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Complete List of Authors:	raghav, neera; kurukshetra university, chemistry Ravish, Indu; Kurukshetra university Kurukshetra, Chemistry

SAR studies of differently functionalized 4'-Phenyl chalcones based compounds as inhibitors of Cathepsins B, H and L

Indu Ravish and Neera Raghav*

Department of Chemistry, Kurukshetra University,

Kurukshetra-136119, Haryana

(INDIA)

*Corresponding author. Tel.: + 919896918277

E-mail address: nraghav.chem@gmail.com

Abstract

Conditions related to the elevated levels of Cathepsin B [3.4.22.1], Cathepsin H [3.4.22.16] and Cathepsin L [3.4.22.15] in various cancerous, rheumatoid arthritis and tissue degenerative disorders impose the designing, synthesis and evaluation of compounds as Cathepsins B, H and L inhibitors. In the present work we report the synthesis and analysis of 36 different compounds belonging to 4 related series as Cathepsins B, H and L inhibitors. After a preliminary screening of synthesized compounds as inhibitors to hydrolysis of endogenous protein substrates at pH 5.0, their inhibitory effects were evaluated on Cathepsins B, H and L. Kinetic studies were carried out to determine the inhibition constant. It was found that the compounds belonging to different classes affected the enzyme activity significantly and is greatly affected by the presence of substituents. Maximum inhibition has been caused by 2b, 2g in case of Cathepsin B and Cathepsin L whereas 4d in case of Cathepsin H where the K_i value of the order of 10^{-8} M, 10^{-9}M and 10^{-6} M respectively has been obtained. The results are correlated with the docking results.

Key words – Cathepsin B, Cathepsin H, Cathepsin L, kinetics studies, N-substituted pyrazolines.

1. Introduction

Cysteine proteases, possessing -CH_2SH group at the active site have become important therapeutic targets for the treatment of a number of diseases. Cysteine proteases of parasites like falcipain, cruzain, amoebepain[1-2] of Plasmodium falciparum. Trypanosoma cruzi and Trypanosoma brucei rhodesiense and Toxoplasma gondii, Entamoeba hystolytica[3-5] are some of the important targets in the development of anti-parasitic agents. Literature reports that inhibitors

of these cysteine proteases have great implications as anti-parasitic agents. N-acylhydrazones[6] have been found to be potent inhibitors of cruzain, a cysteine protease and the compounds showed a comparable magnitude of IC_{50} when tested along with the standard drug nifurtomox. Brak et al.[7] have reported another non-peptidic cruzain inhibitor with a triazoles moiety.

Cathepsins B, H and L, lysosomal cysteine proteases have also emerged as important targets for the development of variety of therapeutic agents because of their involvement in various diseased conditions. Cathepsin B and H have long been reported to be associated with cancer progression because of their ability to degrade extracellular matrices facilitating invasion, angiogenesis and metastasis which is evident from various clinical reports and experimental models[8-11]. Increased levels of these enzymes in tumor state have been predictive factors for cancer patients[12-13]. In addition, elevated levels of cysteine proteases have also been reported in various inflammatory conditions[14] such as periodontitis, rheumatoid arthritis, etc. An imbalance between cellular inhibitors and cathepsin's ratio has been considered as one of the reasons for the elevated levels of this enzymes[15-16]. Therefore, identification of compounds which act as potent inhibitors to cathepsins is a major thrust area in the drug development and chemotherapy. Inhibitors targeting cysteine cathepsins have been used *in-vitro* to show that these enzymes play an important role in tumor invasion[17].

Cathepsin B, a lysosomal cysteine protease possessing carboxydipeptidase and endopeptidase activities[18] responsible for intracellular protein turnover, antigen processing, proenzyme activation and hormone maturation, has received much attention for its potential role in conditions such as cancer[19], muscular dystrophy[20] and rheumatoid arthritis[21]. Cathepsin B has been found to be over expressed in different malignancies like prostrate[22-23], brain[24], lung[25] and colorectal cancers[26]. Inhibitors of cathepsin B have been found effective in reducing the invasive potential of tumor cells[27].

Cathepsin H, another equally important and unique lysosomal cysteine protease with endo aminopeptidase activity has also been found to be associated with various diseases. There is also a growing body of evidence that the level of cathepsin H or cathepsin H-like enzymes[28] is increased in disease states, including breast carcinoma[29], melanoma and tumor metastasis[30]. A cathepsin H-like enzyme has been found in cells from a human melanoma cell line. This enzyme can degrade fibrinogen and fibronectin and thus may, together with other proteases, be involved

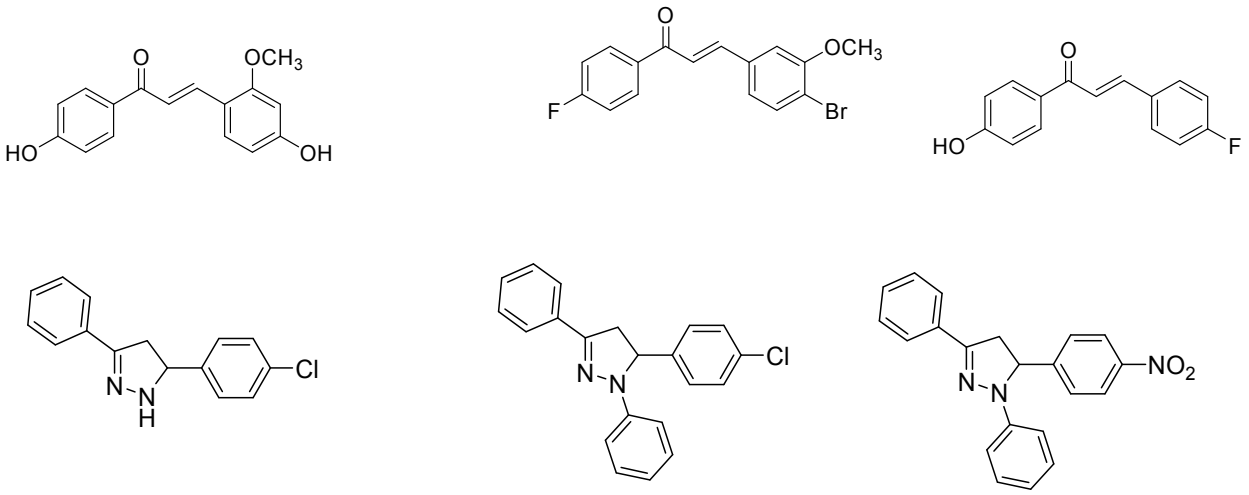
in the destruction of extracellular matrix components, leading to cancer proliferation, migration and metastasis[28]. Deletion of cathepsin H impaired angiogenic switching of the pre-malignant hyperplastic islets significantly and a reduction in the subsequent numbers of tumor has been observed[31].

Cathepsin L is other powerful lysosomal cysteine proteases to degrade many proteins and has been implicated as participating in bone collagen degradation by osteoclasts[32]. Overexpression of cathepsin L and/or abnormal activity has also been implicated in a number of disease states[33]. For example, cathepsin L is responsible for bone resorption through degradation of collagen type I; this deregulation is believed to lead to osteo- and rheumatoid arthritis[34]. In addition, several infective organisms, such as SARS and Ebola viruses, utilize cathepsin L-like proteins for replication in human cells[35].The large number of disease states associated with cathepsin L calls for an understanding of its biological function[33]. Inhibitors of cathepsin L-like cysteine proteases also have potential utility in the treatment of protozoan infections such as trypanosomiasis and malaria[3]. The involvement of cathepsins B, H and L in above mentioned diseased states emphasizes the development of potential inhibitors of these enzymes. As such, the identification of inhibitors of these cathepsins would provide valuable tools to probe the role of these enzymes in biological systems, as well as to provide potential starting points for drug discovery efforts.

A large work has been previously reported on the peptide based inhibitor molecules[36] but due to immulogical problems and gastic instability faced with the use of peptidyl molecules; in this research area, past decade has been focused on the designing and development of non-peptidyl inhibitors of cysteine proteases. Toward this endeavour we have recently reported some non-peptidyl inhibitors of cathepsins B and H. For example acyl hydrazide and triazoles[37], o-hydroxy chalcones and their cyclised derivatives flavones[38], bischalcones based quinozolines as novel inhibitors of Cathepsin B and H[39].

Chalcones chemically known as 1,3-diarylprop-2-en-1-ones, are known for their diverse pharmacological actions including antitumor and anti-inflammatory activities[40]. Literature revealed that chalcones have been found to be potential inhibitors to calpains, cathepsin B and H[41]. Pyrazolines also possess variety of biological activities such as antimicrobial, antifungal, antidepressant, anticonvulsant[42], immunosuppressive[43], antitumor, antiamoebic[44], antiarthritic, cerebroprotective effect and antibacterial[45], properties. Some of these compounds have also anti-inflammatory, anti-diabetic, anaesthetic, analgesic, tranquillizing, muscle relaxant,

psychoanaleptic, antihypertensive activities[46-48] and potent selective activity such as Nitric oxide synthase (NOS) inhibitor and Cannabinoid CB1 receptor antagonists activity[49].



Representative chalcones and pyrazolines as inhibitors of Cathepsins B, H and L

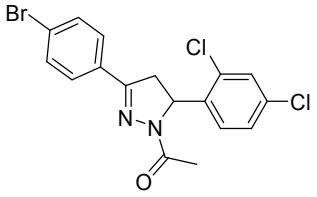
The biological activities such as antitumor and anti-inflammatory possessed by chalcones and pyrazolines and role of cathepsins B, H and L in these diseased conditions motivated us to evaluate the effect of chalcone based pyrazolines on the activity of cathepsin B and H[50] Previously we have reported various compounds as inhibitors of hydrolyzing activities of endogenous protein substrate at pH 5.0, where cysteine proteases are active. Some of these compounds which are identified as inhibitors of these proteases have been found to inhibit cathepsin B and H reversibly[37]. Systematic changes in the structure of inhibitors can lead to a potential therapeutic molecule. It is a known fact that in order to develop an appropriate therapeutic agent, number of molecules synthesized and screened are numerous.

We have previously observed that systematic alteration in the structure of chalcones led to identification of 4'-Phenyl chalcones as activators of one enzyme i.e. acid phosphatase and inhibitors of another enzyme i.e. alkaline phosphatase known to perform similar physiological function[51]. Their simpler structural analogs either had no effect[52-53]or lesser effect on these enzymes[54].

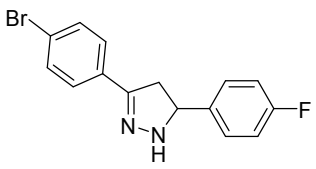
The introduction of phenyl group at 4'-position of chalcones led to such change in the structure that appreciable differential effect of the compounds was observed on acid and alkaline phosphatases. With this background we have observed the effect of 4'-phenyl chalcones and their pyrazoline derivatives on the activities on Cathepsins B, H and L, two significant enzymes emerged as potential drug targets for melanoma therapy.

2. Result and Discussion

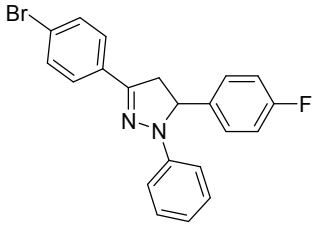
Chalcones and their cyclized derivatives, pyrazolines have a wide potential role as antitumor agents and anti-inflammatory agents. Different pyrazolines (1a, 1b and 1c) have been reported to possess significantly high cytotoxic activity against MCF-7, HCT 116 cell lines[55]. Manna et al has also reported Anti cancer activity of some pyrazolines[56]. 3,5-diaryl pyrazolines (3a) and its N^1-acetylated derivatives (3b) showed potent and selective activity in the NCI 60 human cancer lines panel[57]. Chalcones and simple pyrazolines having potential anticancer activities ignited a thought to evaluate these designed molecules as inhibitors to cysteine proteases, cathepsins B and H, because of over expression and diminished level of inhibitors of these cathepsins in various cancerous conditions[58-62].



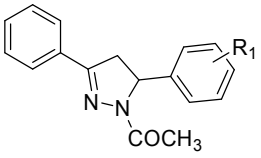
1a



1b

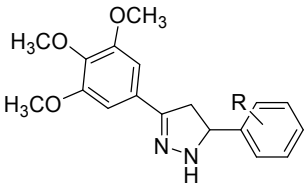


1c



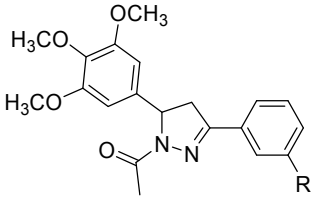
R1= H, 2-OCH_3

2



R= 3-OH, 3-OMe, 4-OMe, 4-Me_2N, 4-Et_2N

3a



R= Br

3b

Preliminary study of some chalcones hydrazones based pyrazolines on the inhibition of endogenous protein substrates have also been reported[63-64]. Some of these pyrazolines inhibited cathepsins B and H appreciably[50]. Based on these observations we in the present work have explored 4'-phenyl substituted chalcones and their pyrazolines derivatives as inhibitors of

proteolytic activity on endogenous protein substrates followed by the detailed enzyme inhibition studies on cathepsins B and H. In addition effect of synthesized compounds was also screened on cathepsin L. To accomplish the project first of all the pyrazolines were synthesized and characterised from respective chalcones (scheme 1) as discussed below.

In the first step, the 4'-phenylchalcones (1a-1i) were obtained by solvent free method between 4'-phenylacetophenone and aromatic aldehydes under basic conditions. In the second step synthesized chalcones on reaction with hydrazine hydrate/ formic acid, phenyl hydrazine hydrate and hydrazine hydrate in alcohol resulted in the formation of N-formylpyrazolines (2a-2i), N-phenylpyrazolines (3a-3i) and pyrazolines (4a-4i) respectively.

The structures of the synthesized compounds were elucidated on the basis of m. pt., ^{1}H NMR and IR spectra. The ring closure is demonstrated by the IR spectra of 2a-2i, 3a-3i and 4a-4i compounds, which showed the characteristic band for NH at 3100-3300cm^{-1} and a band at 1590-1605 cm^{-1} corresponding to C=N stretching. The ^{1}H NMR spectra showed an ABX spin system caused by the coupling of three hydrogen atoms attached to the C-4 and C-5 of the heterocyclic ring. Methylene protons of heterocyclic ring appeared as two double doublets, one at δ = 3.75–3.82 ppm (H_{4a}, J = 17, 12.3 Hz) and the other at 3.05–3.12 ppm (H_{4b}, J = 17, 7.2 Hz). The existence of these double doublets clearly indicates the magnetic non equivalence of these two protons for being adjacent to a chiral center, whose hydrogen have chemical shift at 5.2 ppm (H_{5x}, J = 12.2, 7.2 Hz). These spectral data unequivocally proved the 2-pyrazoline structure. The ^{1}H NMR spectrum of compounds in $CDCl_3$ shows signals at 8.8-9.0 and 8.0-8.85 ppm assignable to CHO protons in 2a-2i and NH protons in 4a-4i respectively. Multiplet signals observed in the 6.6-8.5 ppm region are assigned to protons of phenyl rings. The sharp singlet observed at 3.3- 3.8 and 3.33-3.90 ppm is assigned to diaminomethyl and methoxy protons.

2.1.Biological activity

In-vitro endogenous proteolysis studies at pH 5.0 in goat liver homogenate were carried out for 3 h and 24 h reaction time for preliminary exploration of inhibition by these compounds. It was found that the compounds 1b, 1d, 1e, 2h, 3a, 3f , 3g and 4g inhibited proteolytic activity to 100% at 1 X 10^{-4}M final concentration. A number of cysteine proteases as responsible for this proteolytic activity therefore it is neither feasible nor proper to explore any structural activity relationship between the substituent and enzyme activity at the stage. The only conclusion drawn

from these results is that the inhibition caused by the designed compounds is of reversible type because the inhibition is more at 3h incubation time and less at 24h incubation time. It can be interpreted that either the inhibition is reversed with time or some proteases which are insensitive toward the designed compounds have become active in due course of time. It was evident from experimental data that the compounds inhibited proteolysis of endogenous substrates at this pH. In literature it was found[53] that most of proteolytic activity at this pH is due to cysteine proteases therefore it was thought proper to evaluate their inhibitory effect on cathepsins B, H and L important cysteine proteases. Table-1 presents the results of endogenous proteolytic activity in presence of 4'-phenylchalcones and their pyrazolines derivatives.

2.2. Enzyme Inhibition studies

Inhibitory effects of the synthesized compounds were studied on cathepsins B, H and L. Each derivative was examined at the concentration range of 10^{-4}-10^{-9} M. 4'-phenylchalcones (1a-1i), N- formyl substituted pyrazolines derivatives (2a-2i), N- phenyl substituted pyrazolines derivatives (3a-3i) and N- hydro- substituted pyrazolines derivatives (4a-4i) were tested, all groups showed satisfactory inhibitory effect but the compounds (2a-2i) showed maximum inhibitory effect in case of cathepsins B and L, however (4a-4i) showed maximum inhibitory effect on cathepsin H.

Effect of 4'-Phenyl chalcones (1a-1i) on the activity of cathepsins B, H and L

Figures 1, 2 and 3 show the relationship between the enzyme activity and concentration of substituted chalcones (1a-1i) for cathepsins B, H and L respectively. It has been found that enzymes activity is inhibited and is affected by the substituents present in chalcones. Among the various compounds tested, at 1 X 10^{-5}M concentration 1b and 1d exhibited maximum inhibitory effect in case of cathepsin B and cathepsin H respectively and in case of Cathepsin L 1b shows maximum inhibition at 10^{-9}M.

Effect of N-formyl-2-pyrazolines on the activity of cathepsins B, H and L

On evaluting the inhibitory effect of N-formyl-2-pyrazolines it was found that electron withdrawing substituents exhibited greater inhiitory effect as compared to electron releasing substituents. Activity of cathepsin B was reduced to 54% at 1 X 10^{-7} M and activity of cathepsin L was reduced to 35% for 2b, revealing these compounds as most potent inhibitor among all tested compounds (Table 2). In case of cathepsin H 2d shows maximum inhibition i.e. 32% at 1 X 10^{-5} M.

Effect of N-Phenyl-2-pyrazolines on the activity of cathepsins B, H and L

The experiments were designed to study the effect of N-phenyl-2-pyrazolines on cysteine proteases cathepsins B H and L. It was found that 70% inhibition for cathepsin B activity has been achieved at 1.0 X 10^{-5}M concentration in case of 3b and 58 % in case of 3g. Cathepsin H was maximally inhibited by 3d and 3e i.e. 35% at 1 X 10^{-5}M. Cathepsin L was inhibited by 3b upto 50% at 1 X 10^{-6}M.

Effect of 3-(4-biphenyl)-5-substituted phenyl-2-pyrazolines on cathepsins B, H and L

In case of 3-(4-biphenyl)-5-substituted phenyl-2-pyrazolines a definite pattern of inhibition could not be established. Almost all substituents showed 80-85% inhibition at 1X 10^{-6}M. But in case of cathepsin L 4b showed maximum inhibition i.e. 55% at 1 X 10^{-8}M.

Enzyme kinetic studies

All the synthesized compounds after establishing the inhibitory effect were screened for evaluating the inhbition type and to determine the inhibition constant K_i value. It was found that all the compounds inhibited cathepsin B and Cathepsin L in a competitive manner whereas cathepsin H was inhibited in a non competitive manner except N-phenyl pyrazolines in which competitive inhibition was found (figure 4, 5 & 6). The K_i values have been summarised in table 3.

2.3. SAR studies

Literature reports that cysteine proteases including cathepsins B, H and L play important role in cancer, inflammation and tissue degenerative processes where proteolysis in one or another aspect is an important phase for the spread of these diseases. Targeting these enzymes is therefore one of strategies in the development of new chemotherapeutic agents. *In-vitro* inhibition studies clearly suggest that for Cathepsin B the order of inhibition caused by the different series of compounds was 2a-2i>4a-4i> 1a-1i> 3a-3i, for Cathepsin L 2a-2i>1a-1i>4a-4i >3a-3i whereas Cathepsin H it can be observed that order of inhibition was 1a-1i> 4a-4i >2a-2i> 3a-3i as observed from K_i values. In these enzymes synthesized compounds show differential preference. In Cathepsins B and L the formyl group probably plays an important role and interacts with the nucleophilc thiol group present at the active site. Cathepsin L is sensitive toward α-β unsaturated carbonyl compounds like Cathepsin H. Cysteine of cathepsin H does not seems to interact with this group so effectively. In Cathepsin H the unsaturated carbonyl group seems to play an important role in the binding and inhibition. N-Phenylpyrazolines seems to play

lesser inhibition to both the enzymes as the K_i is in sub micromolar Similar results have been previously obtained in case of 1,3,5-triphenylpyrazolines and related structural analog[48] where phenyl substituent pyrazolines were least inhibitory.

To further explore the structure activity relationship substitution pattern was carefully altered in the designed compounds keeping in mind their differential electronic effects. It can be further observed that for Cathepsin B and Cathepsin L with in the series (1a-1i) the compound 1b greatly influenced enzyme activity with K_i values 7.0 X 10^{-6} and 0.54 X 10^{-9}M, respectively and among pyrazolines (2a-2i) most inhibitory compounds were 2b with K_i values 7.2 X 10^{-8} and 0.26 X 10^{-9}M respectively.

In all the series the electron withdrawing $–NO_2$ group was found to be most inhibitory for Cathepsin B & L. Similar results have previously been reported from our lab for o-hydroxy chalcones and their cyclized derivatives[38] where the K_i values for the most inhibitory compound has been found to be 6.18 X 10^{-8}M. In case of Cathepsin H, we evaluated that halogen substituted pyrazolines were most inhibitory. The results are similar to those reported previously[50] where halogen substituted pyrazolines inhibited Cathepsin H appreciably. However, in series 1 almost similar inhibition was observed invariably depanding on the substituents.

2.4. Molecular Docking

The docking approach used in this study was aimed at identification of compounds that selectively bind to the active site of cathepsin B and modulate the enzymatic activity. Individual binding poses of each compound was assessed and their interactions in the active site of the enzyme were analysed. Docking alterations are based on the interaction force field scoring that includes Vander Waals and electrostatic interactions between active site and ligand. Table 3 represents the data of docking studies of 4'-phenyl chalcones (1a-1i), pyrazolines derivatives (2a-2i), (3a-3i) and (4a-4i) with cathepsin B active site (available through RCSB Protein Data Bank, PDB entry cav2IPP B_PYS.pdb). The binding energy of 4'-phenyl chalcones (1a-1i), pyrazolines derivatives (2a-2i), (3a-3i) and (4a-4i) vary in the range -80.44 to -91.82, -86.39 to -95.35, -96.78 to –101.15 and -94.45 to -98.95 respectively and the binding energies of most inhibitory compounds in each series 1b, 2b, 3b, and 4b show the decreasing in total energy as -83.30, -89.11, -99.42 and -97.74 kcal/mol. On the basis of interaction data of docking experiments, it was observed that all the compounds showed the lesser interaction than the reference leupeptin, a peptidyl inhibitor. The maximum interaction is shown by BANA with a

score of -124.91. Decrease in total energy for leupeptin – cathepsin B has come out be -119.17 of which the contribution of Vander Waal interaction are more with the score of -91.77 as compared to H-bond with a score of -28.00. Leupeptin –cathepsin B binding energy is due to peptide protein interaction. Leupeptin is peptidyl in nature and therefore being a flexible molecule binds effectively with the enzyme active site resulting in higher binding energy. iGemDOCK provide algorithms for flexible docking approach for both ligands and proteins[66] therefore flexible ligands like leupeptin will show a larger decrease in total energy as compared to the molecules under study. These compounds under consideration are smaller in structure and possess lesser flexibility compared to leupeptin. Therefore, the binding energy of title compounds is less than leupeptin, but the in-vitro analysis reveals that the compounds are good inhibitors to cathepsin B. Like leupeptin, which has been reported as competitive inhibitor to cathepsin B the designed compounds show competitive inhibiton.

Fig 5(a, b, c, d) shows the result of the best docking pose of 1b, 2b, 3b and 4b respectively into the active site of Cathepsin B. It can be observed that Cys-29, Trp-30 and Gly-198 have been found to interact with the substrate as well as the compound under consideration. This supports the results obtained during the in-vitro studies because the compounds are evaluated as competitive inhibitors and competes with the binding site of the substrate. The proposed mechanism is shown in scheme 2. The results clearly indicate the significance of the in-vitro inhibition studies.

However in cathepsin H the decrease in total energy for the reference inhibitors 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[65-66] 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 only of – 59.99 of which –43.49 is the Vander Waal interaction and – 16.50 is due to H-bonds.

As listed in table 4 all the designed compounds have been found to show more decrease in ligand-cathepsin H interaction energy than leu-CH_2Cl-cathepsin H. From docking studies the compound 1a was evaluated more inhibitory to cathepsin H in 4’-phenylchalcones show a total decrease in energy as – 81.56 of which – 72.77 is assigned to Vander Waal interactions whereas – 8.79 is of H- bond. The compound 2d was supported to be better inhibitor to cathepsin H in N-

formylpyrazolines because it show a total decrease in energy as –81.94 of which –68.45 is assigned to Vander Waal interactions whereas –13.48 is of H- bond. As evidenced by docking results, among the phenyl pyrazolines, 3d found more inhibitory to cathepsin H in N-phenylpyrazolines show a total decrease in energy as –85.26 of which –81.65 is assigned to Vander Waal interactions whereas –3.61 is of H- bond. However in N- pyrazolines 4d, was evaluated as best inhibitor to cathepsin H showing a total decrease in energy as –83.12 of which –72.92 is assigned to Vander Waal interactions whereas –10.19 is of H- bond.

Figure 6 (a, b, c and d)show the docked poses of the most inhibitory compounds 1d, 2d, 3d and 4d into the aminoacyl binding site of Cathepsin H, respectively. As we can see that the designed compound and the substrate leu-βNA binds at two different sites. Gln-73 and Ser-69 has been found to interact with leu-βNA H_a-H bonds. However Gln-78, Asn-116, Gln-23 interact with the most inhibitory compound in each series. The docked poses and interaction clearly suggest a non-competitive type of inhibition exerted by the compounds on Cathepsin H. *In-vitro* inhibition studies also suggest that expect N-phenylpyrazolines all the series exerted a non-competitive inhibition on Cathepsin H.

In case of Cathepsin L the range of the binding energy of 4'-phenyl chalcones (1a-1i), pyrazolines derivatives (2a-2i), (3a-3i) and (4a-4i) vary from -88.05 to -101.48, -91.34 to -106.21, -99.83 to -115.61 and -94.09 to -106.17 respectively and the most inhibitory compounds in each series 1b, 2b, 3b, and 4b show the decreasing in total energy as –88.05, -105.4, -105.55 and -103.14 kcal/mol. When these docking energies were compared with substrate Z-Phe-Arg-4mβNA and peptidyl inhibitor leupeptin it was found these are very low. For Z-Phe-Arg-4mβNA it was -130.74 kcal/mol and for leupeptin it cause out to be -116.8 kcal/mol. Leupeptin possessing a peptidyl chain exhibits a larger decrease in energy however the extent of inhibition caused by 1b and 2b is of order of 10^{-9}M which is more than leupeptin where the K_i value is reported to be 1.5 X 10^{-9} M[69].

Fig 9(a, b, c, d) shows the result of the best docking pose of 1b, 2b, 3b and 4b respectively into the active site of Cathepsin L. The amino acids Gln-19, leu-69, Asp-162, His-163 have been found to intract with the compounds as well as with substrate Z-Phe-Arg-4mβNA thus indicating a competitive type of inhibition.

Fig 10 (a, b, c) represents the correlation between total energy v/s log K_i of designed compounds for cathepsin B, H and L respectively. It can be observed from fig 10a, that most effective binding of compounds 2b followed by 2g causing maximum inhibitory potential is well correlated in this graph. The series N-formylpyrazolines (2a-2i) are exhibiting maximum inhibitory power followed by N-pyrazolines (4a-4i); 4'-phenyl chalcones (1a-1i) and N-phenylpyrazolines (3a-3i) in that order. The correlation plot for Cathepsin H, 10b, depict the chalcones as most inhibitory series followed by N-pyrazolines, N-formylpyrazolines and N-phenylpyrazolines. The plot of binding energy v/s inhibitory constant for Cathepsin L (10c) details that N-formylpyrazolines are most inhibitory followed by 4'-phenyl chalcones, N-pyrazolines and N-phenylpyrazolines.

In the present work, we have used decrease in total energy of enzyme –legand complex as a measure of 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[70-71].

It may be worth mentioning here that the in silico studies have been used only as supporting tool for enzyme inhibition studies therefore the advanced details of docking studies is not included here. The in –silico predictable behaviour of enzyme-ligand interaction can give an idea about the interaction between these two but cannot substitute the in-solution studies.

In the present study we found that differential effect has been exerted by the related class of compounds on Cathepsin B and H activites. The electron withdrawing $–NO_2$ substitution proved to be a good inhibitor of Cathepsins B & L and chloro and fluoro substituted compounds was more inhibitory to H.

3. Experimental Section

The substrate α-N-benzoyl-D, L-arginine -2-naphthylamide (BANA) / Z-Arg-Arg-4m*β*NA, LeuβNA and Z-Phe-Arg-4mβNA were purchased from Bachem Feinchemikalein AG (Switzerland). Fast Garnet GBC was purchased from Sigma USA. The chromatographic media Sephadex G- 100, CM-Sephadex C-50, DEAE Sephadex, A-50 were supplied by Pharmacia Fine Chemicals, Uppsala (Sweden). All the solutions used were prepared fresh in glass-distilled conductivity water. The source of enzymes were fresh goat liver obtained from a local slaughterhouse. 4'-Phenyl acetophenone and aldehydes were purchased from Himedia, Bombay.

Elisa plate reader was used for measuring absorbance in the visible range. IR spectra were recorded on Horizon 300 MHz spectrometer. ^{1}H NMRspectra were recorded on Bruker 300 MHz instrument. The chemical shifts are expressed in ppm units from an internal TMS standard. Refrigerated ultracentrifuge Remi C-24BL was used for centrifugation purpose under cold conditions.

Melting points were taken in open capillaries and are uncorrected. The progress of the reactions was monitored on silica gel G plates using iodine vapor as visualizing agent.

3.1.Synthesis

3.1.1. Procedure for synthesis of 3-substitutedphenyl-1-(4-biphenyl) propen-1-ones (1a-1i): A series of 1-biphenyl-3-(substituted phenyl)-2-propen-1- ones was synthesized by the grinding of substituted aldehydes (0.01 mole) with 4-phenylacetophenone (0.01 mole) in presence of potassium hydroxide (0.01 mole) with a mortar and pestle. The progress of reaction and the purity of the products were confirmed through TLC. The mixture was poured in ice water, filtered, washed with ethanol, dried and recrystallised with ethanol, yield: 85-90% depending on substitution.

3.1.2. General procedure for synthesis of N-formyl-3-(4-biphenyl)-5-substituted phenyl-2-pyrazolines (2a-2i): Compounds 1a–1i (0.001mole), hydrazine hydrate (0.001 mole) and 15-20 ml of formic acid in ethanol (25 ml). It was then refluxed at 60-70˚C for 12-14 h. The progress of reaction was monitored with the help of TLC. After the completion of reaction, mixture was allowed to cool at room temperature and was kept in refrigerator for 4–5 h. Fine crystals thus separated out were filtered, washed with cold ethanol, dried and recrystallised with ethanol, yield: 75–85% depending on substitution.

3.1.3. General procedure for synthesis of N-phenyl-3-(4-biphenyl)-5-substituted phenyl-2-pyrazolines (3a-3i): Compounds 1a–1i (0.001 mol), phenyl hydrazine hydrate (0.001 mol) and add acetic acid in catalytic amount in ethanol (25 ml) was refluxed at 60-70˚C for 6-8 h. The reaction progress was monitored using TLC. The reaction mixture was allowed to cool at room temperature. Fine crystals thus separated out were filtered, washed with cold ethanol, dried and recrystallised with ethanol, yield: 87–95% depending on substitution.

3.1.4. General procedure for synthesis of 3-(4-biphenyl)-5-substituted phenyl-2-pyrazolines (4a-4i): Compounds 1a–1i (0.001 mol), hydrazine hydrate (0.001 mol) in ethanol (25 ml) was refluxed at 60-70˚C for 6-8 h. The reaction progress was monitored with the help of TLC. Once thereaction was complete the reaction mixture was allowed to cool at room temperature. Fine crystals thus separated out were filtered, washed with cold ethanol, dried and recrystallised with ethanol, yield: 83–90% depending on substitution.

3.2.Proteolytic studies

3.2.1. Preparation of liver homogenate:

Goat liver was purchased freshly from the local slaughter house. The fresh goat liver was first washed with cold isotonic saline solution. The tissue was then homogenized in 0.1M sodium acetate buffer pH 5.5 containing 0.2M NaCl in a mixer-cum-blender to obtain 10% (w/v) homogenate. It was then stored at 4 ºC.

3.2.2. Assay for proteolytic activity:

The proteolytic studies on endogenous protein substrate were carried out at pH 5.0 at 37^{o}C using 0.1 M acetate buffer as the incubation medium. The homogenate was mixed with buffer and was incubated at 37^{o}C for 3 h. 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[72]. The experiment was conducted in triplicate and the results are presented in Table 1.

3.3.Purification of goat liver cathepsin B, **cathepsin H and cathepsin L**

All the purification steps were carried out at 4°C. Cathepsin B, H[50] and L[69] were isolated, separated and purified from goat liver using the following procedure. Goat liver acetone powder was homogenized in cold 0.1 M sodium acetate buffer pH 5.5 containing 0.2 M NaCl and 1mM EDTA followed by Acid-autolysis at pH 4·0 and 30-80% ammonium sulphate fractionation, Sephadex G-100 column chromatography and finally cation-and anion exchange chromatography on CMSephadex C-50 and DEAE-Sephadex A-50. The specific activities of the cathepsin B, cathepsin H and Cathepsin L were 13.46 nmoles/min/mg, 25.56 nmoles/min/mg and 15.42μmoles/min/mg respectively.

3.4.Enzyme inhibition studies

The activities of cathepsin B was estimated at varying concentrations of synthesized 4'-phenylchalcones (1), N-formylpyrazolines (2) and N-phenylpyrazolines (3), pyrazolines (4). First of all, enzyme was equilibrated in 0.1 M 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. After incubation time of 30 min. residual enzyme activity was estimated by the usual enzyme assay[73] at pH 6.0 using α-N-benzoyl-D, L-arginine-2-naphthylamide (BANA) as a substrate.

Similarly for Cathepsin H, the activities were estimated at varying concentrations of synthesized compounds using leuβNA as substrate[67].

The activities for Cathepsin L were estimated at varying concentrations of synthesized compounds using Z-Phe-Arg-4mβNA as substrate[74]. The results are shown in table-1 and figure 1, 2 and 3.

3.5.Determination of K_i values

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. For that, enzyme activity was evaluated at different substrate concentration in presence and absence of a fixed concentration of inhibitor. The enzyme concentration was kept constant in all the experiments. Line-weaver Burk plot was drawn in 1/S and 1/V in presence and absence of inhibitor (figures 4, 5 and 6).

3.6.Drug modeling studies

All docking studies were performed using iGemdock. For these studies small molecular weight ligands were prepared and enzyme structure active site was retrieved from the Protein Data Bank. The structures were prepared using marvin sketch and were saved as MDL Mol File. The structure of cathepsins B, H and L were retrieved from Protein Data Bank (http://www.rcsb.org/) as cav2IPP B_PYS.pdb[75], cav8 PCH-NABpdb[76] and cav3BC3L_CSW[77] respectively. After loading the prepared ligands and the binding site docking experiments were run at drug screening Settings and the results are presented in table 4, 5 & 6 and figures 7, 8 & 9.

4. Conclusions

In the present work we synthesized 4'-phenyl chalcones and their pyrazolines derivatives and screened their effect on cathepsins B, H and L. The synthesized compounds were shown to be potent inhibitors of cathepsins B, H and L. The kinetic studies exposed that all compounds showed reversible competitive inhibition on cathepsin B and L with affinities in subnanomolar range. Except N-phenylpyrazolines all compounds showed reversible non- competitive inhibition on cathepsin H with affinities in the micromolar range. Cathepsin B is more susceptible as compared to cathepsin H. The maximum inhibition was shown by 1-Formyl-3-(4-biphenyl-5-(4'-nitrophenyl)-2-pyrazoline, 2b in case of cathepsins B & L and for Cathepsin H, 2d was found most inhibitory. The results presented in this study can add to the existing knowledge of Cathepsins B, H and L inhibitors not reported earlier.

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The authors have declared no conflict of interest.

Supporting Information

Tables and characterization of synthesized compounds can be found in the supplementary data.

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Figure Captions

Figure 1. The results are depicted as % residual activities of cathepsin B in presence of different concentrations (0.1, 0.2, 0.3, 0.50, 0.75 and 1.0 X 10^{-5} M) of various chalcones (1a-1i), (0.1, 0.2, 0.3, 0.50, 0.75 and 1.0 X 10^{-6} M) of N-formylpyrazolines (2a-2i), (0.1, 0.2, 0.3, 0.50, 0.75 and 1.0 X 10^{-4} M) of N-phenylpyrazolines (3a-3i) and (0.1, 0.2, 0.3, 0.50, 0.75 and 1.0 X 10^{-5} M) of diarylpyrazolines (4a-4i) after an interaction time of 30 min. The experiments were conducted in triplicate and were calculated w.r.t. the control having no compound but an equivalent amount of solvent was added.

Figure 2. The results are depicted as % residual activities of cathepsin H in presence of different concentrations (0.1, 0.2, 0.3, 0.50, 0.75 and 1.0 X 10^{-5} M) of various chalcones (1a-1i), (0.1, 0.2, 0.3, 0.50, 0.75 and 1.0 X 10^{-4} M) of N-formylpyrazolines (2a-2i), (0.1, 0.2, 0.3, 0.50, 0.75 and 1.0 X 10^{-4} M) of N-phenylpyrazolines (3a-3i) and (0.1, 0.2, 0.3, 0.50, 0.75 and 1.0 X 10^{-6} M) of diarylpyrazolines (4a-4i) after an interaction time of 30 min. The experiments were conducted in triplicate and were calculated w.r.t. the control having no compound but an equivalent amount of solvent was added.

Figure 3. The results are depicted as % residual activities of cathepsin L in presence of different concentrations (0.1, 0.2, 0.3, 0.50, 0.75 and 1.0 X 10^{-5} M) of various chalcones (1a-1i), (0.1, 0.2, 0.3, 0.50, 0.75 and 1.0 X 10^{-4} M) of N-formylpyrazolines (2a-2i), (0.1, 0.2, 0.3, 0.50, 0.75 and 1.0 X 10^{-4} M) of N-phenylpyrazolines (3a-3i) and (0.1, 0.2, 0.3, 0.50, 0.75 and 1.0 X 10^{-6} M) of diarylpyrazolines (4a-4i) after an interaction time of 30 min. The experiments were conducted in triplicate and were calculated w.r.t. the control having no compound but an equivalent amount of solvent was added

Figure 4 Effect of compounds 1b, 2b, 3b, 4b on cathepsin B activity respectively. The experiment was done on three different concentrations in triplicate. All compounds showed competitive inhibition.

Figure 5 Effect of compounds 1d, 2d, 3d, 4d on cathepsin H activity respectively. The experiment was done on three different concentrations in triplicate. 3d showed competitive inhibition and other compounds showed non-competitive inhibition.

Figure 6 Effect of compounds 1b, 2b, 3b, 4b on cathepsin L activity respectively. The experiment was done on three different concentrations in triplicate. All compounds showed competitive inhibition.

Figure 7 Docking results showing the alignment of most inhibitory 1b, 2b, 3b and 4b in the active site of cathepsin B (cav2IPP B_PYS.pdb) along with the substrate BANA in 7a, 7b, 7c and 7d respectively. The binding site radius is of 8Å. The results are of docking at drug screening settings. The amino acids Cys-29, Gly-198 and Trp-30 can be observed interacting with inhibitor and amide bond of the substrate BANA through H-bonds as shown in green. The hydrophobic interactions are shown in grey.

Figure 8 Docking results showing the alignment of most inhibitory 1d, 2d, 3d and 4d in the active site of cathepsin H (cav8PCH H_NAG.pdb) along with the substrate leuβNA in 8a, 8b, 8c and 8d respectively. The binding site radius is of 8Å. The results are of docking at drug screening settings. Inhibitor and substrate binds at two different sites. The amino acids Ser-69 and Glu-73 can be observed interacting with the substrate Leu-β-NA and Gln-70, Gln-78, Gln-86 and Asn-112 interact with the inhibitor through H-bonds as shown in green. The hydrophobic interactions are shown in grey.

Figure 9 Docking results showing the alignment of most inhibitory 1b, 2b, 3b and 4b in the active site of cathepsin L (cav3BC3 L_CSW.pdb) along with the substrate Z-Phe-Arg-4mβNA in 9a, 9b, 9c and 9d respectively. The binding site radius is of 8Å. The results are of docking at drug screening settings. Only hydrophobic interactions are visible for except for compound 4b, where Gln-19 interacts through H-bond with the substrate as well as with the compound. Hydrobhobic interactions are exerted by Gln-19, Gln-23, Trp-26, Gly-68, Leu-69, Asp-162, His-163 and Gly-164.

Figure 10 Correlation plot between binding energies and log K_i values for cathepsin B, 10a; cathepsin H, 10b; cathepsin L, 10c. □, △, X and ◇ represents 1a-1i, 2a-2i, 3a-3i and 4a-4i, respectively.

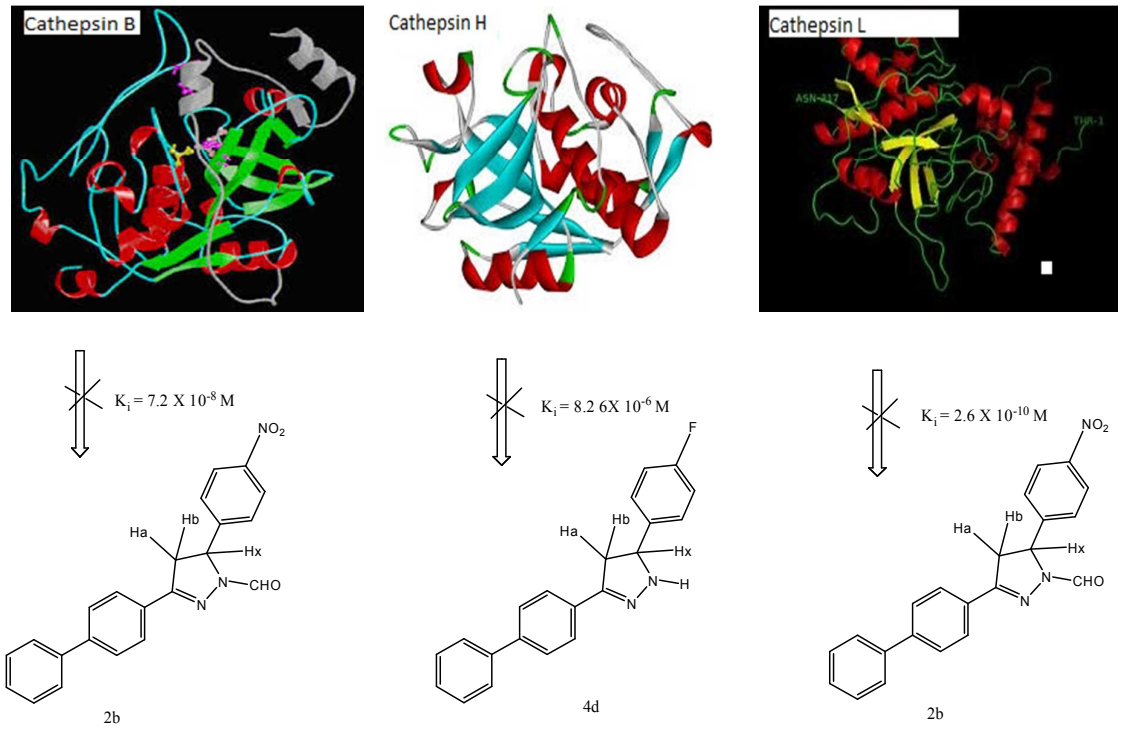
Scheme

Scheme 1: Synthesis of chalcones and their cyclized derivatives.

Scheme 2: Proposed mechanism of inhibition for chalcones.

Graphical Abstract

4’-Phenylchalcones and their cyclised derivatives as novel inhibitors of Cathepsin B, H and L, potential anticancer agents.



Best inhibitors of Cathepsins B, H and L

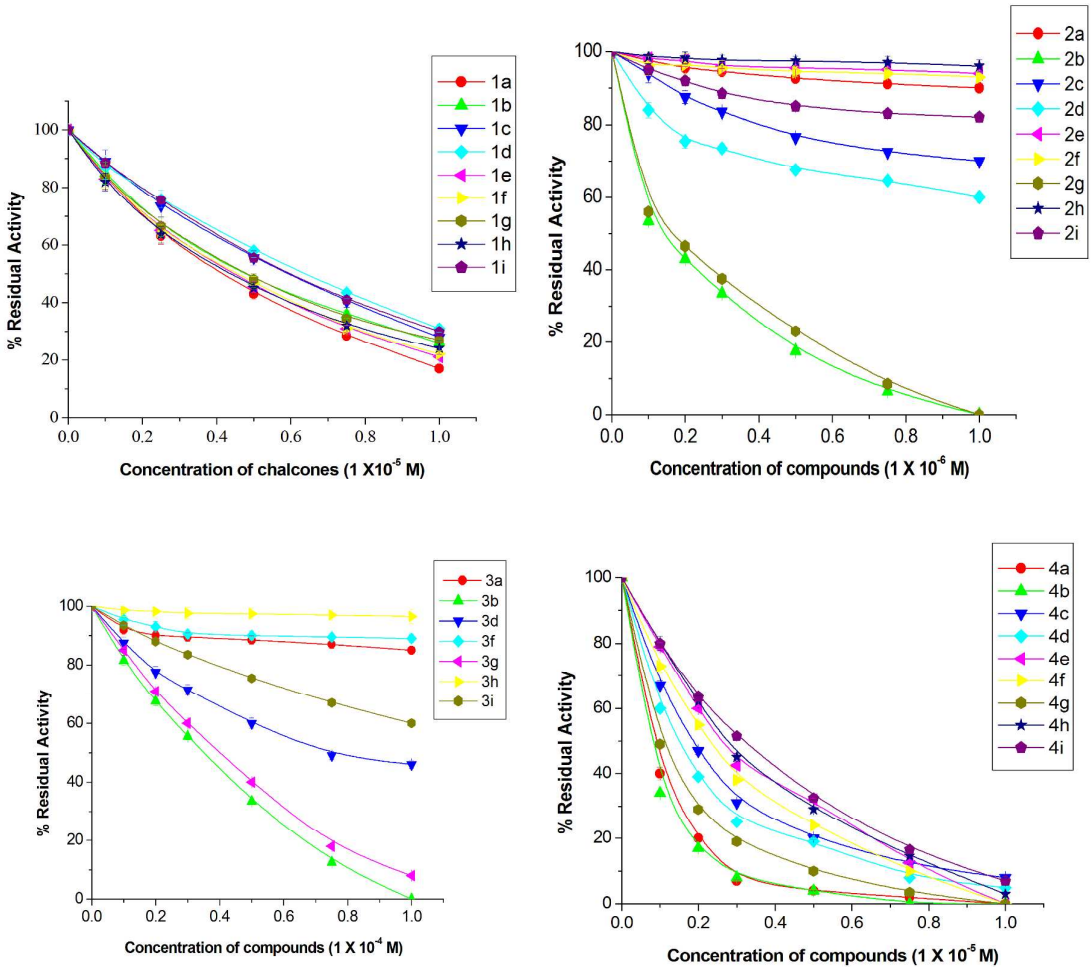
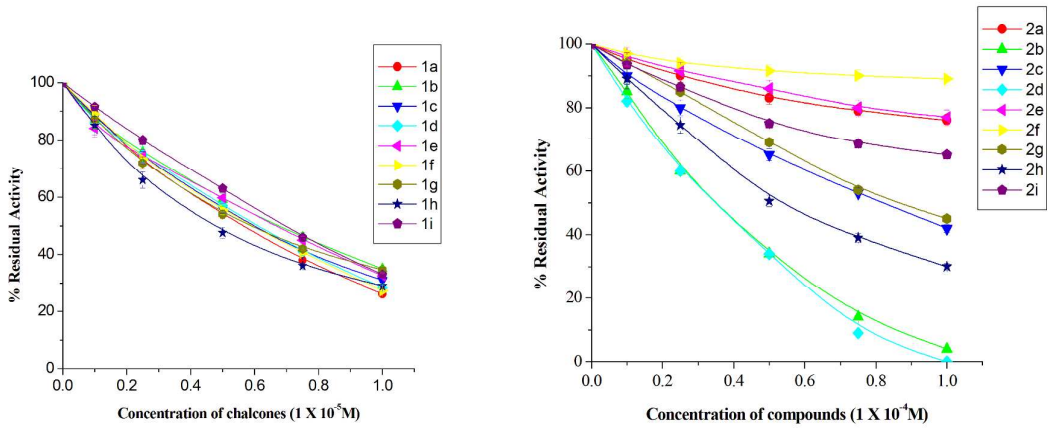

% Residual Activity
Concentration of chalcones (1 X10-5 M)
1a
1b
1c
1d
1e
1f
1g
1h
1i
Concentration of compounds (1 X 10-6 M)
2a
2b
2c
2d
2e
2f
2g
2h
2i
Concentration of compounds (1 X 10-4 M)
3a
3b
3d
3f
3g
3h
3i
Concentration of compounds (1 X 10-5 M)
4a
4b
4c
4d
4e
4f
4g
4h
4i

Figure 1


% Residual Activity
Concentration of chalcones (1 X 10-5M)
1a
1b
1c
1d
1e
1f
1g
1h
1i
Concentration of compounds (1 X 10-4M)
2a
2b
2c
2d
2e
2f
2g
2h
2i

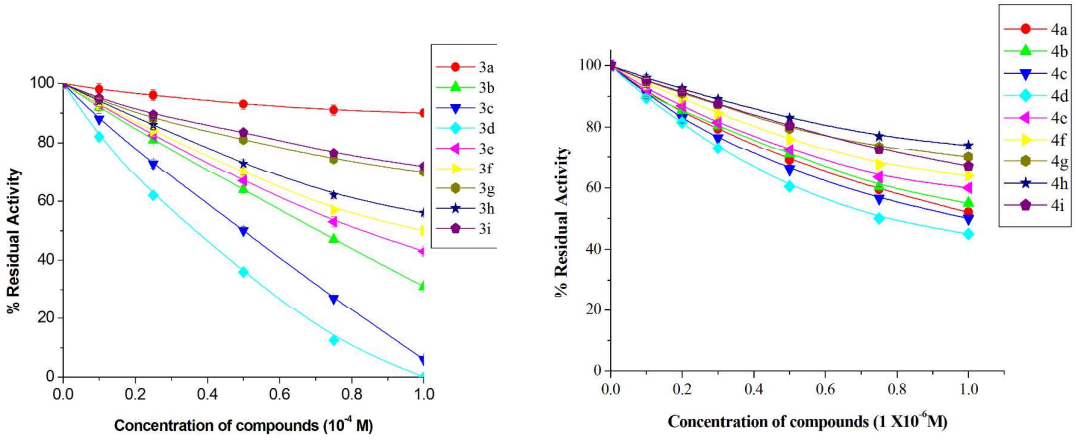


Figure 2

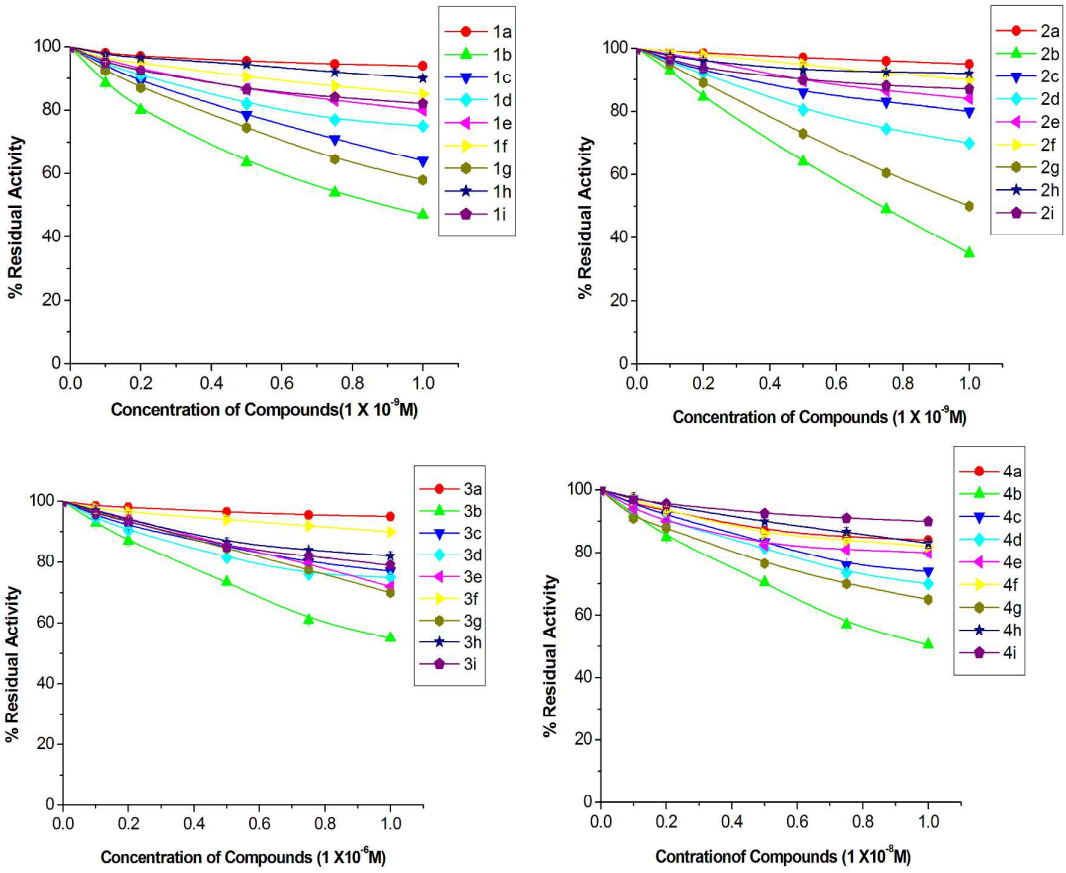


Figure 3

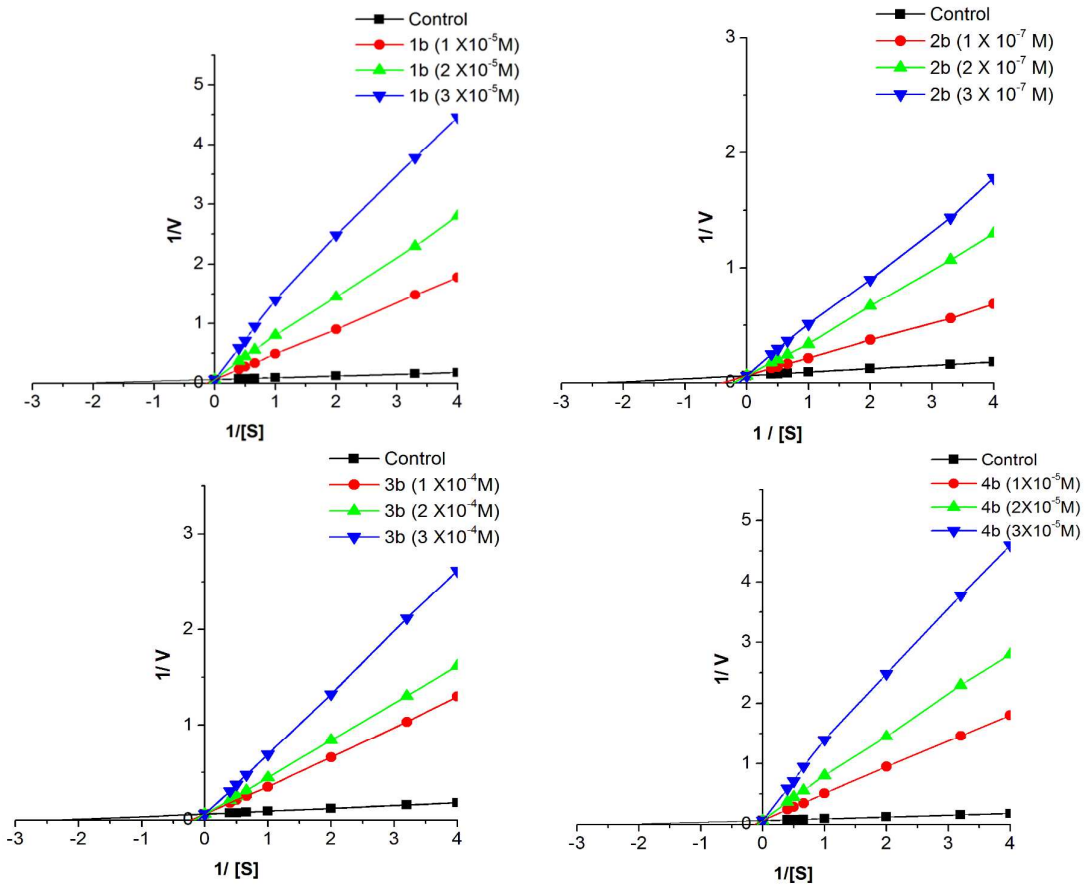
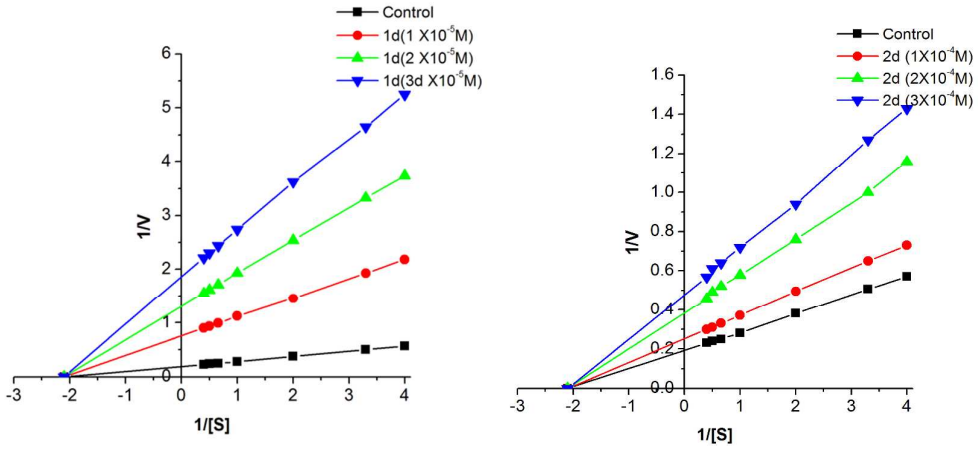

Control
1b (1 X10-5M)
1b (2 X10-5M)
1b (3 X10-5M)
1/V
1/[S]
Control
2b (1 X 10-7 M)
2b (2 X 10-7 M)
2b (3 X 10-7 M)
1/ V
1 / [S]
Control
3b (1 X10-4M)
3b (2 X10-4M)
3b (3 X10-4M)
1/ V
1/ [S]
Control
4b (1X10-5M)
4b (2X10-5M)
4b (3X10-5M)
1/ V
1/[S]

Figure 4


Control
1d(1 X10-5M)
1d(2 X10-5M)
1d(3d X10-5M)
1/V
1/[S]
Control
2d (1X10-4M)
2d (2X10-4M)
2d (3X10-4M)
1/V
1/[S]

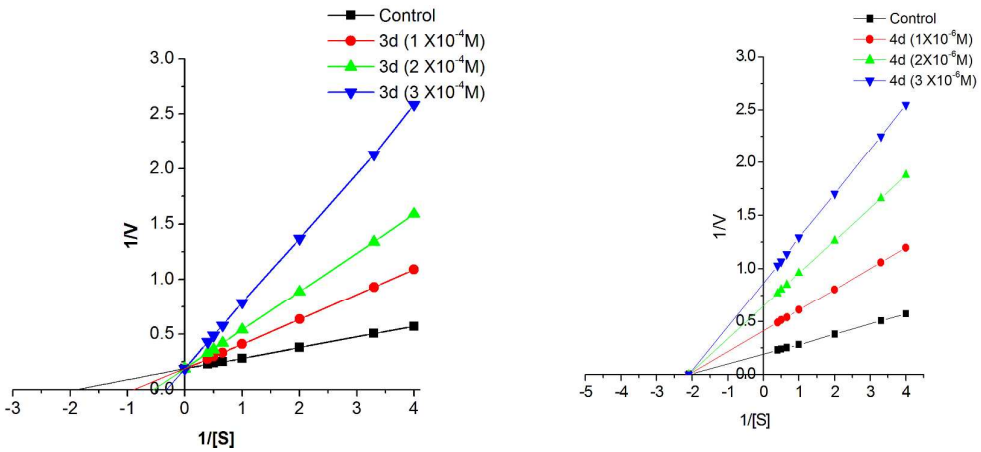


Figure 5

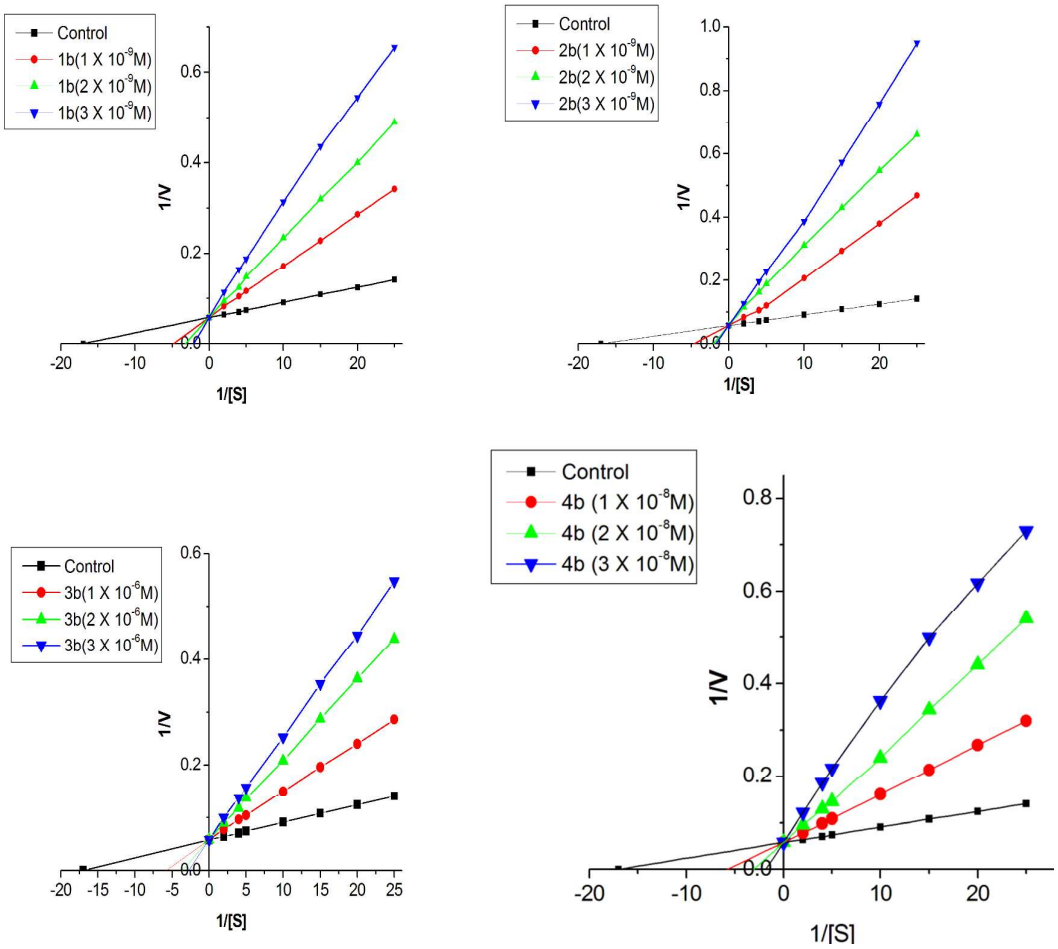
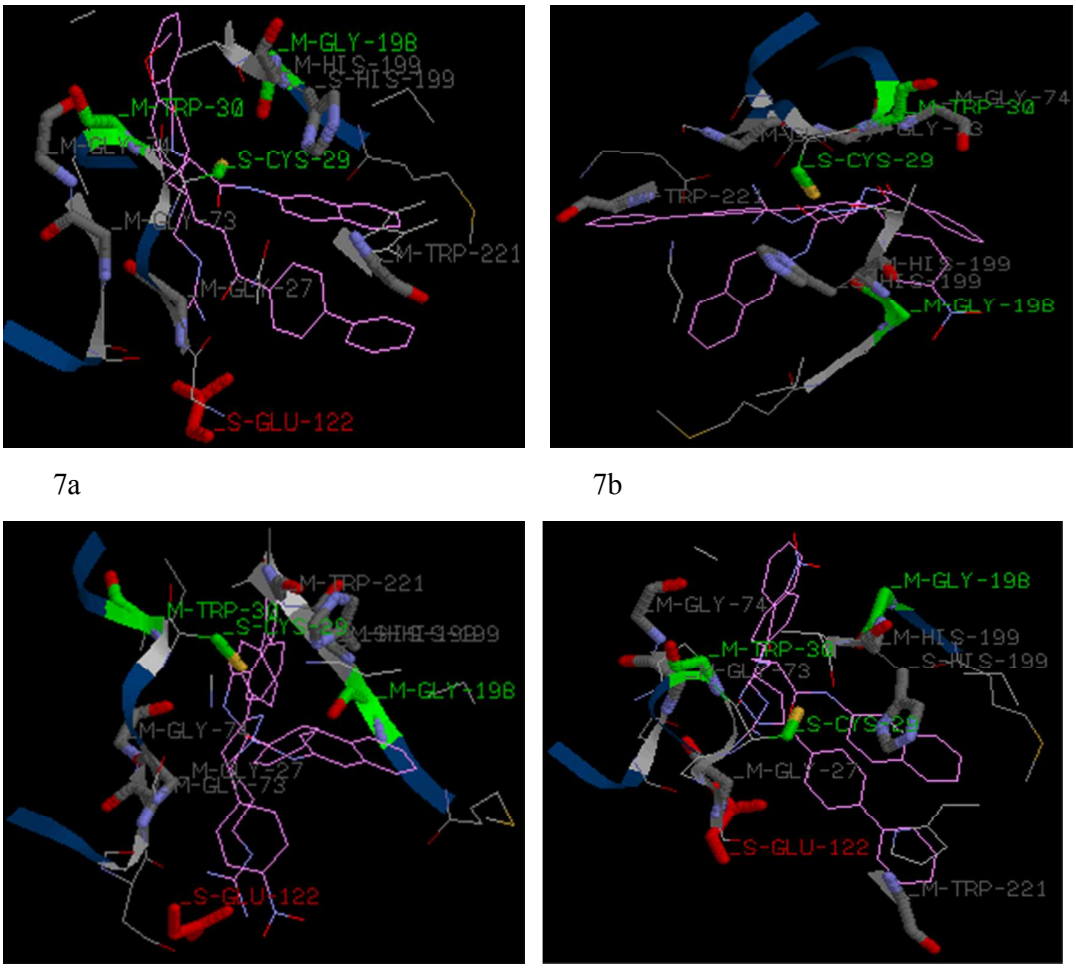


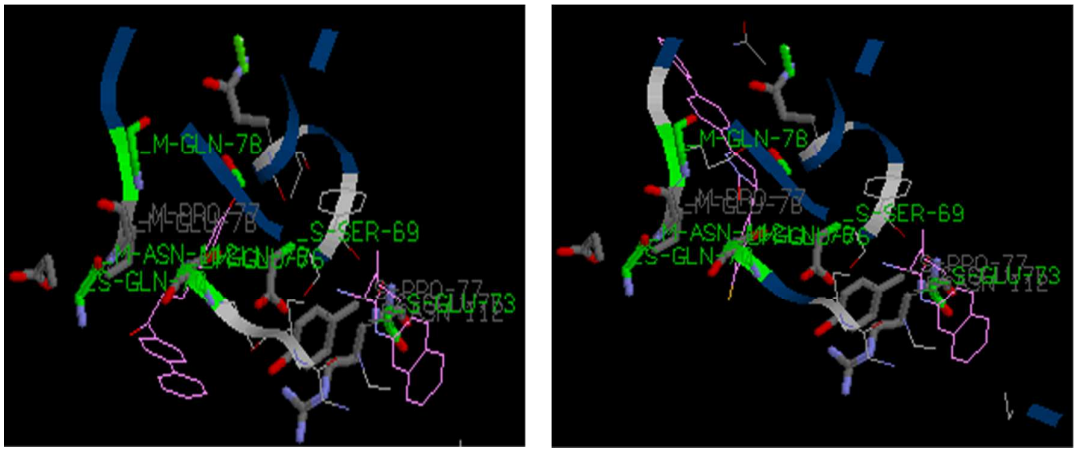
Figure 6



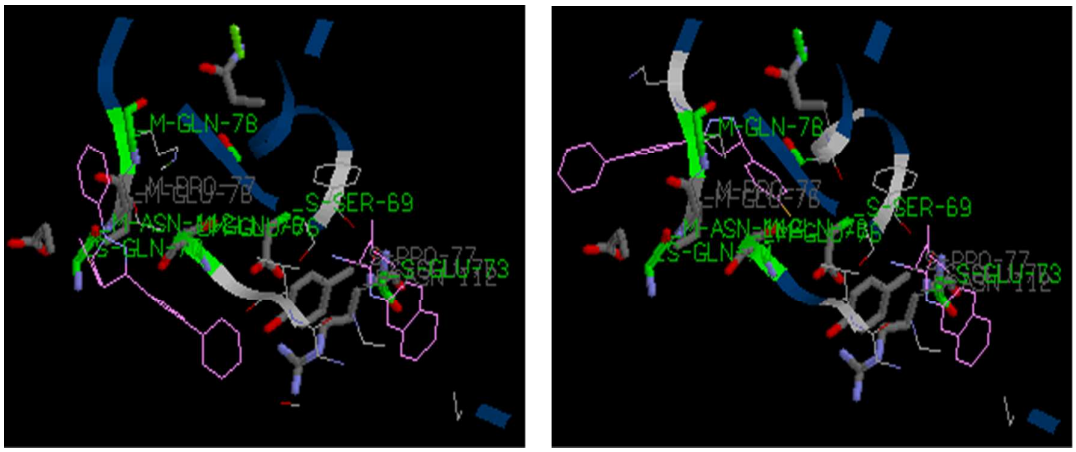
7a 7b

7c 7d

Figure 7

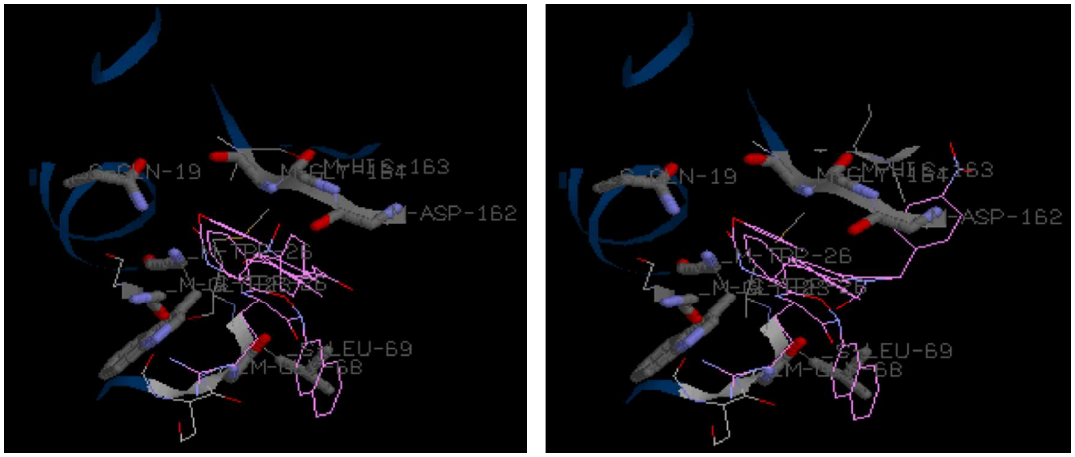


8a 8b

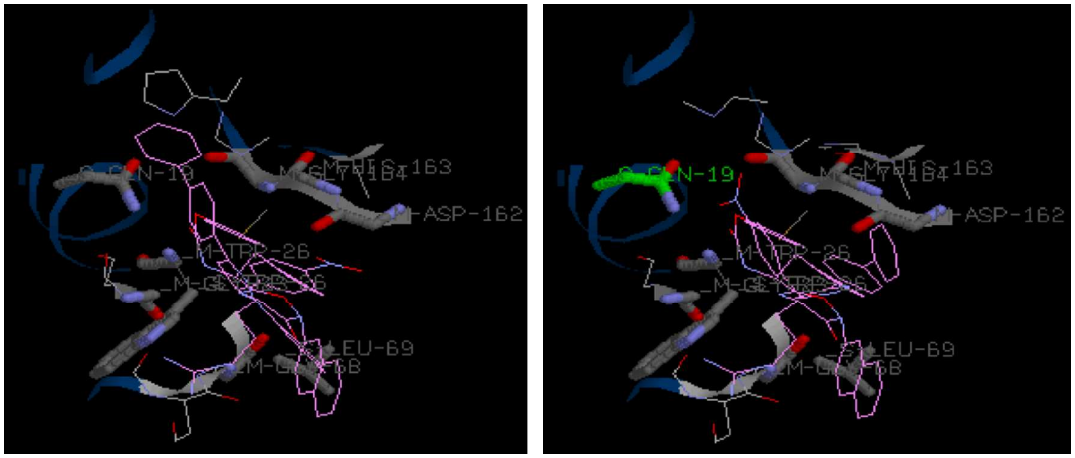


8c 8d

Figure 8

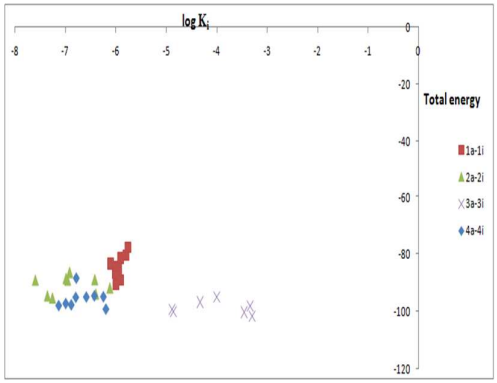


9a 9b

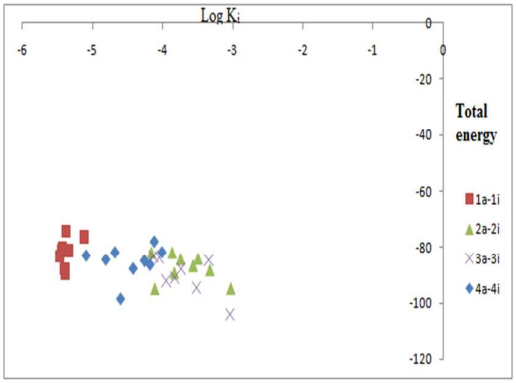


9c 9d

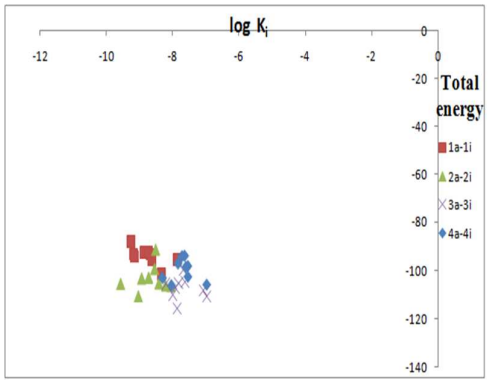
Figure 9



10a

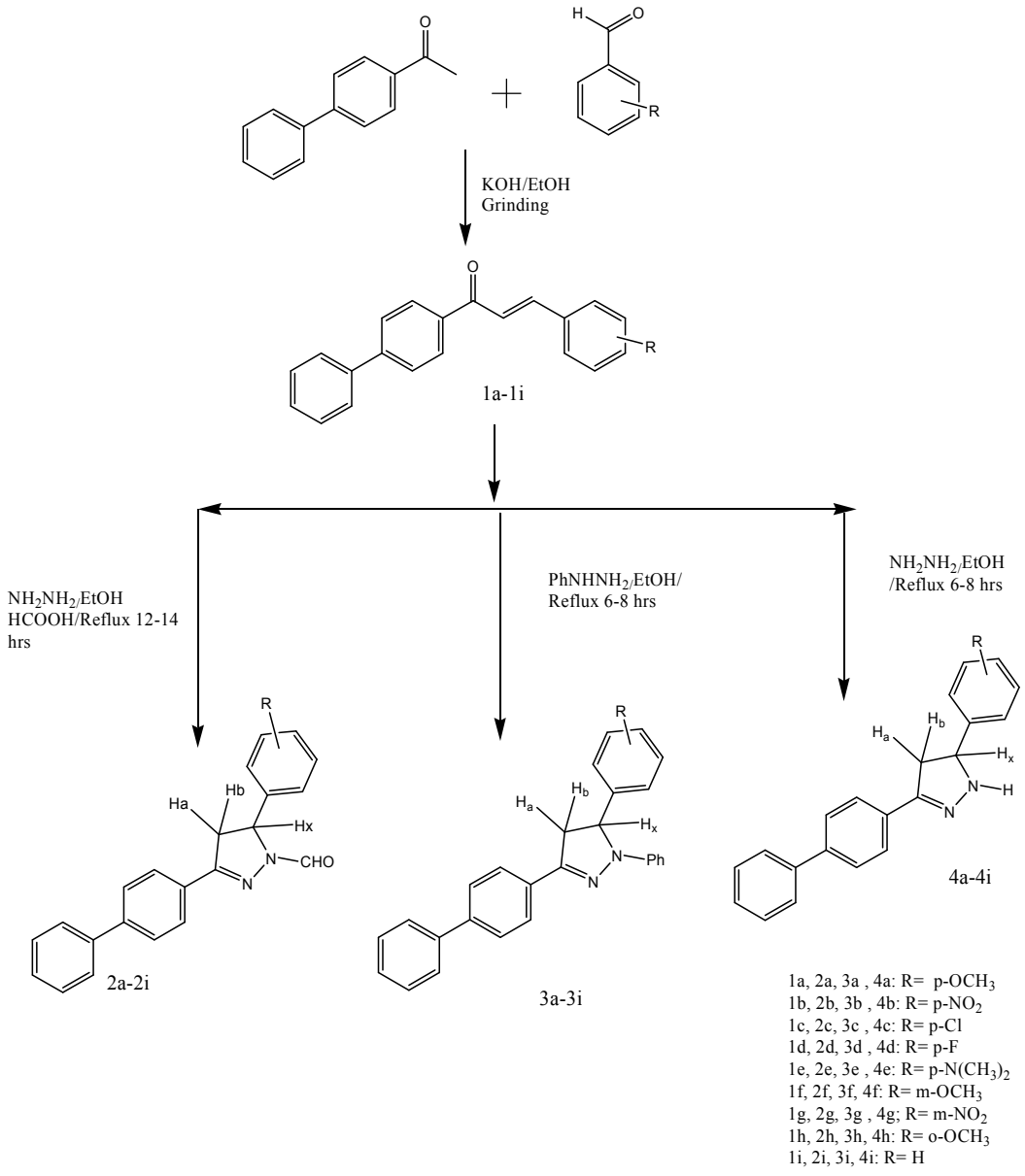


10b

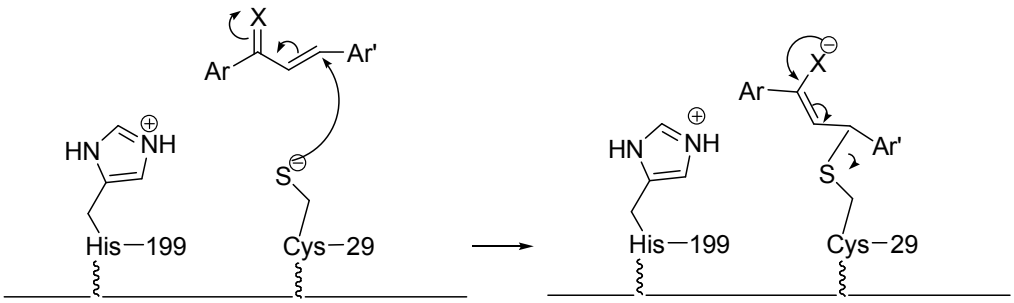


10c

Figure 10


KOH/EtOH
Grinding
1a-1i
NH2NH2/EtOH
HCOOH/Reflux 12-14
hrs
PhNHNH2/EtOH/
Reflux 6-8 hrs
NH2NH2/EtOH
/Reflux 6-8 hrs
2a-2i
3a-3i
4a-4i
1a, 2a, 3a , 4a: R= p-OCH3
1b, 2b, 3b , 4b: R= p-NO2
1c, 2c, 3c , 4c: R= p-Cl
1d, 2d, 3d , 4d: R= p-F
1e, 2e, 3e , 4e: R= p-N(CH3)2
1f, 2f, 3f, 4f: R= m-OCH3
1g, 2g, 3g , 4g; R= m-NO2
1h, 2h, 3h, 4h: R= o-OCH3
1i, 2i, 3i, 4i: R= H

Scheme 1



Chalcone; X =O

Scheme 2