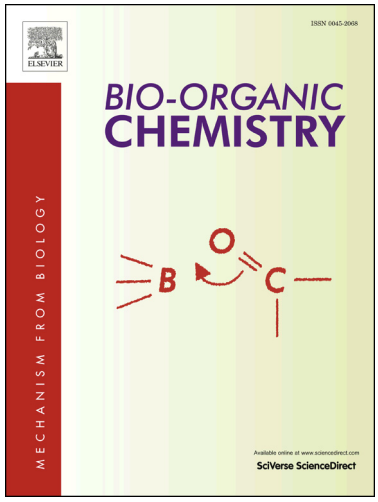

2, 5-Diaryloxadiazoles and their precursors as novel inhibitors of cathepsins B, H and L

Shweta Garg, Neera Raghav

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2, 5-DIARYLOXADIAZOLES AND THEIR PRECURSORS AS NOVEL INHIBITORS OF CATHEPSINS B, H AND L

Shweta Garg and Neera Raghav*

Department of Chemistry, Kurukshetra University, Kurukshetra-136119 (INDIA)

Tel.: +919896918277

E-mail: nraghav.chem@gmail.com

Abstract

High levels of cathepsins indicated in various pathological conditions like arthritis, cancer progressions, atherosclerosis etc. explains the need to explore potential inhibitors of these proteases which can be of great therapeutic significance. We, in the present work, report the synthesis of some 2,5-diaryloxadiazoles from N-subsitutedbenzylidenebenzohydrazides. The synthesized compounds were screened for their inhibitory potential on cathepsins B, H and L. Structure Activity Relationship studies show that 2,5-diaryloxadiazoles were less inhibitory than their precursors. 1i and 2k have been found to be most inhibitory to cathepsins B and L. Their K_i values have been calculated as 11.38×10^{-8} M and 66.4×10^{-8} M for cathepsin B and 4.2×10^{-9} M and 47.31×10^{-9} M for cathepsin L, respectively. However, cathepsin H activity was maximally inhibited by compounds, 1e and 2c with K_i values of 4.4×10^{-7} M and 5.6×10^{-7} M, respectively. Enzyme kinetic studies suggest that these compounds are competitive inhibitors to the enzymes. The results have been compared with docking results obtained using iGemDock.

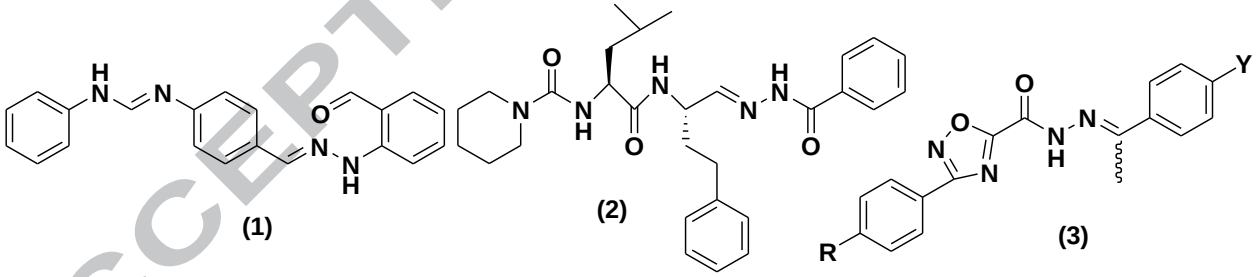
Keywords: 2,5-diaryloxadiazoles, N-subsitutedbenzylidenebenzohydrazides, Cathepsin B, cathepsin H and Cathepsin L inhibitors.

1. Introduction

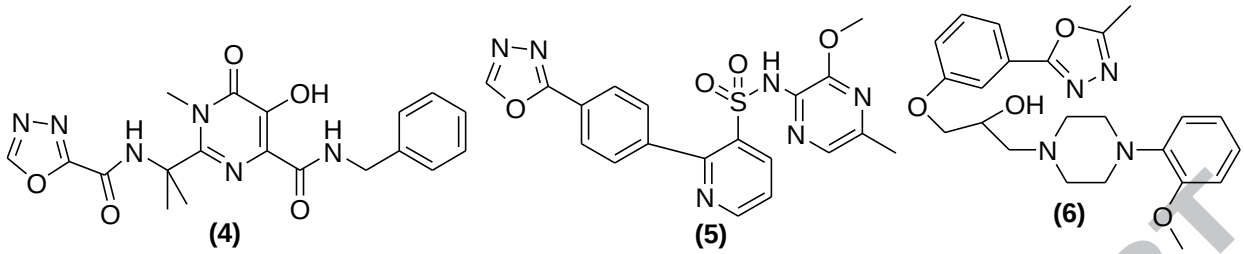
Enzyme inhibition studies have been a great tool in the development of newer medicines. Almost 60% of present drugs act by inhibiting enzymes responsible for pathological conditions. Discovering novel therapeutic targets and identification of their inhibitors can provide different outlook for treatment of diseases. Cathepsins B, H and L, three important lysosomal cysteine proteases have become important therapeutic targets because of their underlying proteolytic role in cell degenerative processes and cleavage of extracellular matrix[1]. Their potential role is not at all deniable in diseases like inflammation[2] particularly arthritis, periodontitis; cell proliferation and tumor growth[3,4], Alzeimers disease[5,6] accentuate the need for development of specific inhibitors of these enzymes which can be of great therapeutic potential. The quest for their

identification is also a result due to a decrease in the concentration of natural inhibitors of these cathepsins which is one of the consequences in the mentioned diseased conditions. In past various peptidyl inhibitors have been synthesized and evaluated as inhibitors to cathepsins[7] but the need for identification of newer therapeutic agents never ends in order to obtain better efficacy than and to avoid untoward effects of available drugs. With oral instability and immunogenic problems faced using peptidyl inhibitors, in recent past research has found its way to discover non-peptidyl inhibitors that can act as therapeutic agents in diseased conditions associated with the cysteine proteases. In this direction, we have reported[8-11] some non-peptidyl inhibitors of cathepsins B and H such as acyl hydrazides and triazoles, o-hydroxy chalcones and their cyclised derivatives and bischalcone based quinozolines and 4'-phenylchalcones and their derivatives as novel inhibitors of cathepsins B and H. Work has also been carried out on semicarbazones and thiosemicarbazones of carbonyl compounds[12] and chalcones[13]. The present work has been proposed on the basis of different biological activities possessed by 2,5-diaryloxadiazoles and N-subsitutedbenzylidenebenzohydrazides and to explore their possible role in the treatment of conditions led due to imbalanced activities of cathepsin B, cathepsin H and cathepsin L such as in cancer, arthritis etc.

The compounds with similar scaffold have been reported as cysteine protease inhibitors; for example, hydrazone, **1** as parasitic cysteine protease inhibitor[14], hydrazone, **2** as cathepsin S Inhibitor[15] and 1,2,4- oxadiazole-N-acylhydrazone[16], **3** as cysteine protease cruzain inhibitor[17].

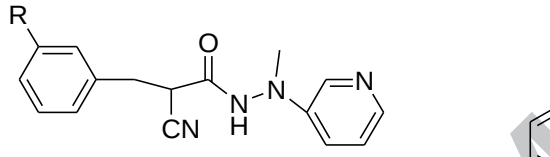


Variety of therapeutically active compounds having Oxadiazole nucleus currently being used in clinical medicine are: HIV – integrase inhibitor Raltegravir®, **4** an antiretroviral drug[18]; Zibotentan®, **5** an anticancer agent[19]; a nitrofuran antibacterial, furamizole; antihypertensive agents tiodazosin and nesapidil, **6**.



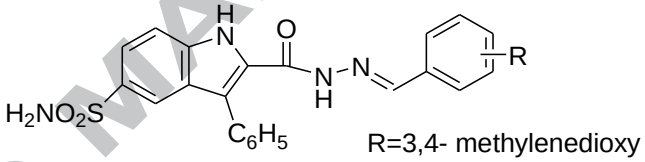
(4) **(5)** **(6)**

N-substituted hydrazides are also of wide interest because of their diverse biological activities and clinical applications. Novel Hydrazide-Hydrazone derivatives, **7** with Antitumor activity[20] Arylidene hydrazides, 3,4-methylenedioxy/4-methyl/4-nitro benzylidene hydrazide[21], **8** showing antidepressant activity, para-subsituted hydrazone, **9** and N-arylidene-2-(2-phenoxyphenyl) acetohydrazides, **10** exhibited good and persistent anti-inflammatory activity[22,23] are reported.



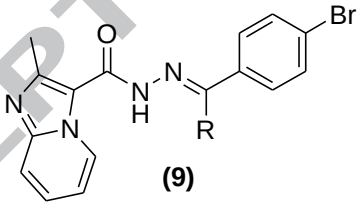
(7)

a) R= H
b) R=Cl
c) R= OCH_3



(8)

R=3,4- methylenedioxy



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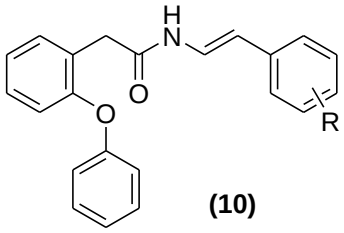


(10)

The present work deals with the synthesis, characterization of some 2**,**5-diaryloxadiazoles from their precursors N-subsitutedbenzylidenebenzohydrazides. It has been observed by our research group that in most of cases open chain analogues have been evaluated as better inhibitors of these enzymes therefore to evaluate potential role of synthesized compounds as cathepsins B, H and L inhibitors we here report the comparative studies of cyclized derivatives as well as their open chain precursors. The results were extended to elaborate the type of inhibition and were also correlated with in-silico studies.

2. Results and Discussion

2.1. Chemistry

N-subsitutedbenzylidenebenzohydrazides were synthesized by the reaction of benzoic hydrazide with substituted benzaldehydes in presence of a few drops of acetic acid as catalyst and 2,5-diaryl-1,3,4-oxadiazole were synthesized by the cyclization of N-subsitutedbenzylidenebenzohydrazides in dichloromethane with the help of iodobenzenediacetate in presence of a few drops of acetic acid (scheme 1). The structure elucidation of compounds was based on the spectral data (IR and ^{1}H NMR). All the synthesized N- substitutedbenzylidenebenzohydrazides show a characteristic IR absorption peak. The IR spectra of N-subsitutedbenzylidenebenzohydrazides showed mainly stretching bands at 1531-1585and 1645-1681 cm^{-1} assigned to imine of Schiff bases and (C=O) functionalities, respectively. In the ^{1}H NMR spectrum, singlets at δ 8.40-8.94 ppm for imine protons were observed. In case of 2,5-diaryl-1,3,4-oxadiazole, above mentioned IR and NMR spectra was missing that confirm the cyclization of N- substitutedbenzylidenebenzohydrazides and formation of 2,5-diaryl-1,3,4-oxadiazole.

2.2. Pharmacological evaluation

2.2.1. Proteolysis of endogenous protein substrates

The effect of synthesized compounds i.e. N- substitutedbenzylidenebenzohydrazides and 2,5-diaryl-1,3,4-oxadiazole, on proteolysis of endogenous protein substrates was observed at pH 5.0, where most of the proteolytic activity is attributed to cysteine proteases[24]. The studies were conducted in homogenate containing endogenous protein substrates for 3h and 24h (Table 1). Some compounds completely inhibited proteolysis at 1×10^{-4}M concentration. Inhibition after 3.0 hr was more than at 24.0 hr (Figure 1) suggesting a reversible type of inhibition. The electron withdrawing nitro- substituted compounds exhibited greater inhibition than electron releasing similarly positioned methyl or methoxy group indicating that inhibition is guided by electronic forces in the synthesized ligands. The inhibition pattern of compounds in series 1 is more or less similar to that of series 2 (Table 1)

The proteolysis studies suggested that open chain precursors are more effective inhibitors as compared to cyclized derivatives; this may be due to the better interaction of imine group with the active site which is lost during cyclization. The inhibition of endogenous proteolytic activity by these compounds at pH 5.0 which is attributable to cysteine proteases opens up further scope

to study these compounds as inhibitors to cathepsins B, H and L.

2.2.2. Effect of synthesized compounds on the activity of Cathepsin B

The activities of cathepsin B were estimated at different concentrations each of synthesized compounds. Effect of N-subsitutedbenzylidenebenzohydrazides **1(a-k)** and 2,5-diaryl-1,3,4-oxadiazole **2(a-k)** on cathepsin B activity is presented in Figure 2(i-ii). Among N-subsitutedbenzylidenebenzohydrazides and 2,5-diaryl-1,3,4-oxadiazole, 1i and 2k with nitro substitution were evaluated as most inhibitory to cathepsin B.

2.2.3. Effect of synthesized compounds on the activity of Cathepsin H

Figure 3 (i-ii) present the effect of N-subsitutedbenzylidenebenzohydrazides **1(a-k)** and 2,5-diaryl-1,3,4-oxadiazole 2**(a-k)** at varying concentrations on cathepsin H activity. In each series N'-(4-chlorophenylmethylene)benzohydrazide, 1e and 2 -phenyl -5-(2-chlorophenyl)-1,3,4-oxadiazole, 2c were evaluated as most inhibitory compounds. It has been found that cathepsin H activity is maximally inhibited by the chloro substituted compounds in both the series with opposing inductive and resonating effect.

2.2.4. Effect of synthesized compounds on the activity of Cathepsin L

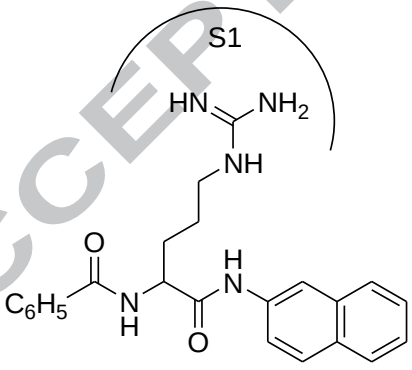
The activities of cathepsin L estimated at varying concentrations of N-subsitutedbenzylidenebenzohydrazides **1(a-k)** and 2,5-diaryl-1,3,4-oxadiazole 2**(a-k)** are presented in figure 4 (i-ii), respectively. Among the various compounds in each series most inhibitory compound to catnepsin B N'-(2-nitrophenylmethylene)benzohydrazide (1i) and 2,5-diaryl-1,3,4-oxadiazoles, 2-phenyl -5-(4-nitrophenyl)- 1,3,4-oxadiazole (2k) were found best inhibitors to cathepsin L also.

2.2.5. Enzyme kinetic studies

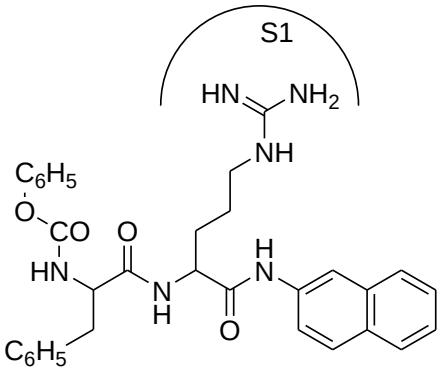
Enzyme kinetic studies are important in order to establish the type of inhibition exhibited by compounds and Lineweaver-Burk plots have been a great tool in differentiating between various reversible inhibitors in an efficient manner, therefore, after ascertaining the inhibition of synthesized compounds on cathepsins B, H and L, experiments were designed to evaluate the type of inhibition and to find out their K_i values. Line-weaver Burk plots drawn in 1/S and 1/V in presence and absence of a fixed concentration of inhibitor at different substrate concentrations for cathepsin B, cathepsin H and cathepsin L are presented in figure 2 (iii, iv), figure 3 (iii, iv) and figure 4(iii, iv), respectively. These results indicated that all compounds exhibited a competitive type of inhibition as evidenced by the plots between 1/V and 1/S, where straight

lines intersecting at the Y-axis with V_{max} remaining constant and changing K_m have been obtained. The K_i values calculated using Line-weaver Burk equation for competitive inhibition has been presented in Table 2.

Among the series of compounds **(1-2)**, it was found that for cathepsin B and cathepsin L, *para*-methyl substituted compounds such as N'-(4-methylphenylmethylene)benzohydrazide (1b) and 2 -phenyl -5-(4-methylphenyl)- 1,3,4-oxadiazole (2b) showed negligible inhibitory effect on enzyme activity. Nitro substituted compounds in both the series were most inhibitory to cathepsin B as well as cathepsin L. N'-(2-Nitrophenylmethylene)benzohydrazide (1i) and 2-phenyl-5-(4-nitro phenyl)-1,3,4-oxadiazole (2k) have been evaluated as best inhibitors with K_i values of 1.138×10^{-7} M and 6.64×10^{-7} M, respectively for cathepsin B. And, for cathepsin L the K_i values were evaluated as 4.2×10^{-9} M and 47.31×10^{-9}M, respectively (Table 2). The inhibition of cathepsins B and L by 1i and 2k can be explained on the basis of the binding sites. The binding sites in proteases designated as S1, S2….Sn corresponding to enzyme site designated as P1, P2….Pn account for the reaction specificities of proteases[25]. The substrate specificity of catehpsins B and L suggest that S1 binding sites of both these enzymes very well accommodate arginine amino acid however S2 is responsible for differentiating the specificities in these two enzymes. For cathepsin L specificity S2 site is described by phenyl alanine whereas in cathepsin B it is again specified by argininine[26]. The similar behavior of cathepsins B and L toward potential inhibitors 1i and 2k, can be due to similar binding site S.



BANA



Z-Phe-Arg-4mβNA

Chloro substituted compounds i.e., N'-(4-chlorophenylmethylene)benzohydrazide (1e) and 2 -phenyl -5-(2-chlorophenyl)- 1,3,4-oxadiazole (2c) showed maximum inhibitory effect on cathepsin H activity with K_i values of 4.4×10^{-7} M and 5.6×10^{-7}, respectively (Table 2).

It can be observed (Table 2) that open chain analogues were potent and selective inhibitor as

compared to their cyclized derivatives for cathepsins B, H and L. The order of inhibition for both of series has been evaluated for cathepsin L > cathepsin B > cathepsin H.

The results when compared with other recently reported non-peptidyl inhibitors of these cathepsins precisely displayed some trends. An important observation was that among various open chain and their cyclized derivatives former have been evaluated as more potent inhibitors. In case of flavanones and flavones when compared with respective chalcones, we observed that open chain precursors were better inhibitors than cyclized derivatives with K_i value of most inhibitory compound in each series was ~6.18x10^{-8}M, 4.8x10^{-7}M and 7.85 x10^{-7}M for cathepsin B and ~2.8 x10^{-7}M, 31.8 x10^{-6}M and 33.7 x10^{-6}M for cathepsin H for chalcone, flavanone and flavones, respectively[9]. In case of acyl hydrazides, the open chain precursors for respective triazoles[8] also have been found to be better inhibitors than cyclized derivatives with K_i value for the most inhibitory compound as 0.64µM, 1.43µM, 2.4 µM, 0.95 µM for cathepsin B and 8.2µM, 8.6µM, 3.3 µM, 2.0 µM for cathepsin H, respectively. Similar results have been observed for chalcone semicarbazones and their pyrazoline derivatives[13] where most inhibitory chalcone semicarbazone and its pyrazoline have been shown a K_i value of ~0.207x10^{-6}M and 9.40 × 10^{-6} M, for cathepsin B, ~2.10x10^{-5}M and 2.5 × 10^{-5} M, for cathepsin H and 0.40 x10^{-10}M and 0.117 × 10^{-8} M, for cathepsin L, respectively.

The cyclized derivatives have been found to be more inhibitory only when a highly susceptible nucleophilic group was introduced at any position in cyclic compound[11,27].

Another important similarity was that nitro compounds have been found to be most inhibitory to cathepsins B and L whereas chloro-substituted compounds have been found best inhibitors to cathepsin H[11,28]. It has been found that in all previously reported non-peptidyl inhibitors of cathepsins under consideration is that cathepsin L has been found to be more prone to inhibition by various inhibitors than cathepsin B and cathepsin H in that order[13,28]. In the present series also, we could find that cathepsin L is inhibited more than cathepsin B and cathepsin H. The results are quite encouraging and support the need to carry out such studies in future so that selective inhibitors of each cathepsin may be searched.

After establishing the in-vitro inhibitory potential of the synthesized compounds in-silico studies were also carried out to correlate the results as discussed below.

2.2.6. Molecular docking experiment

Molecular docking experiments were conducted using iGemDock, an automated drug design system for docking, screening and post analysis and has been successfully used to correlate the *in*

vitro and *in silico* studies of enzymes[8-13]. Using iGemDock, the predicted poses generated can be directly visualized in the active site of the target enzyme and simultaneous analysis can be carried out to predict the affinity of compound with active site by decrease in total energy. The software provides a tool based on docked poses (protein-ligand interactions) and the compound properties[29]. In the present work we have tried to find out a correlation between enzyme inhibition potency and the docking scores.

The interaction data of docking experiments (Table suppl) include total energy and individual energy terms indicating the fitness of a predicted pose in the binding site suggest that the level of interaction is highest for N-subsitutedbenzylidenebenzohydrazides followed by 2,5-diaryloxadiazoles within the active site of cathepsin B and the same order is found in case of cathepsin H and cathepsin L. The results can be correlated with the observed K_i values. The K_i values are lower for open chain analogues suggesting higher interaction of compounds with active site. In cathepsin B, all the compounds showed a lesser interaction than leupeptin, a peptidyl inhibitor of the enzyme. Decrease in total energy for leupeptin-cathepsin B has come out be -127.7 which is almost equal to that of substrate BANA, -125.55. This is due to complementary substrate of peptidyl nature in enzyme active site. Leupeptin binds more effectively with the enzyme active site than any of the synthesized compounds (Table 3) resulting in higher binding energy. Docking methods provide valuable information regarding the binding mode between the ligand and the enzyme active site and can play an important role in understanding the ligand-enzyme interactions. From molecular docking experiments, we observed that all of compounds inhibit the enzyme in a competitive manner and because these compete at the binding site of enzyme with substrate. Figures **5i** and **5ii** show the docked view of most inhibitory compounds, 1i and 2k in the active site of cathepsin B, respectively. It is visible that active site group **Cys-29** and **Gly-198** interact with these compounds as well as with the substrate through H-bonds.

In cathepsin H, the decrease in total energy for the reference inhibitor Leu-CH_2Cl was less as compared to all the designed compounds. Here, it can be seen that though Leu-CH_2Cl is specific inhibitor for cathepsin H[30], but possess only one amino acid residue as compared to leupeptin–cathepsin B. Therefore, the leu-CH_2Cl-cathepsin H interaction causes a decrease in energy of -59.84. Table 3 presents the docking energies of the most potent inhibitory compound in each series which show higher decrease in ligand-cathepsin H interaction energy than **leu-CH_2Cl-**

cathepsin H. The docked view of most inhibitory compounds, **1e** and **2c** in the active site of cathepsin H are presented in figures **5iii** and **5iv**. All these compounds interact with the amino acyl acceptor site of the enzyme. Amino acids **Gln-70 and Gln-78** interact with **1e** and **2c**. These amino acids interact with the compounds through H-bonds as visualized by green coloured ribbon of enzyme. However grey colored Asn-112 and pro-77 interact through vander waals forces of interaction. The compounds can be visualized superimposed with the substrate indicating competitive inhibition of cathepsin H by these compounds, which is in accordance with the enzyme kinetic studies.

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 -102.07. As compared to this the binding energy of most inhibitor compounds of each series are less (Table 3). The docked view of most inhibitory compounds, **1i** and **2k** in the active site of cathepsin L are presented in figures **5v** and **5vi**. From molecular docking experiments, we observed that all of compounds inhibit the enzyme in a competitive manner and because these compete at the binding site of enzyme with substrate. It is clearly observed that **1i** and **2k** interact with **Gln-19**, **Gly-68** and **Gly-164** through hydrogen bonds. A portion of their binding region overlaps with the interaction zone of Z-Phe-Arg-4mβNA suggesting a competitive inhibition that is also observed by enzyme inhibition studies. Fig. 6 presents the correlation plot between K_i value and the docking scores of the most potent inhibitor in each series.

The present study can be helpful in explaining the role of larger contact area between enzyme and ligand in acquiring greater enzyme inhibition. The results are in consensus with the docking data where similarly substituted N-subsitutedbenzylidenebenzohydrazide show a larger decrease in total energy as compared to respective 2,5-diaryloxadiazole. In addition, greater inhibition by N-subsitutedbenzylidenebenzohydrazides can also involve the direct interaction of active site sulfhydryl group of enzymes with either of the nucleophilic carbonyl- or imine- centers present therein which are lacking in cyclized 2,5-diaryloxadiazole derivatives. The study can add to designing of existing non-peptidyl inhibitors that may contribute further to increase the potency and specificity of cysteine protease inhibitors.

In the present work decrease in total energy of enzyme–ligand complex is presented as binding affinity of ligand within the active site of enzyme. However, Zhang et al. have used free energy based approach in understanding such interactions[31].

3. Materials and methods

3.1. Materials

All the chemicals were of analytical grade. Fast Garnet GBC (*o*-aminoazotoluene diazonium salt, α-N-benzoyl-D, L-arginine-2-naphthylamide (BANA), Z-Phe-Arg-4mβNA and Leu-βNA were purchased from Bachem Feinchemikalien AG, Switzerland. Sephadex G-100, CM-Sephadex C-50 and DEAE-Sephadex A-50 were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.The protein sample was concentrated using Amicon stirred cells with YM 10 membrane under nitrogen pressure of 4-5 psi. The source of enzyme was fresh goat liver obtained from local slaughter house.

3.2. Methods

Melting points determined in open capillary tubes are thus uncorrected. All the chemicals and solvents used were of laboratory grade. IR spectra (KBr, cm^{-1}) were recorded on a Perkin-Elmer spectrometer. ^{1}H NMR spectra was recorded on Bruker 300 MHz NMR spectrometer (chemical shifts in δ ppm) using TMS as an internal standard. The purity of the compounds was ascertained by 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). ELISA plate reader was used for measuring absorbance in the visible range.

3.2.1. General Procedure for the synthesis of N-subsitutedbenzylidenebenzohydrazides

To the ethanolic solution of benzoic hydrazide (0.01mol) substituted benzaldehyde (0.011mol) was added and the resulting solution was refluxed in the presence of catalytic amount of acetic acid for 1-2 hours. The solvent was evaporated in air at room temperature. The solid thus obtained was filtered and washed with cold ethanol to get desired product. The structure elucidation of N-subsitutedbenzylidenebenzohydrazides was based on melting point and the spectral data (IR, ^{1}H-NMR and ^{13}C-NMR).

3.2.2. General method for the synthesis of 2,5-diaryl-1,3,4-oxadiazole

To the stirred solution of N-substitutedbenzylidenebenzohydrazides (0.003mol) in dichloromethane, iodobenzenediacetate (0.003mol) was added. The contents were stirred for 2 h and the progress of the reaction was monitored by TLC. The solvent was removed and the residue was taken in petroleum ether and stirred for 30 min. The solid thus obtained was filtered, washed with petroleum ether and dried to afford desired product (scheme 1). The structure elucidation of 2,5 -diphenyl- 1,3,4-oxadiazoles were based on melting point and the spectral data (IR, ^{1}H-NMR and ^{13}C-NMR) which has been provided in the supplementary data.

3.3. Enzymatic studies

3.3.1. 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.

3.3.2. 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. (Figure 1), 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[35]. The experiments were conducted in triplicate and the results are presented in Table 1.

3.3.3. Purification of 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[36]. 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.

Enzyme assays

The purified cathepsin B, H and L were first activated in presence of thiol activators at pH 6.0, pH 7.0 and pH 6.0, respectively. Activated enzyme solution (100µl) was equilibrated with 0.1M phosphate buffer at respective pH containing 1mM EDTA separately for 10 min at 37^{o}C. Cathepsins B, H and L activities were then analyzed using BANA, leu-βNA and Z-Phe-Arg-4mβNA as substrates, respectively. The released β-naphthylamine was measured colorimetrically at 620nm by the use of fast garnet GBC dye using established procedures[8,36]. The experiments were performed in triplicates.

3.4. Pharmacology

3.4.1. Enzyme inhibition studies

Enzyme inhibition studies were carried out in the similar manner as detailed above. To the pre-activated enzyme 20µl of stock solution (5mM prepared in DMSO) of different compounds under study was added separately to the activated enzyme assay mixtures to effect final concentrations as 10^{-4} M in 1 ml assay. After 30 min, 25µl of 100mM substrate stock solution was added to start the reaction. In control experiments, equivalent amount of DMSO was added and percent residual activities were calculated with reference to control (Table 1).

The compounds exhibiting inhibition at 1×10^{-4}M concentration (Table 1) were further studied for their inhibitory effect at their varying concentrations by adding appropriate amount of individual compounds in the reaction mixture separately to effect the final concentration of N-subsitutedbenzylidenebenzohydrazides as 0.1×10^{-5}M, 0.2×10^{-5}M, 0.4×10^{-5}M, 0.6×10^{-5}M, 0.8×10^{-5}M, 1.0×10^{-5}M for cathepsin B; 0.1×10^{-6}M, 0.2×10^{-6}M, 0.4×10^{-6}M, 0.6×10^{-6}M, 0.8×10^{-6}M, 1.0×10^{-6}M for cathepsin L and 0.1×10^{-4}M, 0.2×10^{-4}M, 0.4×10^{-4}M, 0.6×10^{-4}M, 0.8×10^{-4}M, 1.0×10^{-4}M for cathepsin H and 2,5-diaryloxadiazoles as 0.1×10^{-4}M, 0.2×10^{-4}M, 0.4×10^{-4}M, 0.6×10^{-4}M, 0.8×10^{-4}M, 1.0×10^{-4}M for cathepsins B & H and 0.1×10^{-5}M, 0.2×10^{-5}M, 0.4×10^{-5}M, 0.6×10^{-5}M, 0.8×10^{-5}M, 1.0×10^{-5}M for cathepsin L (Figures 2-4(i-ii)).

3.4.2. Enzyme kinetic studies

After establishing the inhibitory action of synthesized compounds on cathepsins B, H and L, experiments were designed to evaluate the type of inhibition and to determine their K_i values. For that, enzyme activity was evaluated at six different substrate concentrations in presence and absence of a fixed concentration of inhibitor. The enzyme concentration was kept constant in all the experiments as detailed previously. The results are presented in figures 2-4(iii-iv). The values represent Mean±S.M.D of at least three individual experiments. The K_i values of compounds were calculated using the Line weaver-Burk equation $K_{m'}=K_m(1+[I]/K_i)$ for competitive inhibition (Table 2).

3.4.3. Molecular Docking 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, cathepsin H and cathepsin L were retrieved from Protein Data Bank as cav2IPP B_PYS.pdb[32], and cav8PCH

H_NAG.pdb[33] and cav3BC3L_CSW[34], 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 presented in table 3 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 5.

3.5. Conclusion

We have evaluated structurally related two series of 22 compounds for their inhibitory activity against cathepsin B, H and L and have identified some synthetic non-peptidyl inhibitors for cathepsin B, H and L. The compounds inhibited the enzyme cathepsin L to maximum extent showing K_i value of the order of nM followed by cathepsin B and were least inhibitory to cathepsin H. In general, the inhibitory potency of compounds was related to the electro-negativity property of substituent. Through enzyme kinetics, it was revealed that all compounds inhibit cathepsin B, H and L in a competitive manner. The *in-vitro* results presented in this study have been compared with *in-silico* studies.

The authors have declared no conflicts of interest.

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REFERENCES

1. Zavasnik-Bergant, T., Turk, B., 2006. Cysteine cathepsins in the immune response. Tissue Antigens. 67, 349–55.
2. Bylaite, M., Moussali, H., Marciukaitiene, I., Ruzicka, T., Walz, M., 2006. Expression of cathepsin L and its inhibitor hurpin in inflammatory and neoplastic skin diseases. Exp Dermatol 15, 110-118.
3. Waghray, A., Keppler, D., Sloane, B. F., Schuger, L., Chen, Y. Q., 2002. Analysis of a truncated form of cathepsin H in human prostate tumor cells. J Biol Chem 277, 11533-11538.

4. Kirschke, H., Eerola, R., Hopsu-Havu, V. K., Bromme, D., Vuorio, E., 2000. Antisense RNA inhibition of cathepsin L expression reduces tumorigenicity of malignant cells. Eur J Cancer 36, 787-795.
5. Yoshiyama, Y., Arai, K., Oki, T., Hattori, T., 2000. Expression of invariant chain and pro-cathepsin L in Alzheimer's brain. Neurosci Lett 290, 125-128.
6. Schechter, I., Ziv, E., 2011. Cathepsins S, B and L with aminopeptidases display beta-secretase activity associated with the pathogenesis of Alzheimer's disease. Biol. Chem. 392, 555–569.
7. Frizler, M., Stirnberg, M., Sisay, M. T., Gütschow, M., 2010. Development of nitrile-based peptidic inhibitors of cysteine cathepsins. Curr Top Med Chem. 10, 294-322.
8. Raghav, N., Singh, M., 2014. Acyl hydrazides and triazoles as novel inhibitors of mammalian Cathepsin B and Cathepsin H. Eur. J. Med. Chem. 77, 231-242.
9. Raghav, N., Garg, S., 2014. SAR studies of o-hydroxychalcones and their cyclised analogs and study them as novel inhibitors of Cathepsin B and Cathepsin H. Eur. J. Pharmaceut. Sci. 60, 55-63.
10. Raghav, N., Singh, M., 2014. Design, synthesis and docking studies of bischalcones based quinazoline-2(1H)-ones and quinazoline-2(1H)-thiones derivatives as novel inhibitors of Cathepsin B and Cathepsin H. Eur. J. Pharmaceut. Sci. 541, 28-39.
11. Raghav, N., Ravish, I., 2015. SAR studies of differently functionalized 4'-phenylchalcone based compounds as inhibitors of Cathepsins B, H and L. RSC Adv. 5, 50440-53.
12. Raghav, N., Kaur, R., 2014. Synthesis and evaluation of some semicarbazone and thiosemicarbazone based cathepsin B inhibitors. Med Chem Res. 23, 4669-79.
13. Raghav, N., Kaur, R., 2015. Chalcones, semicarbazones and pyrazolines as inhibitors of cathepsins B, H and L. Int. J. Biol. Macromol. 80, 710–724.
14. Desai, P. V., Patny, A., Sabnis, Y., Tekwani, B., Gut, J., Rosenthal, P., Srivastava, A., Avery, M., 2004. Identification of novel parasitic cysteine protease inhibitors using virtual screening. J. Med. Chem. 47, 6609-6615.
15. Cywin, C. L., Firestone, R. A., McNeil, D. W., Grygon, C. A., Crane, K. M., White, D. M., Kinkade, P. R., Hopkins, J. L., Davidson, W., Labadia, M. E., Wildeson, J., Morelock, M. M., Peterson, J. D., Raymond, E. L., Brown, M. L., Spero, D. M., 2003. The design of potent hydrazones and disulfides as cathepsin S inhibitors. Bioorg. Med. Chem. 11, 733-

740.

16. Filho, J. M., dos, S., Leite, A. C. L., de Oliveira, B. G., Moreira, D. R. M., Lima, M. S., Soares, M. B. P., Fernanda, L., Leite, C. C., 2009. Design, synthesis and cruzain docking of 3-(4-substituted-aryl)-1,2,4-oxadiazole-N-acylhydrazones as anti-Trypanosoma cruzi agents Bioorg. Med. Chem. 17, 6682-6691.
17. Du, X., Guo, C., Hansell, E., Doyle, P. S., Caffrey, C. R., Holler, T. P., McKerrow, J. H., Cohen, F. E., 2002. Synthesis and structure-activity relationship study of potent trypanocidal thio semicarbazone inhibitors of the trypanosomal cysteine protease cruzain. J. Med. Chem. 2002; 45: 2695-2707.
18. Savarino, A., 2006. A historical sketch of the discovery and development of HIV–1 integrase inhibitors: Review. Expert Opin. Investig. Drugs 15, 1507–1522.
19. James, N. D., Growcott, J. W., 2009. Zibotentan. Drugs Future 34, 624–633.
20. Rafat, M. M., 2011. Novel Synthesis of Hydrazide-Hydrazone Derivatives and Their Utilization in the Synthesis of Coumarin, Pyridine, Thiazole and Thiophene Derivatives with Antitumor Activity. Molecules. 16, 16-27.
21. Ergenç, N., Günay, N. S., 1998. Synthesis and antidepressant evaluation of new 3-phenyl-5-sulfonamidoindole derivatives. Eur. J. Med. Chem. 33, 143-148.
22. Izabella, G. R., Da Silva, K. C. M., Sergio, C., Parrini, Ana Luisa, P., de Miranda, C. A., Fraga, M., Barreori, J. E., 1998. Synthesis and antinociceptive properties of new structurally planned imidazo[1,2-a]pyridine 3-acylarylhydrazone derivatives. Eur. J. Med. Chem. 33, 225-235.
23. Shekarchi, M., Navidpour, L., Khorami, A. R., Partoazar, A., Shafaroodi, H., Rahmanipour, N., Shafiee, A., 2011. Synthesis of N-arylidene-2-(2-Phenoxyphenyl) Acetohydrazides as anti-inflammatory Agents. Iran J Pharm Res. 10, 369–377.
24. Raghav, N., Singh, M., Kaur, R., Suman, Priyanka, 2010. Effect of semicarbazones on endogenous protein hydrolysis in liver homogenate. Asian J. Chem. 22, 7097-7101.
25. Berger, A., Schechter, I., 1967. On the size of active site in proteases. I. Papain. Biochem. Biophys. Res. Commun. 27, 157-162
26. Barrett, A. J., Rawlings, N. D., Woessner J. F. (Eds.), Handbook of Proteolytic Enzymes (2nd Ed), Elsevier, Amsterdam (2004)

27. Garg, S., Raghav, N., 2014. N-formylpyrazolines and N-benzoylpyrazolines as novel inhibitors of mammalian cathepsin B and cathepsin H. J Bioorg. Chem. 57, 43-50.
28. Garg, S., Raghav, N., 2015. Inhibitory potential of some chalcones. RSC Adv. 5, 72937-72949.
29. Yang J.M., 2004. Development and evaluation of a generic evolutionary method for protein-ligand docking, J. Computational Chemistry. 25, 843-857
30. Azaryan, A., Galoyan, A., 1987. Human and bovine brain cathepsin L and cathepsin H: purification, physicochemical properties, and specificity. Neurochem. Res. 12, 207-213.
31. Zhang, Z., Martiny, V., Lagorce, D., Ikeguchi, Y., Alexov, E., Miteva, M. A., 2014. Rational Design of Small-Molecule Stabilizers of Spermine Synthase Dimer by Virtual Screening and Free Energy-Based Approach. PLoS One 9, e1108884.
32. Huber, C. P., Campbell, R. L., Hasnain, S., Hirama, T., To, R., 2013. Crystal structure of the tetragonal form of human liver cathepsin B. (http://www.ebi.ac.uk/pdbe-srv/view/entry/2ipp/citation.html).
33. Guncar, G., Podobnik, M., Pungercar, J., Strukelj, B., Turk, V., Turk, D., 1998. Crystal structure of porcine cathepsin H determined at 2.1 Å resolution: location of the mini-chain C-terminal carboxyl group defines cathepsin H aminopeptidase function. Structure. 6, 51–61.
34. Chowdhary, S. F., Joseph, L., Kumar, S., Tulsidas, S. R., Bhat, S., Ziomek, E., Menard, R. M., Sivaraman, J., Purisima, E. O., 2008. Exploring inhibitor binding at the S' subsites of cathepsin L. J. Med. Chem. 51, 1361-1368.
35. Bradford, M. M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254.
36. Raghav, N., Singh, M., Garg, S., Ravish, I., Kaur, R., Suman, 2015. Ion exchangers: a useful tool for separation and simultaneous purification of lysosomal cysteine proteinases, cathepsins B, H and L. Int. J. pharmaceut. Sci. Res. 6, 2944-2949.

Figure 1: Effect of Substituted N-subsitutedbenzylidenesbenzohydrazides and 2,5-diaryloxadiazoles on the endogenous proteolytic activity for 3 hr and 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 2: Effect of varying concentration of most inhibitory compounds from each series, on cathepsin B activity, figure 2(i-ii), 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 $1x10^{-6}$M and $1x10^{-5}$M concentration of N-subsitutedbenzylidenebenzohydrazides and 2,5-diaryl-1,3,4-oxadiazoles at pH 6.0 figure 2(iii-iv), respectively. The K_m value for control have been found to be $3.64x10^{-4}$ M. The K_i values as calculated from this graph are presented in table 2

Figure 3: Effect of varying concentration of compounds from each series, on cathepsin H activity, figure 3(i-ii), 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 H at varying concentrations of leu-ßNA in presence of $1x10^{-5}$M concentration of N-subsitutedbenzylidenebenzohydrazides and 2,5-diaryl-1,3,4-oxadiazoles at pH 7.0 figure 3(iii-iv),, respectively. The K_m value for control has been found to be $5.34x10^{-4}$M. The K_i values as calculated from this graph are presented in table 2.

Figure 4: Effect of varying concentration of compounds from each series, on cathepsin L activity, figure 4(i-ii), 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 L at varying concentrations of z-Phe-Arg-4mβNA in presence of $1x10^{-6}$M and $1x10^{-5}$M concentration of N-subsitutedbenzylidenebenzohydrazides and 2,5-diaryl-1,3,4-oxadiazoles at pH 6.0,figure 4(iii-iv), respectively. 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 2.

Figure 5: Docking results showing the alignment of most inhibitory compounds along with the substrates. The circles drawn show overlapping of substrate as well as the compound. Here fig. 5(i-ii) show alignment of 1i and 2k, along with BANA in the active site of cathepsin B (cav2IPP B_PYS.pdb[32]), respectively. Fig. 5(iii-iv) show alignment of 1e and 2c, along with LeußNA in the active site of cathepsin H (cav8PCH H_NAG.pdb[33]),

respectively. Fig. 5(v-vi) show alignment of 1i and 2k, along with Z-Phe-Arg-4mβNA in the active site of cathepsin L (cav3BC3L_CSW.pdb[34]), respectively.

Figure 6: Correlation plot between binding energies and log K_i values of most inhibitory compound in each series for cathepsin B, cathepsin H and cathepsin L.

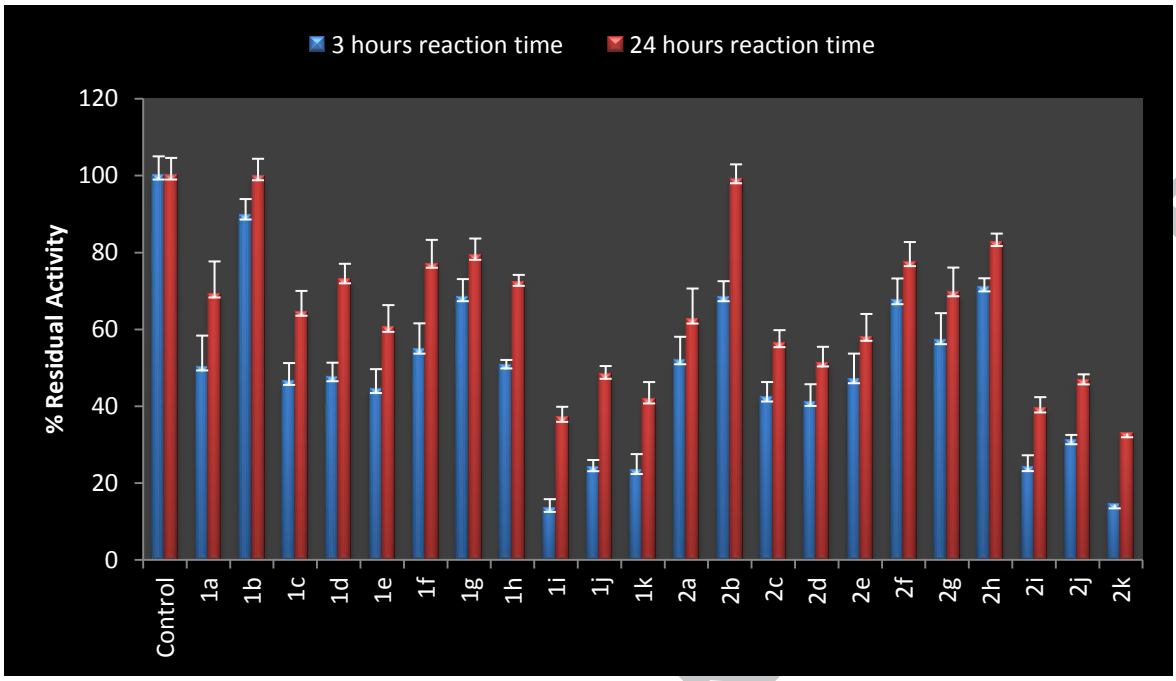


Figure 1

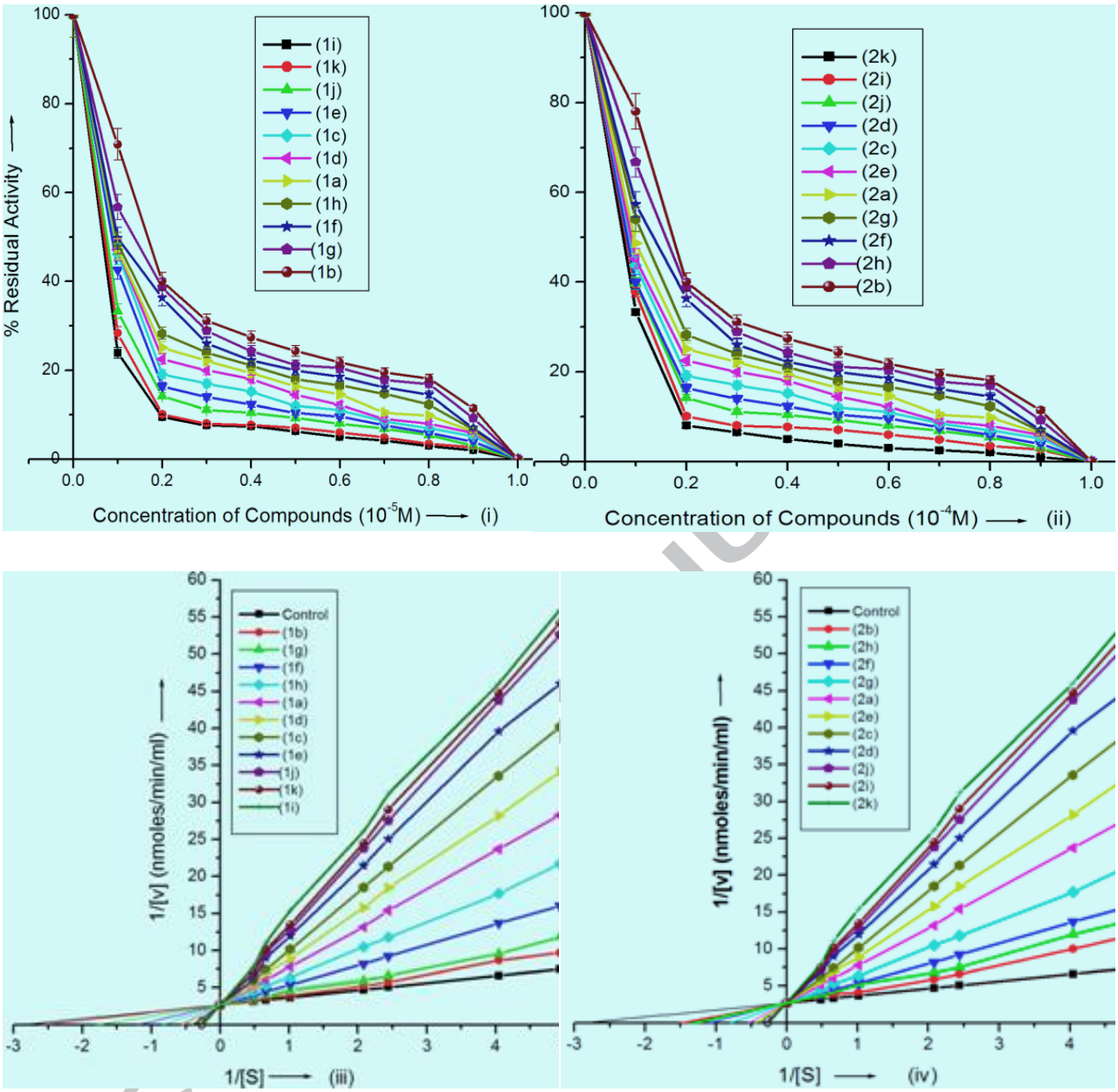

% Residual Activity
(1i)
(1k)
(1j)
(1e)
(1c)
(1d)
(1a)
(1h)
(1f)
(1g)
(1b)
Concentration of Compounds (10^{-5}M) (i)
(2k)
(2i)
(2j)
(2d)
(2c)
(2e)
(2a)
(2g)
(2f)
(2h)
(2b)
Concentration of Compounds (10^{-4}M) (ii)
1/[v] (nmoles/min/ml)
Control
(1b)
(1g)
(1f)
(1h)
(1a)
(1d)
(1c)
(1e)
(1j)
(1k)
(1i)
1/[S] (iii)
Control
(2b)
(2h)
(2f)
(2g)
(2a)
(2e)
(2c)
(2d)
(2j)
(2i)
(2k)
1/[S] (iv)

Figure 2

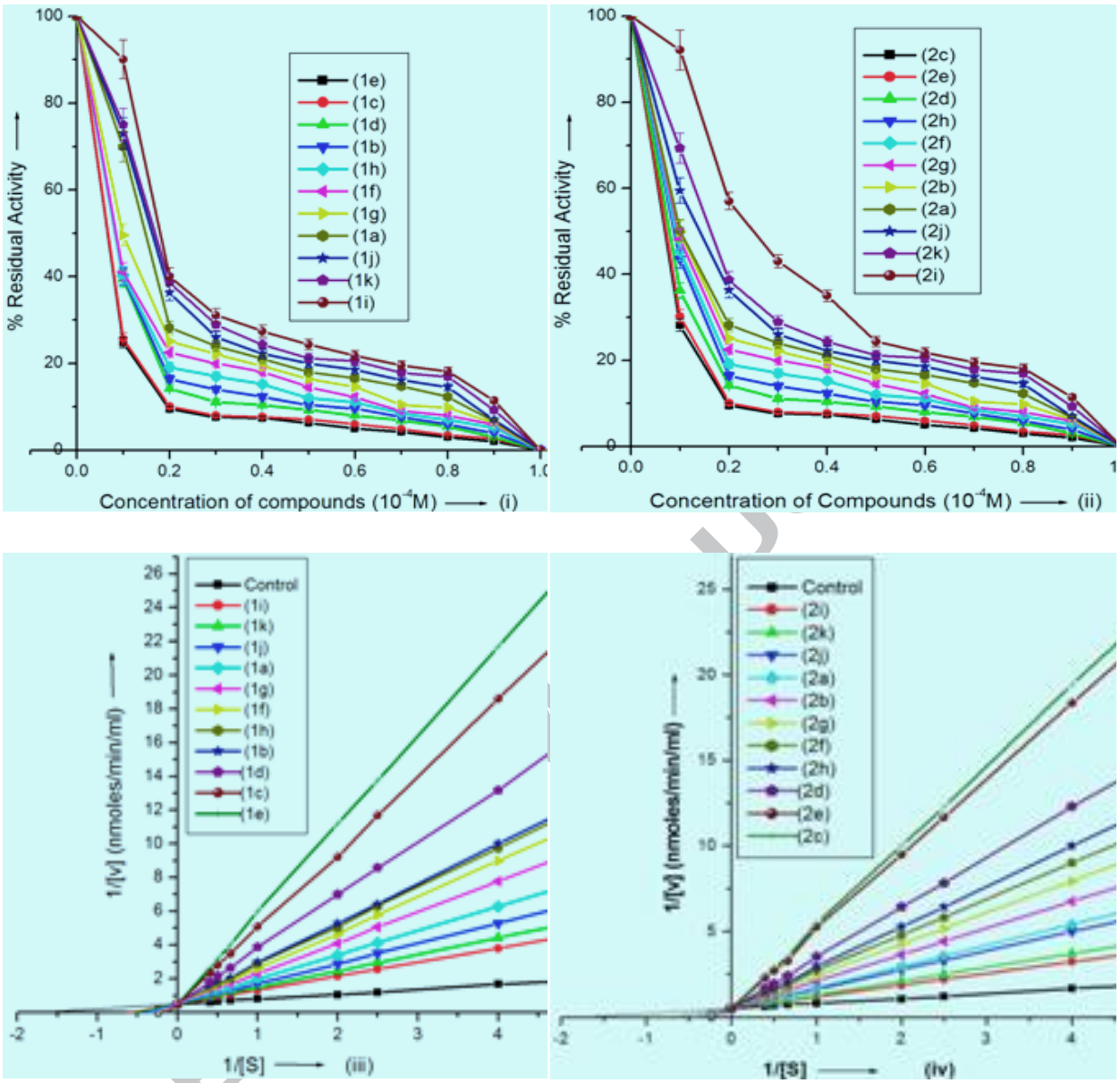

% Residual Activity
Concentration of compounds (10^{-4}M) (i)
(1e)
(1c)
(1d)
(1b)
(1h)
(1f)
(1g)
(1a)
(1j)
(1k)
(1i)
% Residual Activity
Concentration of Compounds (10^{-4}M) (ii)
(2c)
(2e)
(2d)
(2h)
(2f)
(2g)
(2b)
(2a)
(2j)
(2k)
(2i)
1/[v] (nmoles/min/ml)
1/[S] (iii)
Control
(1i)
(1k)
(1j)
(1a)
(1g)
(1f)
(1h)
(1b)
(1d)
(1c)
(1e)
1/[v] (nmoles/min/ml)
1/[S] (iv)
Control
(2i)
(2k)
(2j)
(2a)
(2b)
(2g)
(2f)
(2h)
(2d)
(2e)
(2c)

Figure 3

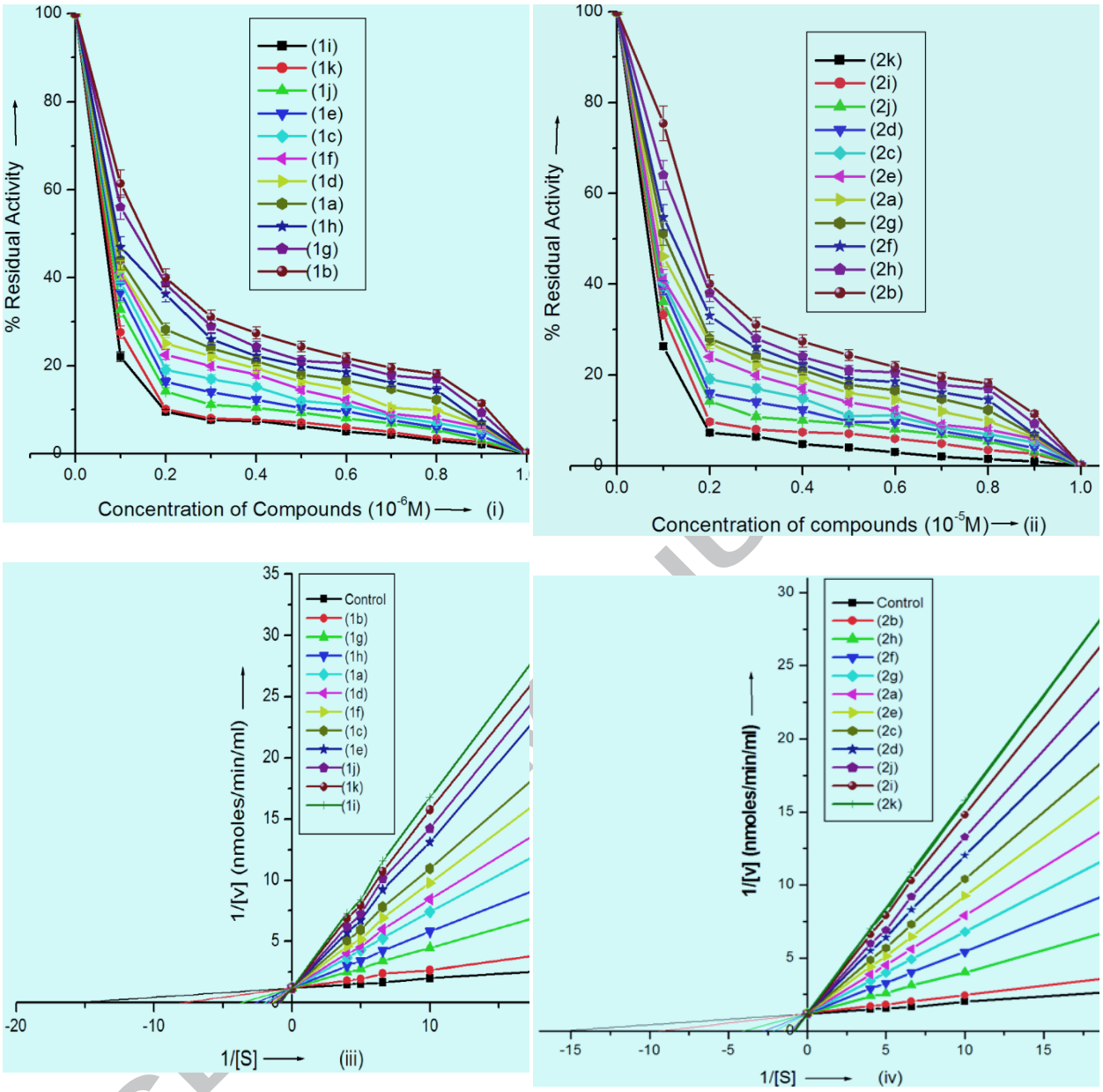

% Residual Activity
Concentration of Compounds (10-6M) (i)
(1i)
(1k)
(1j)
(1e)
(1c)
(1f)
(1d)
(1a)
(1h)
(1g)
(1b)
% Residual Activity
Concentration of compounds (10-5M) (ii)
(2k)
(2i)
(2j)
(2d)
(2c)
(2e)
(2a)
(2g)
(2f)
(2h)
(2b)
1/[v] (nmoles/min/ml)
1/[S] (iii)
Control
(1b)
(1g)
(1h)
(1a)
(1d)
(1f)
(1c)
(1e)
(1j)
(1k)
(1i)
1/[v] (nmoles/min/ml)
1/[S] (iv)
Control
(2b)
(2h)
(2f)
(2g)
(2a)
(2e)
(2c)
(2d)
(2j)
(2i)
(2k)

Figure 4

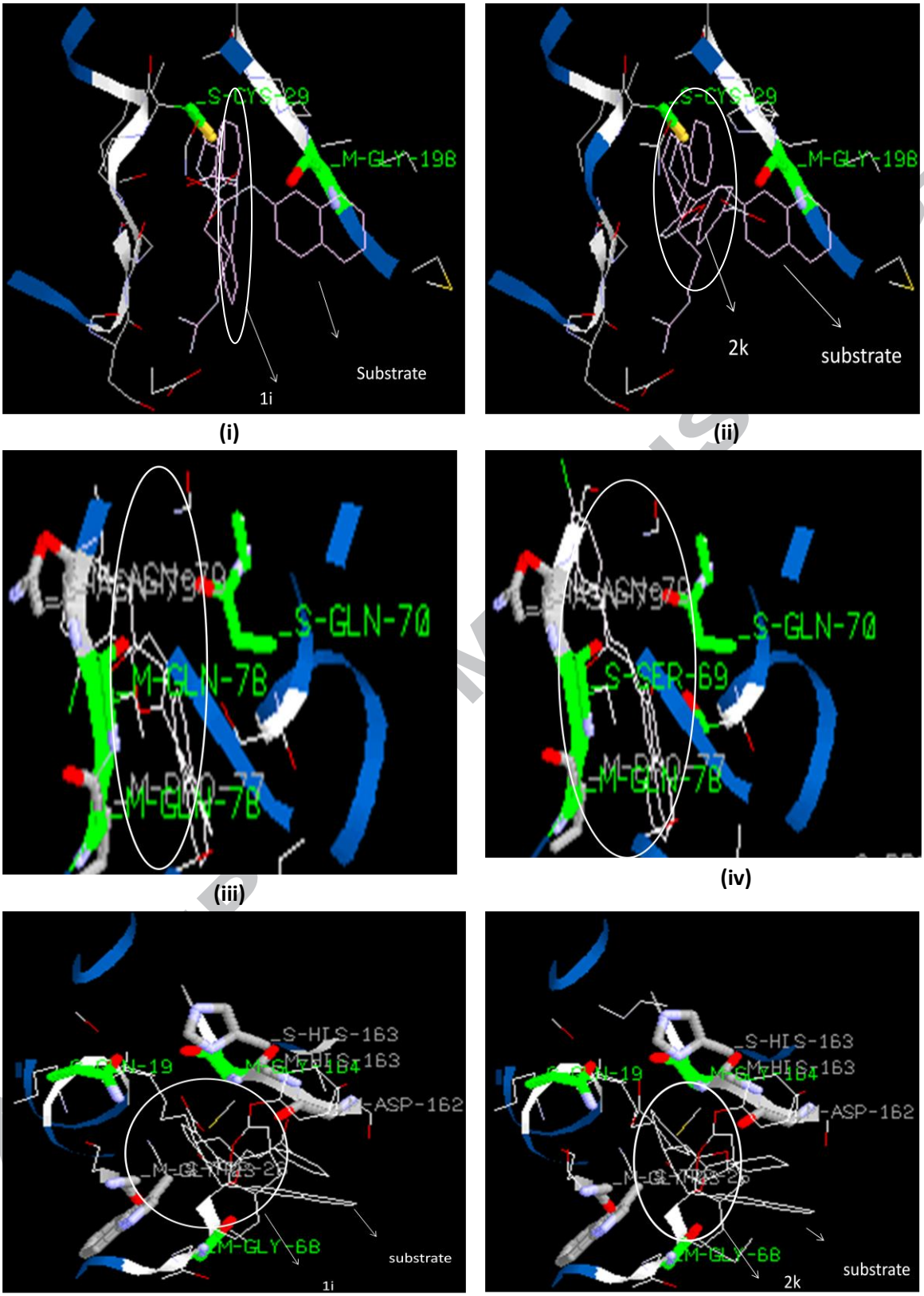

S-CYS-29
M-GLY-198
Substrate
1i
(i)
S-CYS-29
M-GLY-198
2k
substrate
(ii)
S-GLN-70
M-GLN-78
M-GLN-78
(iii)
S-GLN-70
S-SER-69
M-GLN-78
(iv)
S-HIS-163
ASP-162
M-GLY-68
substrate
1i
(v)
S-HIS-163
ASP-162
M-GLY-68
substrate
2k
(vi)

Figure 5

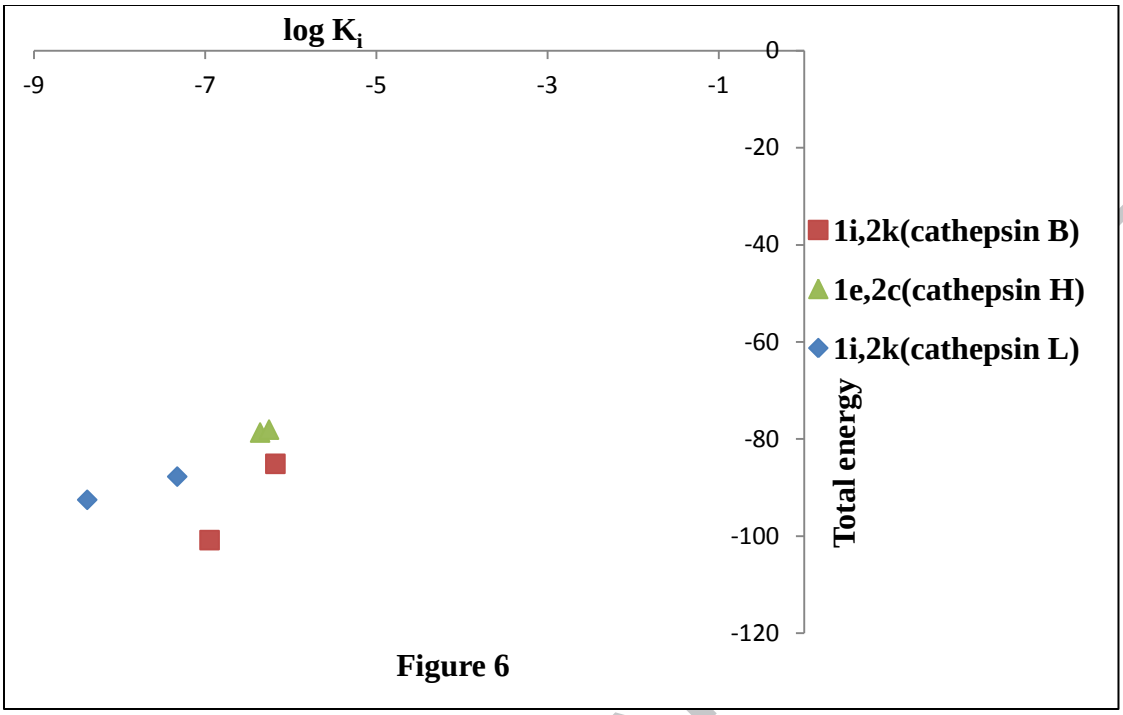
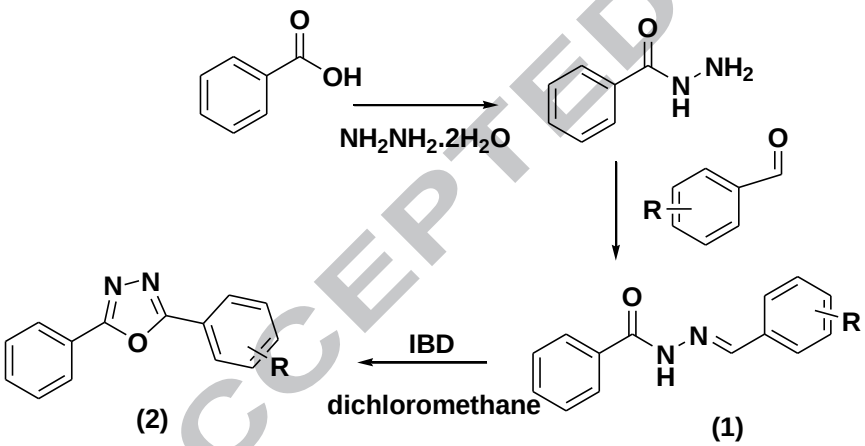


Figure 6

N-subsitutedbenzylidenebenzohydrazides and their cyclized derivatives



Scheme 1

1,2	R
a	H
b	*p*- CH_3
c	*o*-Cl
d	*m*-Cl
e	*p*-Cl
f	*o*-OCH_3
g	*m*-OCH_3
h	*p*- OCH_3
i	*O*-NO_2
j	*m*-NO_2
k	*p*-NO_2

Table-1: Enzyme inhibition studies in presence of Substituted N-subsitutedbenzylidenebenzohydrazides and their derivatives

S.No.	Compound code	(Protease Activity)		Enzyme inhibition studies		
		% residual activity at 10^{-4}M Concentration of compounds		Cathepsin B	Cathepsin H	Cathepsin L
		3 hours incubation	24 hours incubation	% residual activity at (Z)x10^{-5}M Concentration of compounds	% residual activity at (Z)x10^{-5}M Concentration of compounds	% residual activity at (Z)x10^{-6}M Concentration of compounds
	Control	100	100	100	100	100
1.	1a	50.33±5.04	69.32±4.63	47.25±4.69(0.1)	69.90±5.30	44.05±5.23(0.1)
2.	1b	89.60±8.06	99.81±8.38	70.86±6.73(0.1)	39.93±3.59	61.40±7.59(0.1)
3.	1c	46.57±4.33	64.57±4.59	46.12±4.53(0.1)	25.66±2.53	39.11±3.77(0.1)
4.	1d	47.52±4.68	72.99±5.46	47.10±4.67(0.1)	39.45±3.84	41.77±4.02(0.1)
5.	1e	44.44±3.83	60.37±4.09	42.55±4.20(0.1)	24.75±2.43	36.67±3.62(0.1)
6.	1f	54.70±5.22	77.05±5.95	49.70±5.42(0.1)	41.11±4.04	41.24±3.93(0.1)
7.	1g	68.35±6.88	79.11±6.24	56.74±5.64(0.1)	49.59±4.44	56.06±5.57(0.1)
8.	1h	50.85±4.75	72.34±4.54	48.59±4.88(0.1)	40.2±3.99	46.94±4.68(0.1)
9.	1i	13.52±1.21	36.95±1.85	23.89±1.10(0.1)	90.07±8.95	22.07±2.54(0.1)
10.	1j	24.11±2.32	48.15±2.93	33.28±3.13(0.1)	73.01±7.25	32.74±2.86(0.1)
11.	1k	23.38±1.91	41.74±2.32	28.39±2.74(0.1)	75.07±7.30	27.60±2.79(0.1)

12.	**2a**	**51.94±4.18**	**62.53±4.56**	**48.68±4.85**	**50.19±5.02**	**47.13±4.67**
13.	**2b**	**68.35±6.15**	**99.02±8.11**	**78.04±7.59**	**49.89±4.97**	**76.46±7.61**
14.	**2c**	**42.25±4.21**	**56.39±3.92**	**43.75±3.87**	**25.12±2.51**	**41.40±4.21**
15.	**2d**	**41.09±4.07**	**51.37±3.42**	**40.60±3.24**	**36.19±3.61**	**40.20±3.04**
16.	**2e**	**46.99±4.66**	**58.03±4.11**	**45.25±4.51**	**30.98±3.09**	**42.08±4.21**
17.	**2f**	**67.57±6.71**	**77.47±5.99**	**57.32±5.71**	**45.35±4.49**	**55.92±5.48**
18.	**2g**	**57.16±5.69**	**69.63±5.27**	**54.03±5.40**	**48.08±4.79**	**52.09±5.17**
19.	**2h**	**70.90±7.07**	**82.70±6.48**	**66.77±6.67**	**43.49±4.34**	**65.02±6.48**
20.	**2i**	**24.14±2.41**	**39.4±2.24**	**37.71±2.07**	**92.07±9.20**	**34.30±0.22**
21.	**2j**	**31.13±3.12**	**46.70±2.99**	**39.68±2.71**	**59.47±5.94**	**37.10±2.73**
22.	**2k**	**14.44±1.42**	**32.95±1.62**	**33.30±1.29**	**69.33±6.92**	**28.30±1.29**
	leupeptin	--	--	**1.5±0.10(0.1)**	**50.11±5.01(1)**	**90.98±0.88 (0.001)**
	Leu-CH_2-Cl	--	--	**90±8.90 (1)**	**8.11±5.01(1)**	**6.01±0.60 (10)**

The TCA soluble peptides were estimated at 630 nm using Bradford method[31]. The results are presented as % Residual Activity w.r.t. control taken as 100% where no compound was added but an eqvivalent amount of solvent was present. Where no compound was added but an equivalent amount of solvent was present. Cathepsin B, Cathepsin H and Cathepsin L activities were calculated using BANA, Leu-βNA and* Z-Phe-Arg-4mβNA *as substrates at pH 6.0, 7.0 and 6.0, respectively. The enzyme activity was determined at minimum inhibitory concentration of each compound given in parenthesis. The specific activitiy of the Cathepsin B and cathepsin H were ~11.15 nanomoles/min/mg, ~22.91 nanomoles/min/mg and 16.78 nanomoles/min/mg, respectively.

Table-2: K_i values exerted by N-subsitutedbenzylidenebenzohydrazides and their derivatives on Cathepsin B, Cathepsin H and Cathepsin L

S. No.	Code of compound	Cathepsin B K_i (10^{-7}M)	Cathepsin H K_i (10^{-7}M)	Cathepsin L K_i (10^{-9}M)
1.	1a	2.90	21.40	13.00
2.	1b	87.20	12.60	95.90
3.	1c	2.10	6.80	7.30
4.	1d	2.60	11.30	8.63
5.	1e	1.90	4.40	6.34
6.	1f	8.30	14.00	8.14
7.	1g	1.80	16.80	28.60
8.	1h	4.40	13.30	19.76
9.	1i	1.13	48.40	4.20
10.	1j	1.40	36.40	5.86
11.	1k	1.20	41.60	5.53
12.	2a	26.90	27.20	124.00
13.	2b	113.00	25.50	1357.0
14.	2c	16.17	5.60	92.10
15.	2d	12.81	11.30	70.20
16	2e	19.21	10.00	99.30
17	2f	68.50	14.70	216.00
18	2g	44.40	16.10	144.00
19	2h	92.20	13.30	337.00
20	2i	7.90	66.90	57.00
21	2j	10.00	38.50	63.40
22	2k	6.64	49.60	47.31

The data present the K_i values calculated using Lineweaver – Burk equation for competitive inhibition evidenced through Lineweaver – Burk plots drawn between 1/V and 1/S. The experiments were conducted using cathepsin B, H and L preparation having specific activities 11.15, 22.91 and 16.78 nmoles/min/mg, respectively.

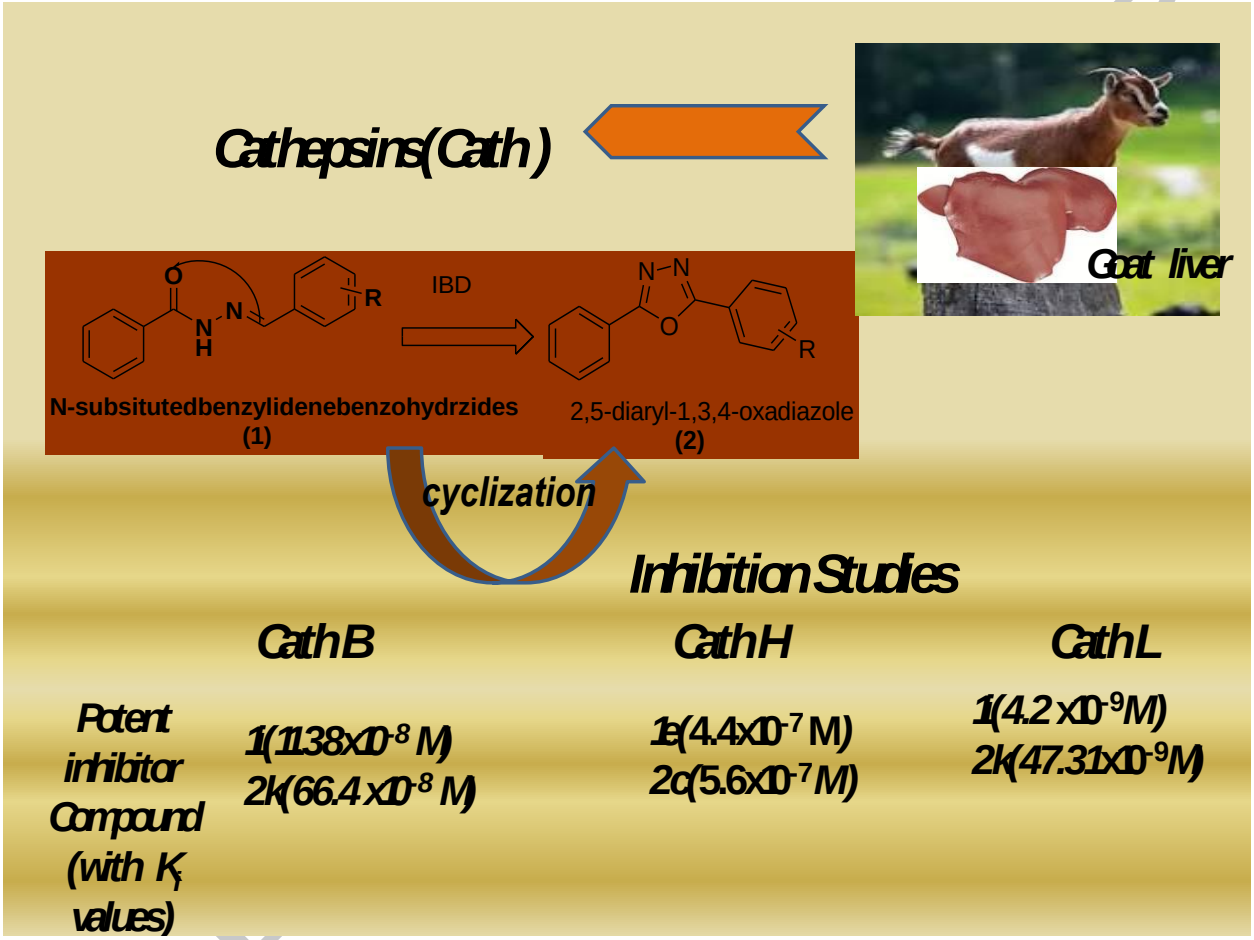
Table-3: Docking energies of Cathepsins B, H and L in presence of most inhibitory compounds and their analogues

Compound	Total Energy (kcalmol^{-1})	VDW	H Bond	Electronic
CATHEPSIN B				
BANA	**-125.56**	**-89.68**	**-32.21**	**-3.67**
Leupeptin	**-127.72**	**-96.85**	**-30.87**	**0**
1i	**-100.84**	**-69.33**	**-32.76**	**1.25**
2k	**-85.12**	**-67.02**	**-18.35**	**0.25**
CATHEPSIN H				
leuCH_2Cl	**-59.84**	**-43.88**	**-15.96**	**0**
LeuβNA	**-76.89**	**-67.30**	**-9.60**	**0**
1e	**-78.67**	**-65.27**	**-13.40**	**0**
2c	**-78.07**	**-64.07**	**-14.00**	**0**
CATHEPSIN L				
Z-Phe-Arg-4mβNA	**-127.24**	**-94.83**	**-28.90**	**-3.50**
Leupeptin	**-102.07**	**-34.83**	**-65.06**	**-2.17**
1i	**-92.53**	**-79.54**	**-13.00**	**0**
2k	**-87.75**	**-70.44**	**-17.71**	**0**

The results are one of the docking experiments run using iGemdock under drug screening settings. The active sites were retrieved from Protein Data Bank; cathepsin B as cav2IPP B_PYS.pdb[29], cathepsin H as cav8PCH H_NAG.pdb[30] and cathepsin L as cav3BC3L_CSW[31] and the ligands were loaded as MDL mol files.

Graphical Abstract

N-subsitutedbenzylidenebenzohydrazides and their cyclized derivatives i.e, 2,5-diaryl-1,3,4-oxadiazole are reported as novel inhibitors of cathepsin B, cathepsin H and cathepsin L with potency in nanomolar range.



Highlights

- The manuscript adds to the existing knowledge of some non-peptidyl inhibitors of Cathepsins B, H and L.
- The synthesized compounds exhibited differential effect on Cathepsin B, Cathepsin H and Cathepsin L.
- The present work details about the comparative account of precursors and their derivatives. The results are compared with in -silico studies.