RSC Advances

PAPER

View Article Online
View Journal | View Issue

Cite this: *RSC Adv.*, 2015, **5**, 50440

SAR studies of differently functionalized 4′-phenylchalcone based compounds as inhibitors of cathepsins B, H and L†

Indu Ravish and Neera Raghav*

Received 8th January 2015
Accepted 7th April 2015

DOI: 10.1039/c5ra00357a

www.rsc.org/advances

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 motivate the design, synthesis and evaluation of compounds as cathepsin 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 cathepsin B, H and L inhibitors. After a preliminary screening of the synthesized compounds as inhibitors to the 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 constants. It was found that the compounds belonging to different classes affected the enzyme activity significantly, and the activity was greatly affected by the presence of substituents. Maximum inhibition was caused by **2b** and **2g** in the cases of cathepsin B and cathepsin L, and **4d** in the case of cathepsin H, where K_i values of the order of 10^{-8} M, 10^{-9} M and 10^{-6} M, respectively, were obtained. The results are correlated with the docking results.

1. Introduction

Cysteine proteases, possessing a $-CH_2SH$ group at the active site, have become important therapeutic targets for the treatment of a number of diseases. The cysteine proteases of parasites, such as falcipain, cruzain and amoebepain[1,2] of *Plasmodium falciparum*, *Trypanosoma cruzi*, *Trypanosoma brucei rhodesiense*, *Toxoplasma gondii* and *Entamoeba hystolytica*[3–5] are some of the important targets in the development of anti-parasitic agents. The 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 against the standard drug nifurtomox. Brak *et al.*[7] reported another non-peptidic cruzain inhibitor with a triazole moiety.

Cathepsins B, H and L, lysosomal cysteine proteases, have also emerged as important targets for the development of a variety of therapeutic agents because of their involvement in various disease 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 the 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 the ratio of cellular inhibitors and cathepsin has been considered as one of the reasons for the elevated levels of these enzymes.[15,16] Therefore, the identification of compounds which act as potent inhibitors to cathepsins is a major thrust area in 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 overexpressed 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 endoaminopeptidase 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

Department of Chemistry, Kurukshetra University, Kurukshetra-136119, Haryana, India. E-mail: nraghav.chem@gmail.com; Tel: +91 9896918277

† Electronic supplementary information (ESI) available: Tables and characterization of synthesized compounds can be found in the ESI. See DOI: 10.1039/c5ra00357a

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 cathespin H impaired angiogenic switching of the pre-malignant hyperplastic islets significantly, and a reduction in the subsequent number of tumors was observed.[31]

Cathepsin L is another of the powerful lysosomal cysteine proteases which degrades 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 the degradation of collagen type I; this deregulation is believed to lead to osteo- and rheumatoid arthritis.[34] In addition, several infectious organisms, such as the 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 the above mentioned disease states emphasizes the need for 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 amount of work has been previously reported on peptide based inhibitor molecules,[36] but due to the immunological problems and gastric instability encountered with the use of peptidyl molecules, in the past decade this research area 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] and bischalcone based quinozolines, as novel inhibitors of cathepsin B and H.[39]

The chalcones chemically known as 1,3-diarylprop-2-en-1-ones (Scheme 1) are known for their diverse pharmacological activities, including antitumor and anti-inflammatory activities.[40] The literature has revealed that chalcones have been found to be potential inhibitors to calpains and cathepsin B and H.[41] Pyrazolines (Scheme 1) also possess a 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, and antihypertensive activities[46–48] and potent selective activity such as nitric oxide synthase (NOS) inhibitor and cannabinoid CB1 receptor antagonist activity.[49]

The biological activities, such as antitumor and anti-inflammatory activity, possessed by chalcones and pyrazolines and the role of cathepsins B, H and L in these disease 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 hydrolysis activity on endogenous protein substrates 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 structures of inhibitors can lead to a potential therapeutic molecule. It is a known fact that in order to develop an appropriate therapeutic agent, the number of molecules synthesized and screened is great.

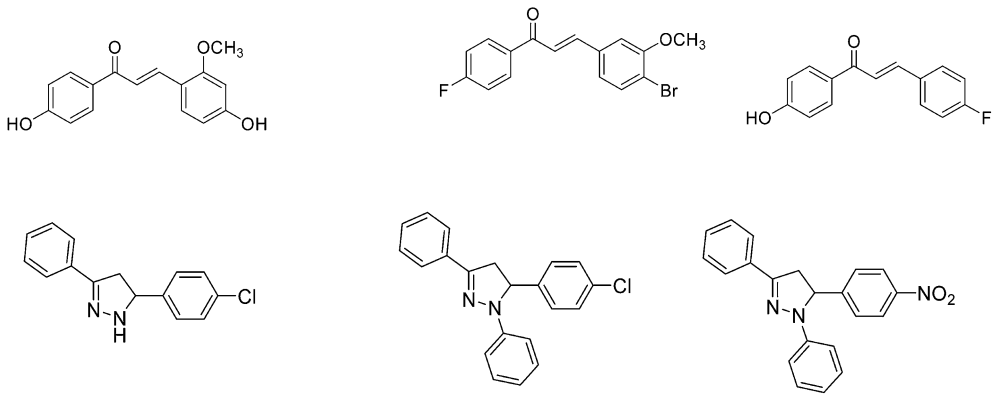
We have previously observed that systematic alterations in the structure of chalcones led to the identification of 4′-phenylchalcones as activators of one enzyme *i.e.* acid phosphatase and inhibitors of another enzyme *i.e.* alkaline phosphatase, known to perform similar physiological functions.[51]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

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

2. Results and discussion

Chalcones and their cyclized derivatives, pyrazolines, has wide potential roles as antitumor agents and anti-inflammatory agents (Scheme 2). Different pyrazolines (**1a**, **1b** and **1c**) have been reported to possess significantly high cytotoxic activity against MCF-7 and HCT 116 cell lines.[55] Manna *et al.* has also reported anticancer activity of some pyrazolines.[56] 3,5-Diaryl pyrazolines (**3a**) and their 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 the overexpression and diminished level of inhibitors of these cathepsins in various cancerous conditions.[58–62]

Preliminary studies of some chalcone hydrazone 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 pyrazoline derivatives as inhibitors of proteolytic activity on endogenous protein substrates, followed by detailed enzyme inhibition studies on cathepsins B and H. In addition the effect of the synthesized compounds was also screened on cathepsin L. To accomplish the project, first of all



Scheme 1 Representative chalcones and pyrazolines as inhibitors of cathepsins B, H and L.

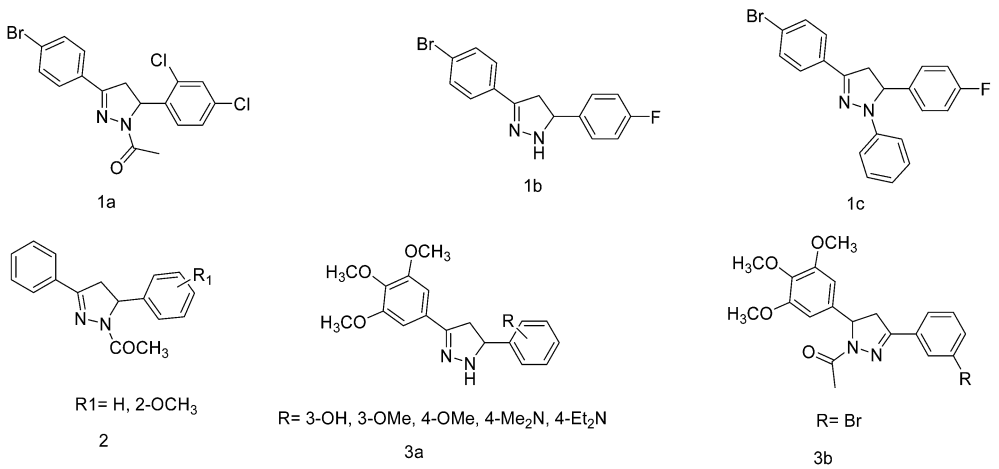
the pyrazolines were synthesized and characterised from the respective chalcones (Scheme 3) as discussed below.

In the first step, the 4′-phenylchalcones (**1a–1i**) were obtained by a solvent free method from 4′-phenylacetophenone and aromatic aldehydes under basic conditions. In the second step, reaction of the synthesized chalcones 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.

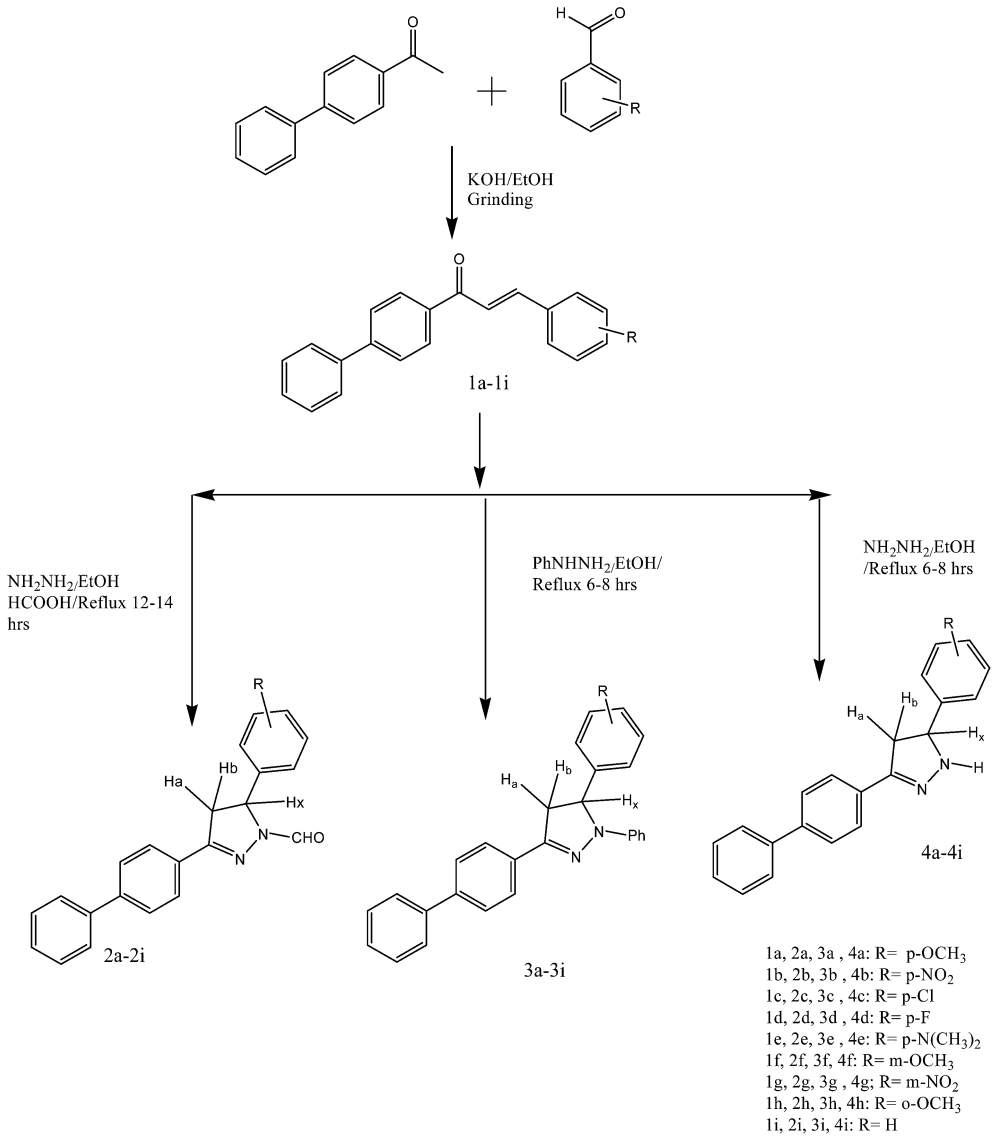
The structures of the synthesized compounds were elucidated on the basis of mp, ^{1}H NMR and IR spectra. The ring closure is demonstrated by the IR spectra of compounds **2a–2i**, **3a–3i** and **4a–4i**, which showed the characteristic band for NH at 3100–3300 cm^{-1} and a band at 1590–1605 cm^{-1} 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. The methylene protons of the heterocyclic ring appeared as two double doublets, one at $\delta = 3.75$–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 nonequivalence of these two protons due to being adjacent to a chiral center, whose hydrogen has a chemical shift of 5.2 ppm (H_{5x}, $J = 12.2$, 7.2 Hz). These spectral data unequivocally proved the 2-pyrazoline structure. The ^{1}H NMR spectra of the compounds in $CDCl_3$ show 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. The multiplet signals observed in the 6.6–8.5 ppm region are assigned to the protons of the phenyl rings. The sharp singlets observed at 3.3–3.8 and 3.33–3.90 ppm are assigned to the 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 the preliminary exploration of inhibition by these compounds. It was found that the compounds **1b**, **1d**, **1e**, **2h**, **3a**, **3f**, **3g** and **4g** inhibited 100% of the proteolytic activity at a final concentration of 1×10^{-4} M. A number of cysteine proteases are 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 this stage. The only conclusion drawn from these results is that the inhibition caused by the designed compounds is of reversible type



Scheme 2 Some pyrazolines as antitumor and anti-inflammatory agents.



Scheme 3 Synthesis of chalcones and their cyclized derivatives.

because the inhibition is greater at **3h** incubation time and less at 24 h 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 the due course of time. It was evident from experimental data that the compounds inhibited proteolysis of endogenous substrates at this pH. In the 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 S1† presents the results of the endogenous proteolytic activity in the presence of 4′-phenylchalcones and their pyrazoline derivatives.

2.2. Enzyme inhibition studies

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

2.2.1. Effect of 4′-phenylchalcones (1a–1i) on the activity of cathepsins B, H and L. Fig. 1–3 show the relationship between the enzyme activity and concentration of the substituted chalcones (**1a–1i**) for cathepsins B, H and L respectively. It has been found that enzyme activity is inhibited and is affected by the substituents present in chalcones. Among the various compounds tested, at 1×10^{-5} M concentration **1b** and **1d** exhibited the maximum inhibitory effect in case of cathepsin B and cathepsin H respectively, and in the case of cathepsin L **1b** shows maximum inhibition at 10^{-9} M.

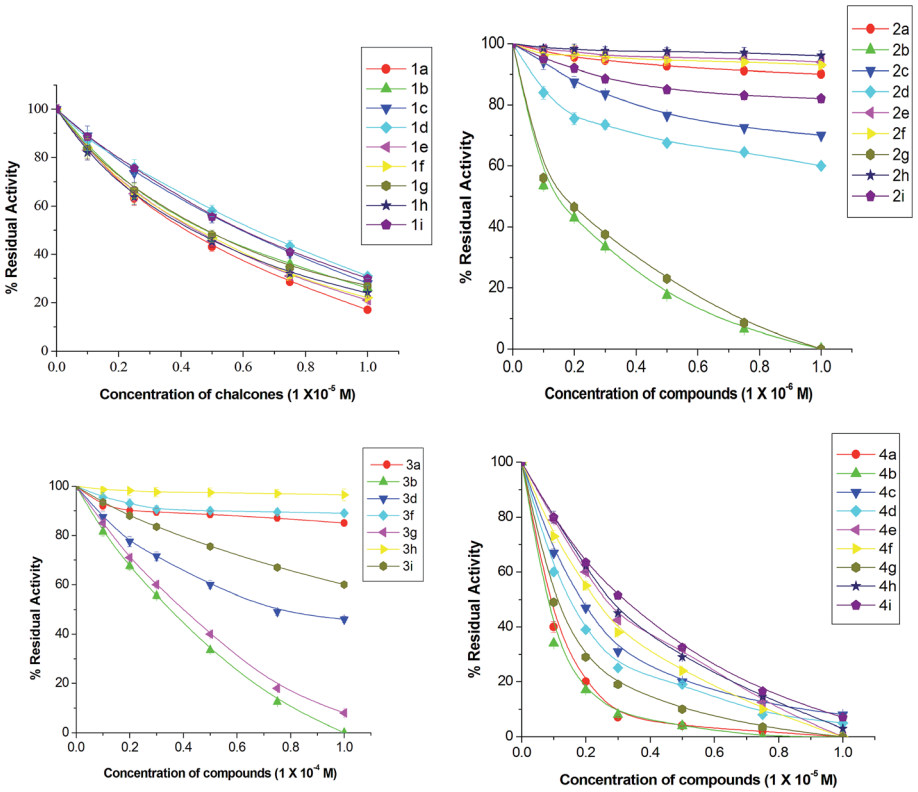


Fig. 1 The results are depicted as the % residual activities of cathepsin B in the presence of different concentrations (0.1, 0.2, 0.3, 0.50, 0.75 and 1.0×10^{-5} M) of various chalcones (**1a–1i**), 0.1, 0.2, 0.3, 0.50, 0.75 and 1.0×10^{-6} M of *N*-formylpyrazolines (**2a–2i**), 0.1, 0.2, 0.3, 0.50, 0.75 and 1.0×10^{-4} M of *N*-phenylpyrazolines (**3a–3i**) and 0.1, 0.2, 0.3, 0.50, 0.75 and 1.0×10^{-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.

2.2.2. Effect of *N*-formyl-2-pyrazolines on the activity of cathepsins B, H and L. On evaluating the inhibitory effect of *N*-formyl-2-pyrazolines it was found that electron withdrawing substituents exhibited a greater inhibitory effect as compared to electron releasing substituents. The activity of cathepsin B was reduced to 54% at 1×10^{-7} M, and the activity of cathepsin L was reduced to 35% for **2b**, revealing this compound as the most potent inhibitor among all tested compounds (Table S2†). In the case of cathepsin H, **2d** shows the maximum inhibition *i.e.* 32% at 1×10^{-5} M.

2.2.3. 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 the cysteine proteases, cathepsins B, H and L. It was found that 70% inhibition for cathepsin B activity was achieved at 1.0×10^{-5} M concentration in the case of **3b** and 58% in case of **3g**. Cathepsin H was maximally inhibited by **3d** and **3e** *i.e.* 35% at 1×10^{-5} M. Cathepsin L was inhibited by **3b** up to 50% at 1×10^{-6} M.

2.2.4. Effect of 3-(4-biphenyl)-5-substituted phenyl-2-pyrazolines on cathepsins B, H and L. In the case of the 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 1×10^{-6} M. But in the case of cathepsin L, **4b** showed the maximum inhibition, *i.e.* 55% at 1×10^{-8} M.

2.2.5 Enzyme kinetic studies. All the synthesized compounds, after establishing the inhibitory effect, were screened to evaluate the inhibition 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 noncompetitive manner except for in the case of *N*-phenyl pyrazolines, in which competitive inhibition was found (Fig. 4–6). The K_i values have been summarised in Table S3.†

2.3. SAR studies

The literature reports that cysteine proteases, including cathepsins B, H and L, play important roles in cancer, inflammation and tissue degenerative processes, where proteolysis in one aspect or another is an important phase for the spread of these diseases. Targeting these enzymes is therefore one of the 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 it was **2a–2i** > **1a–1i** > **4a–4i** > **3a–3i**, whereas for cathepsin H it can be observed that the order of inhibition was **1a–1i** > **4a–4i** > **2a–2i** > **3a–3i** as observed from the K_i values. In these enzymes, the synthesized compounds show different tendencies. In

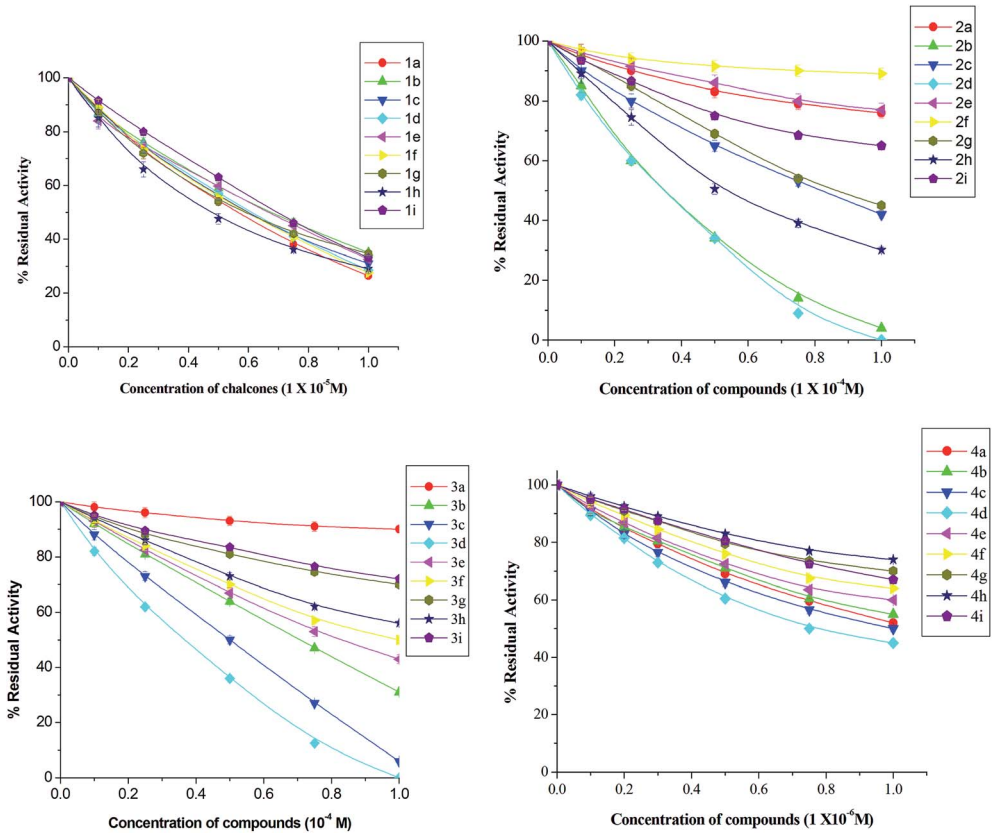


Fig. 2 The results are depicted as the % residual activities of cathepsin H in the presence of different concentrations (0.1, 0.2, 0.3, 0.50, 0.75 and 1.0×10^{-5} M) of various chalcones (**1a–1i**), 0.1, 0.2, 0.3, 0.50, 0.75 and 1.0×10^{-4} M of *N*-formylpyrazolines (**2a–2i**), 0.1, 0.2, 0.3, 0.50, 0.75 and 1.0×10^{-4} M of *N*-phenylpyrazolines (**3a–3i**) and 0.1, 0.2, 0.3, 0.50, 0.75 and 1.0×10^{-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.

cathepsins B and L the formyl group probably plays an important role and interacts with the nucleophilic thiol group present at the active site. Cathepsin L is sensitive toward α–β unsaturated carbonyl compounds like cathepsin H. The cysteine of cathepsin H does not seem 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 seem to cause less inhibition to both the enzymes as the K_i value is sub-micromolar. Similar results have been previously obtained in the case of 1,3,5-triphenylpyrazolines and related structural analogs[48] where the phenyl substituent pyrazolines were least inhibitory.

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

In all the series the electron withdrawing $-NO_2$ group was found to be most inhibitory for cathepsin B and L. Similar results have previously been reported from our lab for *o*-hydroxy chalcones and their cyclized derivatives,[38] where the K_i value for the most inhibitory compound has been found to be 6.18×10^{-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, nearly the same inhibition was observed invariably, regardless of the substituents.

2.4. Molecular docking

The docking approach used in this study was aimed at the identification of compounds that selectively bind to the active site of cathepsin B and modulate the enzymatic activity. The individual binding poses of each compound were assessed, and their interactions in the active site of the enzyme were analysed. Docking alterations are based on the interaction force field scoring, which includes van der Waals and electrostatic interactions between the active site and ligand. Table S3† represents the data of the docking studies of the 4′-phenylchalcones (**1a–1i**) and the pyrazoline derivatives (**2a–2i**), (**3a–3i**) and (**4a–4i**) with the cathepsin B active site (available through RCSB Protein Data Bank, PDB entry cav2IPP B_PYS.pdb). The binding energies

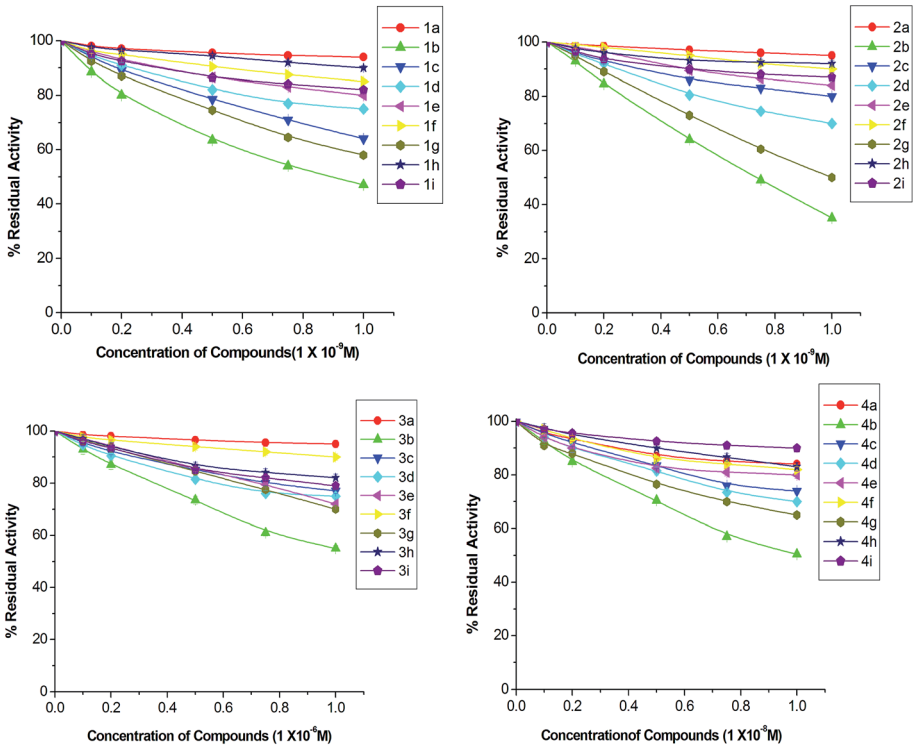


Fig. 3 The results are depicted as the % residual activities of cathepsin L in the presence of different concentrations (0.1, 0.2, 0.3, 0.50, 0.75 and 1.0×10^{-5} M) of various chalcones (**1a–1i**), 0.1, 0.2, 0.3, 0.50, 0.75 and 1.0×10^{-4} M of *N*-formylpyrazolines (**2a–2i**), 0.1, 0.2, 0.3, 0.50, 0.75 and 1.0×10^{-4} M of *N*-phenylpyrazolines (**3a–3i**) and 0.1, 0.2, 0.3, 0.50, 0.75 and 1.0×10^{-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.

(kcal mol^{-1}) of the 4′-phenylchalcones (**1a–1i**) and pyrazoline derivatives (**2a–2i**), (**3a–3i**) and (**4a–4i**) vary in the ranges −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 the most inhibitory compounds in each series **1b**, **2b**, **3b**, and **4b** show decreases in total energy of −83.30, −89.11, −99.42 and −97.74, respectively. On the basis of the interaction data of the docking experiments, it was observed that all the compounds showed less interaction than the reference leupeptin, a peptidyl inhibitor. The maximum interaction is shown by BANA with a score of −124.91. The decrease in total energy for leupeptin–cathepsin B came out to be −119.17, of which the contributions of the van der Waals interactions was greatest with a score of −91.77 as compared to the H-bond with a score of −28.00. The leupeptin–cathepsin B binding energy is due to peptide–protein interaction. Leupeptin is peptidyl in nature, and therefore, being a flexible molecule, it binds effectively with the enzyme active site, resulting in a higher binding energy. iGemDOCK provides 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. The compounds under consideration are smaller in structure and possess lower flexibility compared to leupeptin. Therefore, the binding energy of the 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 a competitive inhibitor to cathepsin B, the designed compounds show competitive inhibition.

Fig. 7(a–d) shows the results of the best docking pose of **1b**, **2b**, **3b** and **4b**, respectively, in 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 to be competitive inhibitors and to compete with the binding site of the substrate. The proposed mechanism is shown in Scheme 4. The results clearly indicate the significance of the *in vitro* inhibition studies.

However for 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 although leu-CH_2Cl is a specific inhibitor for cathepsin H[65,66] it possesses only one amino acid residue in contrast to leupeptin–cathepsin B. Therefore the leu-CH_2Cl–cathepsin H interaction causes a decrease in energy of only −59.99, of which −43.49 is the van der Waals interaction and −16.50 is due to H-bonds.

As listed in Table S4† all the designed compounds have been found to show a greater decrease in ligand–cathepsin H interaction energy than leu-CH_2Cl–cathepsin H. From docking studies, the compound **1a** was evaluated to be the most inhibitory to cathepsin H among the 4′-phenylchalcones, showing a

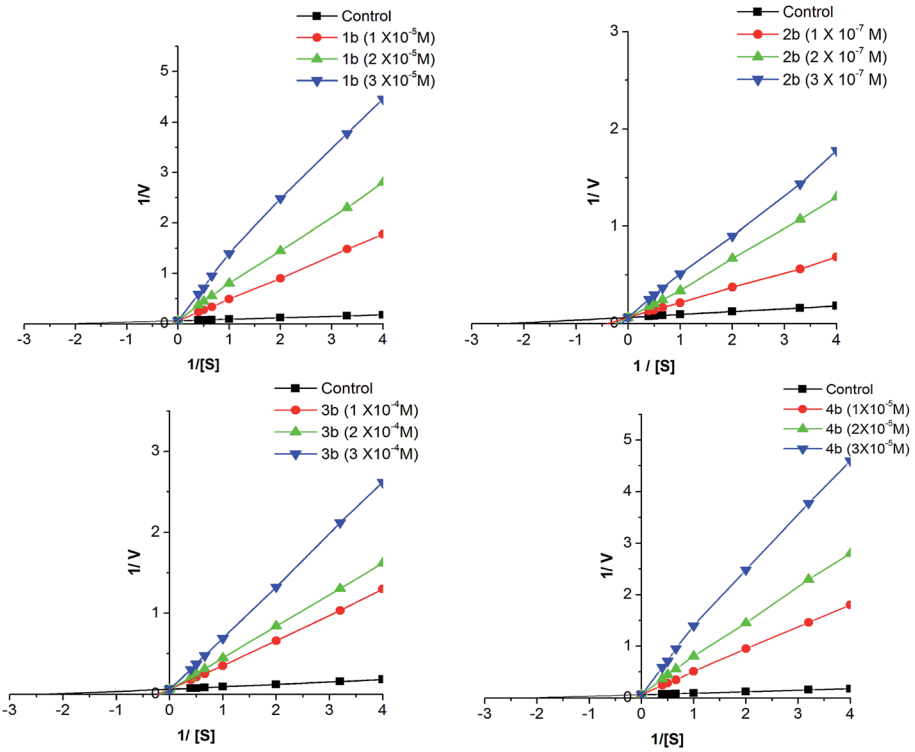


Fig. 4 Effect of the compounds **1b**, **2b**, **3b** and **4b** on cathepsin B activity. The experiment was done in three different concentrations in triplicate. All compounds showed competitive inhibition.

total decrease in energy of −81.56, of which −72.77 is assigned to van der Waals interactions whereas −8.79 is from H-bonding. The compound **2d** was supported as being the best inhibitor to cathepsin H among the *N*-formylpyrazolines, because it showed a total decrease in energy of −81.94, of which −68.45 is assigned to van der Waals interactions whereas −13.48 is from H-bonding. As evidenced by the docking results, among the phenylpyrazolines, **3d** was found to be most inhibitory to cathepsin H, showing a total decrease in energy of −85.26, of which −81.65 is assigned to van der Waals interactions whereas −3.61 is from H-bonding. However among the *N*-pyrazolines, **4d** was evaluated to be the best inhibitor to cathepsin H, showing a total decrease in energy of −83.12, of which −72.92 is assigned to van der Waals interactions whereas −10.19 from H-bonding.

Fig. 8(a–d) shows the docked poses of the most inhibitory compounds **1d**, **2d**, **3d** and **4d** in the aminoacyl binding site of cathepsin H, respectively. As can be seen, the designed compound and the substrate leu-βNA bind at two different sites. Gln-73 and Ser-69 have been found to interact with leu-βNA H_a–H bonds. However Gln-78, Asn-116 and Gln-23 interact with the most inhibitory compound in each series. The docked poses and interactions clearly suggest that a non-competitive type of inhibition is exerted by the compounds on cathepsin H. *In vitro* inhibition studies also suggest that all the *N*-phenylpyrazoline series exerted a non-competitive inhibition on cathepsin H.

In the case of cathepsin L the ranges of the binding energies of the 4′-phenylchalcones (**1a–1i**) and pyrazoline 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 a decrease in total energy of −88.05, −105.4, −105.55 and −103.14 kcal mol^{-1}. When these docking energies were compared with those of the substrate Z-Phe-Arg-4mβNA and the peptidyl inhibitor leupeptin, it was found that they are very low. For Z-Phe-Arg-4mβNA it was −130.74 kcal mol^{-1}, and for leupeptin it came out to be −116.8 kcal mol^{-1}. Leupeptin, possessing a peptidyl chain, exhibits a larger decrease in energy; however, the extent of the inhibition caused by **1b** and **2b** is of the order of 10^{-9} M, which is greater than that of leupeptin, where the K_i value is reported to be 1.5×10^{-9} M.[71]

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

Fig. 10(a–c) represents the correlation between the total energy *vs.* log K_i of the designed compounds for cathepsin B, H and L respectively. It can be observed from Fig. 10(a), that the most effective binding of compound **2b** followed by **2g** causing maximum inhibitory potential is well correlated in this graph. The *N*-formylpyrazoline series (**2a–2i**) exhibits maximum inhibitory power, followed by the *N*-pyrazolines (**4a–4i**), the 4′-phenylchalcones (**1a–1i**) and *N*-phenylpyrazolines (**3a–3i**) in that order. The correlation plot for cathepsin H, **10b**, depicts the chalcones as the most inhibitory series followed by *N*-pyrazolines, *N*-formylpyrazolines and *N*-phenylpyrazolines. The plot of

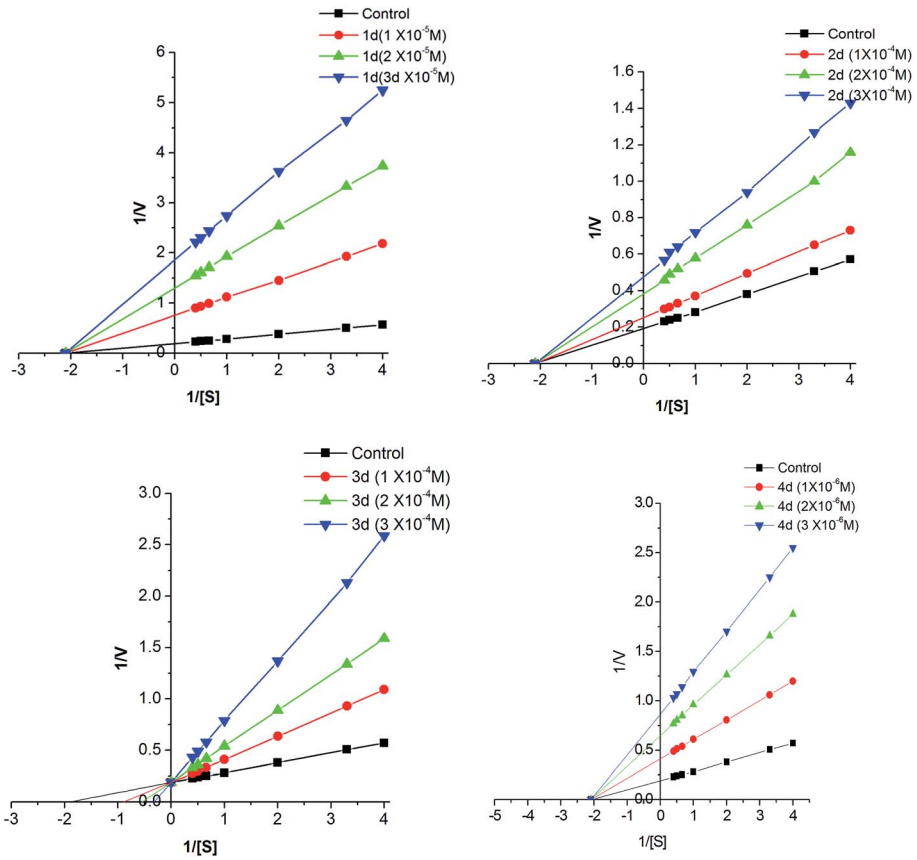


Fig. 5 Effect of the compounds **1d**, **2d**, **3d** and **4d** on cathepsin H activity. The experiment was done in three different concentrations in triplicate. **3d** showed competitive inhibition, and the other compounds showed non-competitive inhibition.

binding energy *vs.* inhibitory constant for cathepsin L (Fig. 10(c)) shows that *N*-formylpyrazolines are the most inhibitory, followed by 4′-phenylchalcones, *N*-pyrazolines and *N*-phenylpyrazolines.

In the present work, we have used the decrease in total energy of the enzyme–ligand complex as a measure of the binding affinity of the ligand within the active site of the enzyme. However, Zhang *et al.* used a free energy based approach in understanding such interactions.[68–70]

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 the docking studies are not included here. The *in silico* predicted behaviour of the enzyme–ligand interaction can give an idea about the interaction between these two, but cannot substitute for the in-solution studies.

In the present study we found that different effects have been exerted by the related classes of compounds on cathepsin B and H activities. The electron withdrawing $-NO_2$ substitution proved to be a good inhibitor of cathepsins B and L, and the chloro- and fluoro-substituted compounds were more inhibitory to H.

3. Experimental section

The substrates α-*N*-benzoyl-d,L-arginine-2-naphthylamide (BANA)/Z-Arg-Arg-4mβNA, 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 and 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 the enzymes was fresh goat liver obtained from a local slaughterhouse. 4′-Phenyl acetophenone and aldehydes were purchased from Himedia, Bombay. An Elisa plate reader was used for measuring absorbance in the visible range. IR spectra were recorded on a Horizon 300 MHz spectrometer. ^{1}H NMR spectra were recorded on a Bruker 300 MHz instrument. The chemical shifts are expressed in ppm units from an internal TMS standard. The refrigerated ultracentrifuge Remi C-24BL was used for centrifugation purposes 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 the visualizing agent.

3.1. Synthesis

3.1.1. Procedure for syntheses 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 mol) with 4-phenyl-acetophenone (0.01 mol) in the presence of potassium

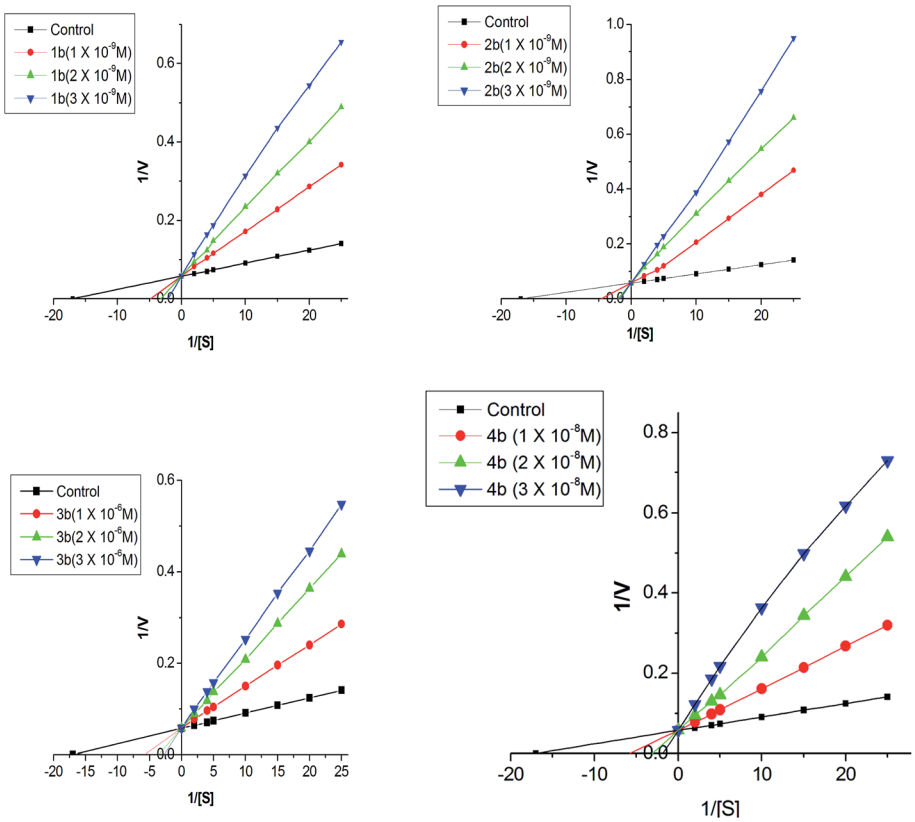


Fig. 6 Effect of the compounds **1b**, **2b**, **3b** and **4b** on cathepsin L activity. The experiment was done in three different concentrations in triplicate. All compounds showed competitive inhibition.

hydroxide (0.01 mol) with a mortar and pestle. The progress of the 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 syntheses of *N*-formyl-3-(4-biphenyl)-5-substituted phenyl-2-pyrazolines (2a–2i). Compounds **1a–1i** (0.001 mol), hydrazine hydrate (0.001 mol) and 15–20 ml of formic acid in ethanol (25 ml) were 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 the reaction, the mixture was allowed to cool at room temperature and was kept in a refrigerator for 4–5 h. The fine crystals that 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 syntheses 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 added acetic acid in a catalytic amount in ethanol (25 ml) were 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. The fine crystals that 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 syntheses of 3-(4-biphenyl)-5-substituted phenyl-2-pyrazolines (4a–4i). Compounds **1a–1i** (0.001 mol) and hydrazine hydrate (0.001 mol) in ethanol (25 ml) were refluxed at 60–70 °C for 6–8 h. The reaction progress was monitored with the help of TLC. Once the reaction was complete the reaction mixture was allowed to cool at room temperature. The fine crystals that 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 a local slaughterhouse. The fresh goat liver was first washed with cold isotonic saline solution. The tissue was then homogenized a in 0.1 M sodium acetate buffer at pH 5.5 containing 0.2 M NaCl in a mixer-*cum*-blender to obtain 10% (w/v) homogenate. It was then stored at 4 °C.

3.2.2. Assay for proteolytic activity. The proteolytic studies on the endogenous protein substrate were carried out at pH 5.0 at 37 °C using 0.1 M acetate buffer as the incubation medium. The homogenate was mixed with the buffer and was incubated at 37 °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

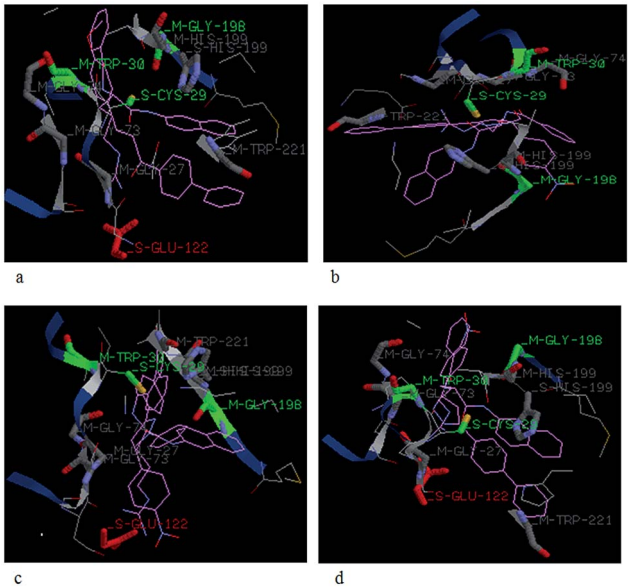
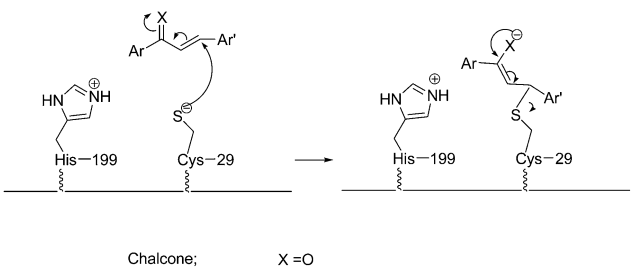


Fig. 7 Docking results showing the alignment of the most inhibitory compounds, **1b**, **2b**, **3b** and **4b**, in the active site of cathepsin B (cav2IPP B_PYS.pdb), along with the substrate BANA in a, b, c and d, respectively. The binding site radius is 8 Å. The results are for docking at drug screening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



Scheme 4 Proposed mechanism of inhibition for chalcones.

supernatant using the Bradford method.[72] The experiment was conducted in triplicate and the results are presented in Table S1.†

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 1 mM 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 a CMSephadex C-50 and DEAE-Sephadex A-50. The specific activities of the cathepsin B, cathepsin H and cathepsin L were 13.46 nmol per min mg, 25.56

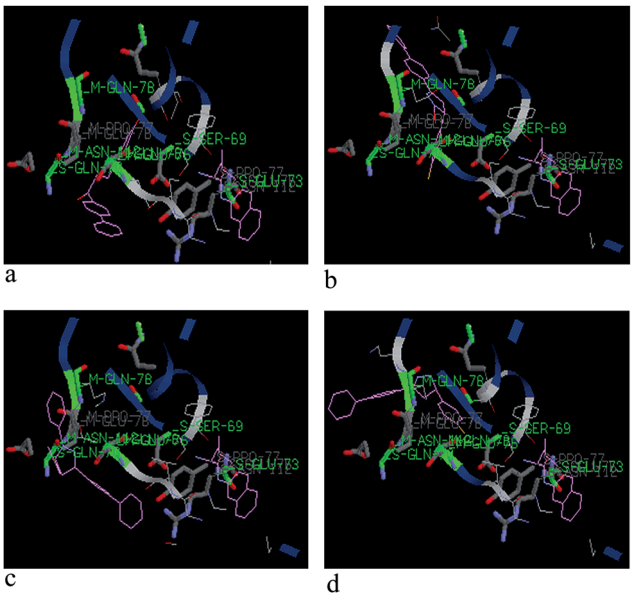


Fig. 8 Docking results showing the alignment of the most inhibitory compounds, **1d**, **2d**, **3d** and **4d**, in the active site of cathepsin H (cav8PCH H_NAG.pdb) along with the substrate leuβNA in a, b, c and d, respectively. The binding site radius is 8 Å. The results are for docking at drug screening settings. The inhibitor and substrate bind 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.

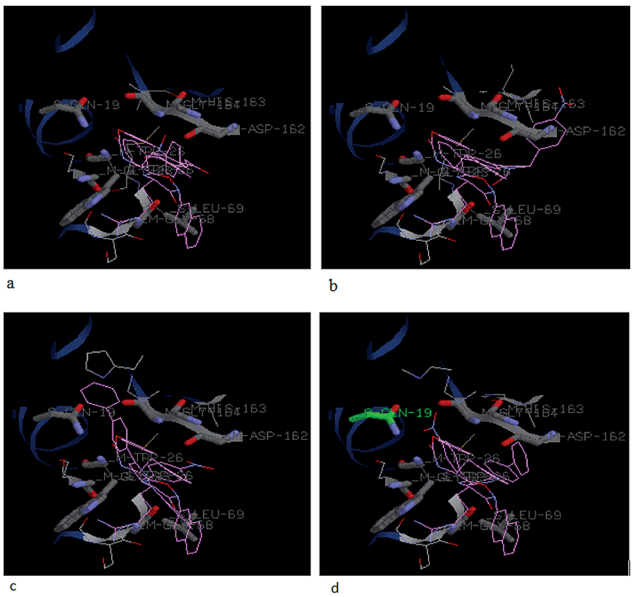


Fig. 9 Docking results showing the alignment of the most inhibitory compounds, **1b**, **2b**, **3b** and **4b**, in the active site of cathepsin L (cav3BC3 L_CSW.pdb) along with the substrate Z-Phe-Arg-4mβNA in a, b, c and d, respectively. The binding site radius is 8 Å. The results are for docking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

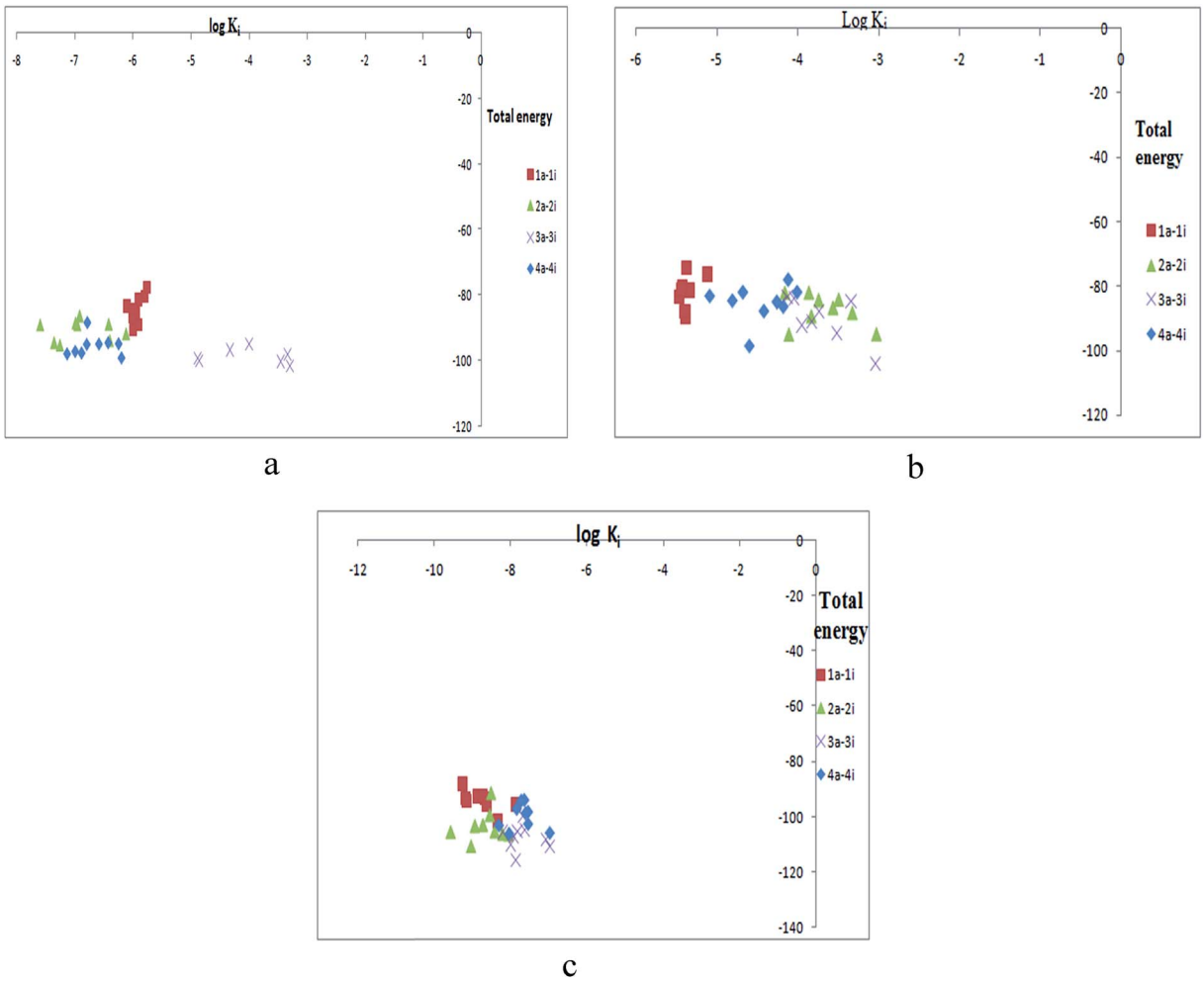


Fig. 10 Correlation plot between binding energies and log K_i values for cathepsin B (a), cathepsin H (b), and cathepsin L (c). □, Δ, × and ◇ represent **1a–1i**, **2a–2i**, **3a–3i** and **4a–4i**, respectively.

nmol per min per mg and 15.42 μmol per min per mg respectively.

3.4. Enzyme inhibition studies

The activities of cathepsin B were estimated at varying concentrations of synthesized 4′-phenylchalcones (**1**), *N*-formylpyrazolines (**2**), *N*-phenylpyrazolines (**3**) and pyrazolines (**4**). First of all, the enzyme was equilibrated in 0.1 M phosphate buffer of pH 6.0 at 37 °C. The stock solutions of the compounds were prepared in DMSO. Then an appropriate amount of the individual compounds was added to the reaction mixture separately to effect the final concentration of each compound. After an incubation time of 30 min, the 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 the synthesized compounds using leuβNA as the substrate.[67]

The activities for cathepsin L were estimated at varying concentrations of the synthesized compounds using Z-Phe-Arg-4mβNA as the substrate.[74] The results are shown in Table S1† and Fig. 1–3.

3.5. Determination of K_i values

After establishing the inhibitory action of the 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, the enzyme activity was evaluated at different substrate concentrations in the presence and absence of a fixed concentration of the inhibitor. The enzyme concentration was kept constant in all the experiments. A Lineweaver–Burk plot was drawn of $1/S$ and $1/V$ in the presence and absence of the inhibitor (Fig. 4–6).

3.6. Drug modeling studies

All docking studies were performed using iGemdock. For these studies the small molecular weight ligands were prepared and the enzyme structure active site was retrieved from the Protein Data Bank. The structures were prepared using marvin sketch and were saved as MDL Mol Files. The structure of cathepsins B,

H and L were retrieved from the 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 with drug screening settings, and the results are presented in Table S4–S6† and Fig. 7–9.

4. Conclusions

In the present work we synthesized 4′-phenylchalcones and their pyrazoline 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 revealed that all compounds showed reversible competitive inhibition on cathepsin B and L with affinities in the subnanomolar range. Except for the *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 and 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.

Conflicts of interest

The authors have declared no conflict of interest.

Acknowledgements

Among the authors, Indu Ravish is thankful to UGC New Delhi, India for the award of SRF and also to Kurukshetra University, Kurukshetra for providing necessary research laboratory facilities.

References

1 X. Du, C. Guo, E. Hansell, P. S. Doyle, G. R. Caffrey, T. P. Holler, J. H. McKerrowand and F. E. Cohen, *J. Med. Chem.*, 2002, **45**, 2695–2707.
2 G. Aguiree, L. Boiani, H. Cerecetto, M. Fernandez, M. Gonzalez, A. Denicola, L. Otero, D. Gambino, C. Rigol, C. Olea-Azar and M. Faundez, *Bioorg. Med. Chem.*, 2004, **12**, 4885–4893.
3 N. Fujii, J. P. Mallari, E. J. Hansell, Z. Mackey, P. Doyle, Y. M. Zhou, J. Gut, P. J. Rosenthal, J. H. Mckerrow and R. K. Guy, *Bioorg. Med. Chem. Lett.*, 2005, **15**, 121–123.
4 P. T. Romulo, S. C. Cristiane, S. P. Carla, G. de Lima Jose, R. de Faria Antonio, J. A. Antonio, J. T. de Melo Edesio and J. S. Goes Alexander, *Bioorg. Med. Chem. Lett.*, 2005, **15**, 2575–2578.
5 D. C. Greenbaum, Z. Mackey, E. Hansell, P. Doyle, J. Gut, C. R. Affrey, J. Lehrman, P. J. Rosenthal, J. H. McKerrow and K. Chibale, *J. Med. Chem.*, 2004, **47**, 3212–3219.
6 N. C. Romeiro, G. Aguirre, P. M. Hernández, M. González, H. Cerecetto, I. Aldana, S. Pérez-Silanes, A. Monge, E. J. Barreiro and L. M. Lima, *Bioorg. Med. Chem.*, 2009, **17**, 641–652.
7 K. Brak, I. D. Kerr, K. T. Barrett, K. N. Fuchi, M. Debnath, M. K. Ang, J. C. Engel, J. H. McKerrow, P. S. Doyle, L. S. Brinen and J. A. Ellman, *J. Med. Chem.*, 2010, **53**, 1763–1773.
8 L. O. Carlos and M. Lynn, *Nat. Rev. Cancer*, 2007, **7**, 800–808.
9 T. Nomura and N. Katunuma, *J. Med. Invest.*, 2005, **52**, 1–9.
10 J. A. Joyce, A. Baruch, K. Chehade, N. Meyer-Morse, E. Giraudo, F. Y. Tsai, D. C. Greenbaum, J. H. Hager, M. Bogyo and D. Hanahan, *Cancer Cell*, 2004, **5**, 443–453.
11 M. M. Mohamed and B. F. Sloane, *Nat. Rev. Cancer*, 2006, **6**, 764–775.
12 J. Kos and T. T. Lah, *Oncol. Rep.*, 1998, **5**, 1349–1361.
13 M. J. Duffy, *Clin. Cancer Res.*, 1996, **2**, 613–618.
14 I. Assfalg-Machleidt, M. Jochum, D. Nast-Kolb, M. Siebeck, A. Billing, T. Joka, G. Rothe, G. Valet, R. Zauner, H.-P. Scheuber and W. Machleidt, *Biol. Chem. Hoppe-Seyler*, 1990, **371**, 211–222.
15 H. Fritz, M. Jochum, R. Geiger, K. H. Duswald, H. Dittmer, H. Kortmann, S. Neumann and H. Lang, *Folia Histochem. Cytobiol.*, 1986, **24**, 99–115.
16 M. Jochum, K. H. Duswald, S. Neumann, J. Witte, H. Fritz and U. Seemüller, Proteinases and their inhibitors in inflammation: basic concepts and clinical implications, in *Proteinase inhibitors: medical and biological aspects*, ed. N. Katunuma, H. Umezawa and H. Holzer, Japan Scientific Societies Press, Tokyo, Springer, Berlin, 1983, pp. 85–95.
17 C. Jedeszko and B. F. Sloane, *Biol. Chem.*, 2004, **385**, 1017–1027.
18 H. Kirschke, A. J. Barrett and N. D. Rawlings, *Protein Profile*, 1995, **2**, 1587–1643.
19 B. F. Sloane, K. Moin, E. Krepela and J. Rozhin, *Cancer Metastasis Rev.*, 1990, **9**, 333–352.
20 N. Katunuma and E. Kominami, *Rev. Physiol., Biochem. Pharmacol.*, 1987, **108**, 1–20.
21 Z. Werb, Proteinases and matrix degradation, in *Textbook of Rheumatology*, ed. W. N. Keller, E. D. Haris, S. Ruddy and C. S. Sledge, W. B. Saunder Co., Philadelphia, 1989, pp. 300–321.
22 A. A. Sinha, D. F. Gleason and O. F. Deleon, *Anat. Rec.*, 1993, **235**, 233–240.
23 A. A. Sinha, J. L. Morgan and R. J. Buus, *Anticancer Res.*, 2007, **27**, 3135–3141.
24 S. A. Rempel, M. L. Rosenblum and T. Mikkelsen, *Cancer Res.*, 1994, **54**, 6027–6031.
25 E. Krepela, E. Kasafirek, K. Novak and J. Viklicky, *Neoplasma*, 1990, **37**, 61–70.
26 M. J. Murnane, K. Sheahan, M. Ozdemirli and S. Shuja, *Cancer Res.*, 1991, **51**, 1137–1142.
27 A. Premzl, V. Zavasnik-Bergant, V. Turk and J. Kos, *Exp. Cell Res.*, 2003, **15**, 206–214.
28 H. Tsushima, A. Ueki, Y. Matsuoka, H. Mihara and V. K. Hopsu-Havu, *Int. J. Cancer*, 1991, **48**, 726–732.
29 D. Gabrijelcic and V. Turk, *et al.*, *Eur. J. Clin. Chem. Clin. Biochem.*, 1992, **30**, 69–74.

30 A. Schweiger, B. Stabuc, T. Popovic, V. Turk and J. Kos, *J. Immunol. Methods*, 1997, **201**, 165–232.
31 V. Gocheva, X. Chen, C. Peters, T. Reinhecke and J. A. Joyce, *Biol. Chem.*, 2010, **391**, 937–945.
32 N. Katunuma, *J. Bone Miner. Metab.*, 1997, **15**, 1–8.
33 O. Vasiljeva, T. Reinheckel, C. Peters, D. Turk, V. Turk and B. Turk, *Curr. Pharm. Des.*, 2007, **13**, 385.
34 R. E. Esser, R. A. Angelo, M. D. Murphey, L. M. Watts, L. P. Thornburg, J. T. Palmer, J. W. Talhouk and R. E. Smith, *Arthritis Rheum.*, 1994, **37**, 236.
35 G. Simmons, D. N. Gosalia, A. J. Rennekamp, J. D. Reeves, S. L. Diamond and P. Bates, *Proc. Natl. Acad. Sci. U. S. A.*, 2005, **102**, 11876.
36 M. Frizler, M. Stirnberg, M. T. Sisay and M. Gütschow, *Curr. Top. Med. Chem.*, 2010, **10**, 294–322.
37 N. Raghav and M. Singh, *Eur. J. Med. Chem.*, 2014, **77**, 231–242.
38 N. Raghav and S. Garg, *Eur. J. Pharm. Sci.*, 2014, **60**, 55–63.
39 N. Raghav and M. Singh, *Eur. J. Pharm. Sci.*, 2014, **54**, 28–39.
40 V. Prabhakar, R. Balasubramanian, P. Sathe, M. Krishna and A. Juvekar, *Int. J. Tumor Ther.*, 2014, **3**, 1–9, DOI: 10.5923/j.ijtt.20140301.01.
41 S. H. Kim, E. Lee, K. H. Baek, H. B. Kwon, H. Woo, E. S. Lee, Y. Kwon and Y. Na, *Bioorg. Med. Chem. Lett.*, 2013, **23**(3), 320–3324.
42 Z. Ozdemir, H. B. Kandilci, B. Gumusel, U. Calis and A. A. Bilgin, *Eur. J. Med. Chem.*, 2007, **42**, 373–379.
43 J. G. Lombardino and I. G. J. Otterness, *Med. Chem.*, 1981, **24**, 830–834.
44 A. Budakoti, M. Abid and A. Azam, *Eur. J. Med. Chem.*, 2006, **41**, 63–70.
45 A. Sid, *et al.*, *Eur. J. Med. Chem.*, 2011, **2**, 311–313.
46 S. S. Parmar, B. R. Pandey, C. Dwivedi and R. D. Harbinson, *J. Pharm. Sci.*, 1974, **63**, 1152.
47 N. Soni, K. Pande, R. Kalsi, T. K. Gupta, S. S. Parmar and J. P. Barthwal, *Res. Commun. Chem. Pathol. Pharmacol.*, 1987, **56**, 129.
48 G. Turan-Zitouni, P. Chevallet, F. S. Kilic and K. Erol, *Eur. J. Med. Chem.*, 2000, **35**, 635.
49 Y. Rajendra Prasad, A. Lakshmana Rao, K. Prasoona, K. Murali and P. Ravi Kumar, *Bioorg. Med. Chem. Lett.*, 2005, **15**, 5030.
50 N. Raghav and M. Singh, *Bioorg. Med. Chem.*, 2014, **22**, 4233–4245.
51 N. Raghav and I. Ravish, *Med. Chem. Res.*, 2015, **24**, 152–160.
52 N. Raghav, S. Jangra, M. Singh, R. Kaur and A. Rohilla, *Int. J. Appl. Biol. Pharm. Technol.*, 2011, **2**, 193–198.
53 N. Raghav, S. Jangra, M. Singh, R. Kaur and A. Rohilla, *Int. J. Pharm. Pharm. Sci.*, 2012, **4**, 288–292.
54 N. Raghav and M. Singh, *Med. Chem. Res.*, 2014, **24**, 1781–1788.
55 Z. H. Ismaeil, F. M. A. Soliman and S. H. Abd-El Monem, *Am. J. Sci.*, 2011, **7**, 756–767.
56 F. Manna, F. Chimenti, R. Fioravanti, A. Bolasco, D. Secci, P. Chimenti, C. Ferlini and G. Scambia, *Bioorg. Med. Chem. Lett.*, 2005, **15**, 4632–4635.
57 C. Congiu, V. Onnis, L. Vesci, M. Castorina and C. Pisano, *Bioorg. Med. Chem.*, 2010, **18**, 6238–6248.
58 K. M. Bell-McGuinn, A. L. Garfall, M. Bogyo, D. Hanahan and J. A. Joyce, *Cancer Res.*, 2007, **67**, 7378–7385.
59 B. Friedrich, K. Jung, M. Lein, I. Türk, B. Rudolph, G. Hampel, D. Schnorr and S. A. Loening, *Eur. J. Cancer*, 1999, **35**, 138–144.
60 I. Berdowska, M. Siewiński, A. Zarzycki-Reich, J. Jarmułowicz and L. Noga, *Med. Sci. Monit.*, 2001, **7**, 675–679.
61 M. Budihna, P. Strojan, L. Smid, J. Skrk, I. Vrhovec, A. Zupevc, Z. Rudolf, M. Zargi, M. Krasovec, B. Svetic, N. Kopitar-Jerala and J. Kos, *Biol. Chem. Hoppe-Seyler*, 1999, **377**, 385–390.
62 J. Kos, B. Werle, T. Lah and N. Brunner, *Int. J. Biol. Markers*, 2000, **15**, 84–89.
63 M. Singh and N. Raghav, *Int. J. Pharm. Pharm. Sci.*, 2013, **5**, 80–86.
64 M. Singh and N. Raghav, *Int. J. Pharm. Pharm. Sci.*, 2013, **5**, 365–368.
65 N. Raghav, R. Kaur, M. Singh, Suman and Priyanka, *Asian J. Chem.*, 2010, **22**, 7097–7101.
66 J. M. Yang and C. C. Chen, *Proteins*, 2004, **55**, 288–304.
67 N. Raghav, R. C. Kamboj, S. Parnami and H. Singh, *Indian J. Biochem. Biophys.*, 1995, **32**, 279–285.
68 Z. Zhang, V. Martiny, D. Lagorce, Y. Ikeguchi, E. Alexov and M. A. Miteva, *PLoS One*, 2014, **9**, e1108884.
69 Z. Zhang, S. Witham, M. Petukh, G. Moroy, M. Miteva, Y. Ikeguchi and E. Alexov, *J. Am. Med. Informat. Assoc.*, 2013, **20**, 643–651, DOI: 10.1136/amiajni-2012-001505.
70 J. M. Yang, Y. F. Chen, T. W. Shen, B. S. Kristal and D. F. Hsu, *J. Chem. Inf. Model.*, 2005, **45**, 1134–1146.
71 R. C. Kamboj, N. Raghav, R. Sadana and H. Singh, *Res. Bull. Panjab Univ., Sci.*, 2001, **51**, 41–59.
72 M. M. Bradford, *Anal. Biochem.*, 1976, **72**, 248–254.
73 R. C. Kamboj, S. Pal and H. Singh, *J. Biosci.*, 1990, **15**, 397–408.
74 R. C. Kamboj, S. Pal, N. Raghav and H. Singh, *Biochimie*, 1993, **75**, 873–878.
75 C. P. Huber, R. L. Cambell, S. Hasnain, T. Hirama and R. To, 2013, http://www.ebi.ac.uc\pdbe–srv/view/entry/2ipp\citation.Html.
76 G. Guncar, M. Podobrik, J. Pungercar, B. Strukelj, V. Turk and D. Turk, *Structure*, 1998, **6**, 51–61.
77 S. F. Chowdhary, L. Joseph, S. Kumar, S. R. Tulsidas, S. Bhat, E. Ziomek, R. M. Menard, J. Sivaraman and E. O. Purisima, *J. Med. Chem.*, 2008, **51**, 1361–1368.