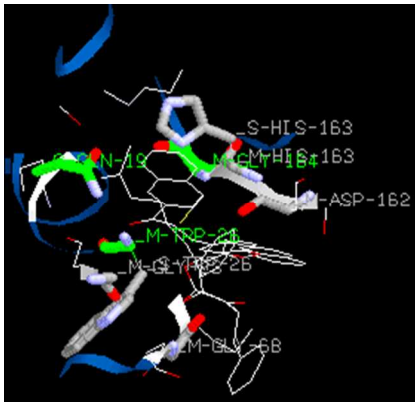

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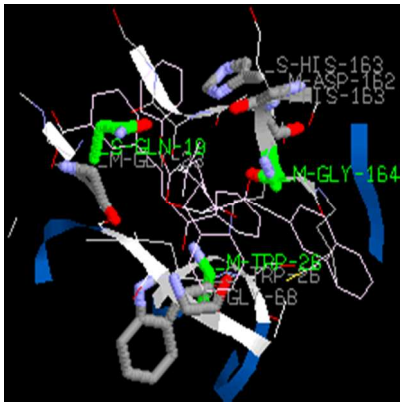

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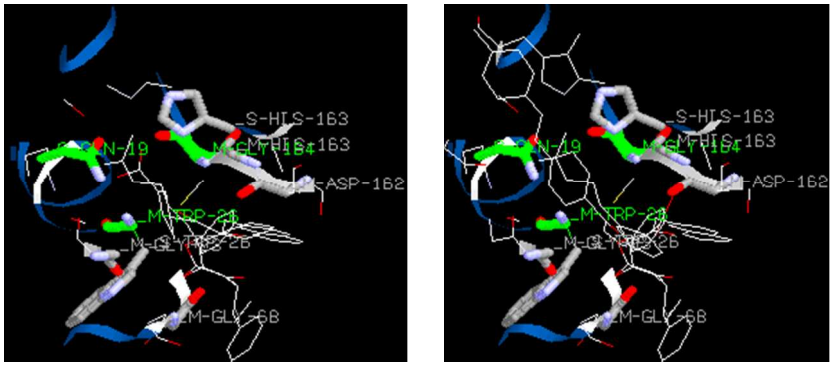

6x **6xi**

Figure 6