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N-formylpyrazolines and N-benzoylpyrazolines as novel inhibitors of mammalian cathepsin B and cathepsin H

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ABSTRACT

Cathepsins, intracellular proteases, are known to be involved in a number of physiological processes ranging from degradation of extracellular proteins, prohormone processing, progressions of atherosclerosis, etc. High levels of cathepsins have been indicated in various pathological conditions like arthritis, cancer and other tissue degenerative disorders. One of the reasons attributed to these high levels is decrease in inhibitor concentration. Therefore, the work on the identification of small molecular weight compounds as inhibitors of cysteine proteases is of great therapeutic significance. Exploring this work in the same direction, we here present the synthesis of substituted N-formylpyrazolines and N-benzoylpyrazolines and study these as inhibitors to cysteine proteases. After a preliminary screening of the compounds as inhibitors to cysteine proteases in general, studies were carried out to study their inhibitory effects on cathepsin B and cathepsin H. SAR studies show that N-formylpyrazolines were better inhibitors than N-benzoylpyrazolines. The most potent inhibitors among the two series were nitro substituted compounds 1i and 2i with K_i values of $\sim 1.1 \times 10^{-9}$ M and 19.5×10^{-8} M for cathepsin B and K_i values of $\sim 5.19 \times 10^{-8}$ M and 9.8×10^{-7} M for cathepsin H, respectively. Docking experiments showing interaction between N-formylpyrazolines and N-benzoylpyrazolines with enzyme active sites structures also provided useful insights.

1. Introduction

Cysteine proteases are the center of attention in synthetic protease inhibitor development for chemotherapy of a number of diseases. It has been revealed through literature studies that cathepsin B is by far the most abundantly expressed mammalian cathepsin. Increased expression of cathepsin B levels in patients with heart failure suggests its role in the genesis and development of cardiac disease [1]. Furthermore, cathepsin B is negatively correlated with other pathological conditions like pancreatitis [2,3] osteoarthritis [4], gastric cancer [5], oral cancer metastasis [6], colorectal cancer [7] and ovarian cancer [8,9]. Cathepsin H, an aminopeptidase, is also associated with various pathological conditions like human fibrous meningioma [10], colorectal cancer [11], arthritis [12], human prostate tumor [13] and lung cancer [14].

Literature survey suggests that a large work has been accomplished on peptides or peptidyl analogues as inhibitors to cysteine proteases [15,16]. Various compounds such as flavones [17] (I), aldehyde [18] (II), acyloxymethyl ketone [19] (III), cyclopropenones [20] (IV) and nitriles [21] (V) as peptidyl or thiadiazoles [22] (VI) and epoxide [23] (VII) have also been reported as (peptidyl and non peptidyl) inhibitors of cathepsin B are shown in Fig. 1.

Peptidyl inhibitors are not considered to be viable drug candidates for treating diseases like cancer, apoptosis, etc. because of gastric instability or the possibility of immunogenic reactions. Keeping in view the involvement of cathepsins B and H in different inflammatory and cancerous conditions research on non-peptidyl drugs has become an important aspect in drug research and development [24,25].

Low molecular weight compounds with different functionalities have also been identified as cysteine protease inhibitors and their potential use as anti parasitic agents [26–29] has been reported.

Pyrazole nucleus having two aryl substituent increased *in vitro* cytotoxic activity against human cancer cell lines [30]. Pyrazolines have also been reported to possess antibacterial [31], antifungal [32], antiviral [33], antiparasitic [34], antitubercular [35], insecticidal agents [36], antipyretic [37], diuretic [38], antidiabetic [39], tranquillizing [40], muscle relaxant [41], psychoanaleptic [42], anticonvulsant [43], antihypertensive [44], antidepressant [45] and anticancer [46] activities. They have also been found to be Nitric oxide synthase (NOS) inhibitors and had shown Cannabinoid CB1 receptor antagonist activity [47].

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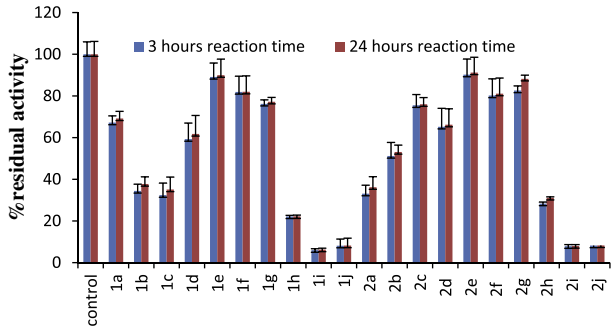


Fig. 1. Effect of substituted N-formylpyrazolines and N-benzoylpyrazolines on the endogenous proteolytic activity for 3 h and 24 h reaction. The data in each bar represents the % residual activity in presence of individual compound w.r.t. control taken as 100.

We have previously reported the effect of some semicarbazones, thiasemicarbazones, pyrazoles and pyrazolines as inhibitors of endogenous proteolytic activities [48–53]. Recently we have reported bischalcones and their derivatives [54]; acylhydrzides [55]; o-hydroxychalcones and their cyclized derivatives [56] as novel inhibitors of cathepsins B and H. Evaluation of pyrazolines as anti-inflammatory and anticancer agents and role of cathepsins B and H in these diseased conditions emphasize the importance of study of pyrazoline derivatives as inhibitors [57] of these enzymes. Toward this endeavor, in the present study we report the synthesis and screening of N-formyl and N-benzoylpyrazolines as cathepsins B and H inhibitors not reported earlier. The present study may provide new therapeutic opportunities in cancer treatment. The results are further compared with *in silico* studies which support the postulation that synthesized compounds may act as enzyme inhibitors.

2. Materials and methods

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

2.1. General procedure

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

2.2. Synthesis of compounds

2.2.1. General method for the synthesis of N-formylpyrazolines [58]

A mixture of chalcone (0.01 moles), hydrazine hydrate (0.02 moles) and formic acid (0.01 moles) was refluxed for 6 h in absolute ethanol (50 mL). The solution was kept for cooling at room temperature, and the solid formed was filtered off, washed with water, dried and recrystallized from ethanol.

2.2.2. General method for the synthesis of N-benzoylpyrazolines [59]

Equimolar mixture of chalcone and benzoic hydrazide was refluxed for 6 h in absolute ethanol in the presence of a few drops of acetic acid as catalyst. The solution was kept for cooling at room temperature, and the solid formed was filtered off, washed with water, dried and recrystallized from ethanol.

The characterization of all the compounds has been done on the basis of literature melting points, IR and ^1H NMR spectral data which has been provided in the Supplementary data.

2.3. Proteolytic studies

2.3.1. Preparation of liver homogenate

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

2.3.2. Assay for proteolytic activity

The proteolytic activity was estimated at pH 5.0, 37 °C using 0.1 M acetate buffer as the incubation medium. The homogenate prepared above was incubated with the buffer at 37 °C for 3 h and 24 h, separately. The reaction was stopped by the addition of TCA and the resulting solution was centrifuged to precipitate proteins. The acid soluble proteins were quantitated in the supernatant using Bradford method [60]. The experiments were conducted in triplicate and the results are presented in Table 1 (Fig. 1).

2.3.3. Purification of goat liver cathepsin B and cathepsin H

All the purification steps were carried out at 4 °C. Cathepsin B and H were isolated, separated and purified as reported previously [54] including the steps of acetone powder preparation, homogenization, acid-autolysis, 30–80% ammonium sulfate fractionation, molecular sieve chromatography on Sephadex G-100 column chromatography and finally ion-exchange chromatographies on CM-Sephadex C-50 and DEAE Sephadex A-50 column. The enzymes were sufficiently pure to carry out enzyme assays in order to see the inhibitory effects of synthesized compounds with the help of specific synthetic substrates. The purity of enzymes after the above mentioned procedure has been checked by electrophoresis to a single band homogeneity and has been reported [61,62]. The specific activities of the cathepsin B and cathepsin H obtained were ~11.15 and ~22.91 nmol/min/mg, respectively.

2.3.4. Effect of synthesized compounds on the activity of cathepsin B and cathepsin H

The activities of cathepsin B were estimated at varying concentrations of synthesized compounds (Fig. 2a and b), separately. First of all, enzyme was equilibrated in 0.1 M phosphate buffer, pH 6.0 at 37 °C. The stock solutions of compounds were prepared in DMSO. Appropriate amount of individual compounds at different concentrations were added in the reaction mixture separately. After incubation time of 30 min. residual enzyme activity was estimated by the usual enzyme assay [54] at pH 6.0 using a-N-benzoyl-D,L-arginine-2-naphthylamide (BANA) as substrate. The experiments were performed in triplicate for each concentration and averaged before further calculations. The % activity in each case has been calculated with respect to the control where no compound was added but an equivalent amount of solvent was present. The results are presented in Table 1. Similar experiments

Table 1
Enzyme inhibition studies in presence of substituted N-formylpyrazolines and N-benzoylpyrazolines.

S. no.	Compound name	(Protease activity)		Enzyme inhibition studies			
		% Residual activity at 10^{-4} M concentration of compounds		Cathepsin B		Cathepsin H	
		3 h incubation	24 h incubation	% Residual activity at $(Z) \times 10^{-6}$ M concentration of compounds	K_i (10^{-8} M)	% Residual activity at $(Z) \times 10^{-5}$ M concentration of compounds	K_i (10^{-8} M)
	Control	100	100	100		100	
1.	N-formyl-3,5-diphenylpyrazoline (1a)	67.2 ± 5.9	69.20 ± 6.2	67.21 ± 6.6 (0.1)	2.27 ± 0.21	76.78 ± 6.9 (0.1)	17.4 ± 1.74
2.	N-formyl-5-(-2′-chlorophenyl)-3-phenylpyrazoline (1b)	34.43 ± 3.2	37.75 ± 3.4	57.12 ± 3.5 (0.1)	1.80 ± 0.17	61.38 ± 4.9 (0.1)	13.5 ± 1.35
3.	N-formyl-5-(-3′-chlorophenyl)-3-phenylpyrazoline (1c)	32.40 ± 3.2	35.10 ± 3.4	34.610 ± 0.3 (0.1)	1.46 ± 0.14	49.57 ± 3.0 (0.1)	13.0 ± 1.30
4.	N-formyl-5-(-4′-chlorophenyl)-3-phenylpyrazoline (1d)	52.29 ± 5.8	61.77 ± 6.0	66.25 ± 5.7 (0.1)	2.11 ± 0.20	69.38 ± 6.1 (0.1)	13.7 ± 1.32
5.	N-formyl-5-(-2′-methoxyphenyl)-3-phenylpyrazoline (1e)	89.11 ± 7.7	89.87 ± 8.9	91.82 ± 0.9 (0.1)	6.68 ± 0.66	92.97 ± 8.6 (0.1)	38.7 ± 3.86
6.	N-formyl-5-(-3′-methoxyphenyl)-3-phenylpyrazoline (1f)	81.92 ± 6.7	82.00 ± 7.7	89.76 ± 8.3 (0.1)	4.55 ± 0.45	86.03 ± 8.0 (0.1)	27.2 ± 2.70
7.	N-formyl-5-(-4′-methoxyphenyl)-3-phenylpyrazoline (1g)	76.20 ± 7.5	77.20 ± 7.6	82.87 ± 0.7 (0.1)	4.48 ± 0.44	80.06 ± 7.6 (0.1)	22.1 ± 2.20
8.	N-formyl-5-(-2′-nitrophenyl)-3-phenylpyrazoline (1h)	22.17 ± 1.8	22.24 ± 2.1	28.89 ± 0.2 (0.1)	1.41 ± 0.14	30.20 ± 2.7 (0.1)	9.63 ± 0.96
9.	N-formyl-5-(-3′-nitrophenyl)-3-phenylpyrazoline (1i)	6.000 ± 0.5	6.280 ± 0.6	10.410 ± 0.9 (0.1)	0.11 ± 0.01	14.15 ± 1.0 (0.1)	5.19 ± 0.51
10.	N-formyl-5-(-4′-nitrophenyl)-3-phenylpyrazoline (1j)	8.140 ± 0.7	8.20 ± 0.7	23.47 ± 1.1 (1.0)	1.13 ± 0.11	27.64 ± 1.4 (1.0)	5.80 ± 0.58
11.	N-benzoyl-3,5-diphenylpyrazoline (2a)	33.10 ± 3.2	36.20 ± 3.6	40.17 ± 3.3 (1.0)	115.2 ± 11.0	66.19 ± 6.6 (1.0)	320 ± 3.20
12.	N-benzoyl-5-(-2′-chloro phenyl)-3-phenylpyrazoline (2b)	51.19 ± 4.1	53.10 ± 5.0	29.28 ± 2.8 (1.0)	77.5 ± 7.75	55.13 ± 4.4 (1.0)	189 ± 0.18
13.	N-benzoyl-5-(-3′-chloro phenyl)-3-phenylpyrazoline (2c)	75.51 ± 6.5	76.01 ± 3.3	27.51 ± 2.7 (1.0)	67.7 ± 6.76	44.20 ± 3.5 (1.0)	181 ± 1.80
14.	N-benzoyl-5-(-4′-chloro phenyl)-3-phenylpyrazoline (2d)	65.19 ± 5.2	66.20 ± 3.2	32.54 ± 3.0 (1.0)	92.5 ± 9.25	62.47 ± 5.5 (1.0)	301 ± 3.00
15.	N-benzoyl-5-(-2′-methoxyphenyl)-3-phenylpyrazoline (2e)	90.21 ± 8.9	91.21 ± 7.6	82.66 ± 5.9 (1.0)	490.1 ± 49.0	79.37 ± 7.6 (1.0)	965 ± 9.61
16.	N-benzoyl-5-(-3′-methoxy phenyl)-3-phenylpyrazoline (2f)	80.20 ± 7.5	81.10 ± 7.4	58.17 ± 5.2 (1.0)	159.4 ± 15.90	76.06 ± 7.0 (1.0)	503 ± 50.1
17.	N-benzoyl-5-(-4′-methoxy phenyl)-3-phenylpyrazoline (2g)	82.59 ± 8.0	88.20 ± 7.5	51.85 ± 4.0 (1.0)	127.7 ± 12.77	69.69 ± 6.7 (1.0)	410 ± 41.1
18.	N-benzoyl-5-(-2′-nitrophenyl)-3-phenylpyrazoline (2h)	28.34 ± 2.2	31.08 ± 1.8	26.46 ± 2.1 (1.0)	57.2 ± 5.72	34.77 ± 2.9 (1.0)	144 ± 1.42
19.	N-benzoyl-5-(-3′-nitrophenyl)-3-phenylpyrazoline (2i)	7.90 ± 0.8	8.000 ± 0.5	20.77 ± 1.8 (1.0)	19.5 ± 1.95	27.49 ± 2.7 (1.0)	98 ± 9.80
20.	N-benzoyl-5-(-4′-nitrophenyl)-3-phenylpyrazoline (2j)	8.20 ± 0.8	8.27 ± 0.7	21.46 ± 2.1 (1.0)	28.7 ± 2.87	28.77 ± 2.6 (1.0)	127 ± 12.65

The TCA soluble peptides were estimated at 630 nm using Bradford method [48] and the results are the mean and S.M.D. of the experiment conducted in triplicate and is calculated as % residual protease activity in 0.1% liver homogenate w.r.t. control where no compound was added but an equivalent amount of solvent was present. Cathepsin B and cathepsin H activities were calculated using BANA and Leu-βNA as substrates at pH 6.0 and 7.0, respectively. The enzyme activity was determined at minimum inhibitory concentration of each compound given in parenthesis. The specific activity of the cathepsin B and cathepsin H were ~11.15 nmol/min/mg and ~22.91 nmol/min/mg, respectively. In order to determine K_i values, experiments were conducted in triplicates in presence and absence of a fixed concentration of different compound, separately. The results were then plotted between 1/V and 1/S to obtain Line-weaver Burk plots and all the compounds were established as competitive inhibitors. The K_i values were calculated using Line-weaver Burk equations for competitive inhibition.

were designed to evaluate the effect of varying concentrations of synthesized compounds separately on cathepsin H using LeuβNA as substrate [54] at pH 7.0 (Fig. 2c and d).

2.3.5. Kinetic studies of synthesized compounds on cathepsin B and cathepsin H

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

2.4. Drug modeling studies

All docking studies were performed using iGemdock. For these studies, small molecular weight ligands and enzyme active site structure is required. The structure of cathepsin B [63] and cathepsin H [64] were retrieved from Protein Data Bank as (cav2IPP B_PYS.pdb), and (cav8PCH H_NAG.pdb), respectively. The active site box was selected as such provided in the pdb file of 8 Å radius excluding the reference ligands. The structures were prepared in Marvin sketch, minimized and were saved as MDL Mol File. The prepared ligands and the binding site were loaded in the iGemdock software and docking was started by setting the GA-parameters at drug screening setting with population size 200, generation 70 and no of solutions 3. The results presented in Table 2 pertain to the interaction data. Fitness is the total energy in kcal/mol of a predicted pose in the binding site. The empirical scoring function of iGemdock is the sum total of Van der Waal, H-bonding and electrostatic energy. The best docked pose of the substrates in the active site of cathepsins B and H along with the most inhibitory or the reference compounds are shown in Fig. 4.

3. Result and discussion

All the synthesized substituted N-formylpyrazolines and substituted N-benzoylpyrazolines (Scheme 1) show a characteristic IR absorption peak. The structure elucidation of compounds was based on the spectral data (IR & ^{1}H NMR). The IR spectra showed mainly stretching bands at 1610–1580, 1600–1450 and 2950–2800 cm^{-1} assigned to (C═N), aromatic (C═C) and (C—H) functionalities, respectively. In the ^{1}H NMR spectrum, an ABX pattern was observable; H_a, H_b and H_x appear as double doublets at δ 3.10–3.30, 3.75–3.80 and 5.40–5.50 ppm with (J_{ab} = 16.2–18.0 Hz, J_{ax} = 4.5–7.2 Hz, J_{bx} = 10.8–12.3 Hz). The protons of the aromatic ring were observed at 7.03–7.76 ppm in case of N-benzoylpyrazolines and the formyl proton appeared as a singlet at 8.90 ppm in case of N-benzoylpyrazolines.

3.1. Effect of synthesized compounds on in vitro endogenous proteolysis in liver homogenate

Table 1 presents the results of proteolytic studies conducted at pH 5.0 on endogenous protein substrate in presence of different

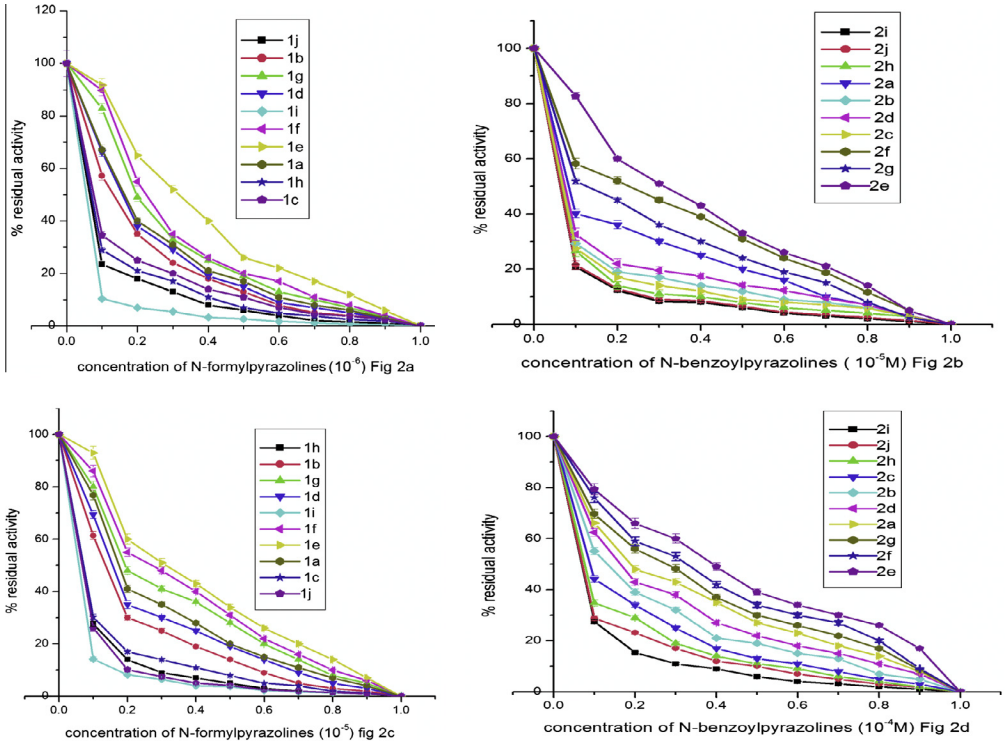


Fig. 2. Effect of N-formylpyrazolines, 1a–1j, (a) and N-benzoylpyrazolines, 2a–2j, (b), on cathepsin B activity. Effect of N-formylpyrazolines, 1a–1j, (c) and N-benzoylpyrazolines, 2a–2j, (d) on cathepsin H activity. Results are mean of experiments conducted in triplicate. % Residual activities are presented w.r.t. control which contain equivalent amount of solvent.

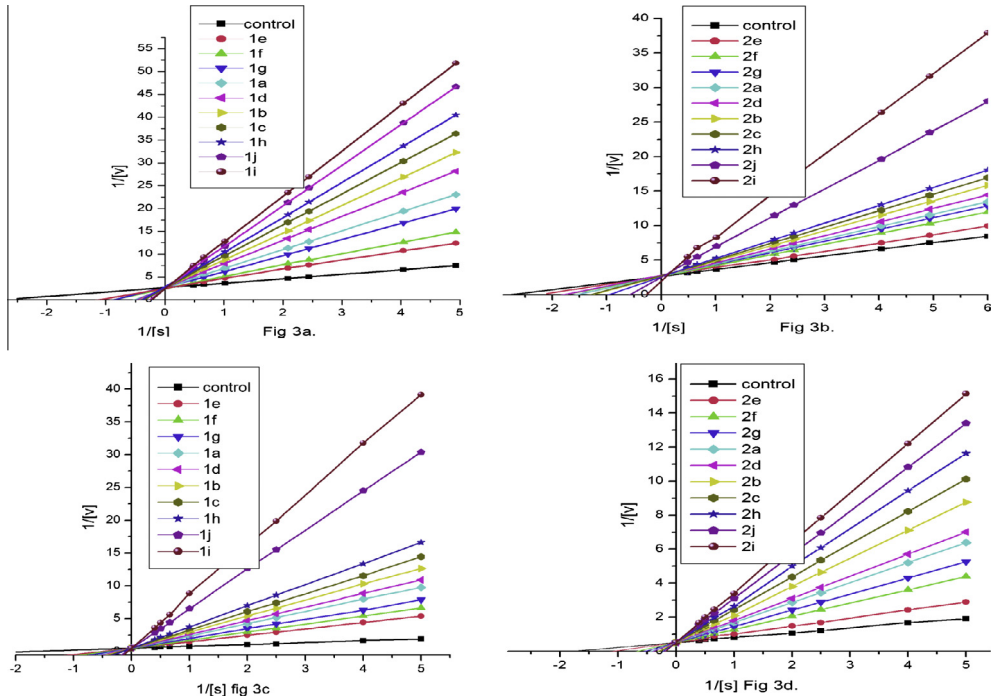


Fig. 3. Line-weaver Burk plot for cathepsin B at varying concentrations of BANA in presence of 1×10^{-7} M concentration of N-formylpyrazolines (a), 1×10^{-6} M concentration of N-benzoylpyrazolines (b), respectively at pH 6.0. The K_m value for control have been found to be 3.64×10^{-4} M. Line-weaver burk plot for cathepsin H on varying concentrations of leu-ßNA in presence of 1×10^{-6} M concentration of N-formylpyrazolines (c), 1×10^{-5} M concentration of N-benzoylpyrazolines (d), respectively at pH 7.0. The K_m value for control have been found to be 5.34×10^{-4} M. The K_i values as calculated from this graph are presented in Table 1.

compounds separately at 10^{-4} M. It can be observed that proteolytic activity is inhibited appreciably in presence of these compounds. Further, it was found that the inhibition was more at 3.0 h and less at 24.0 h suggesting that inhibition caused by the compounds is of reversible type because initial inhibition caused by the compounds is reversed when incubated for a longer time. The compounds bearing nitro group led to a dramatic decrease in proteolytic activity and showed higher inhibition than those of the corresponding

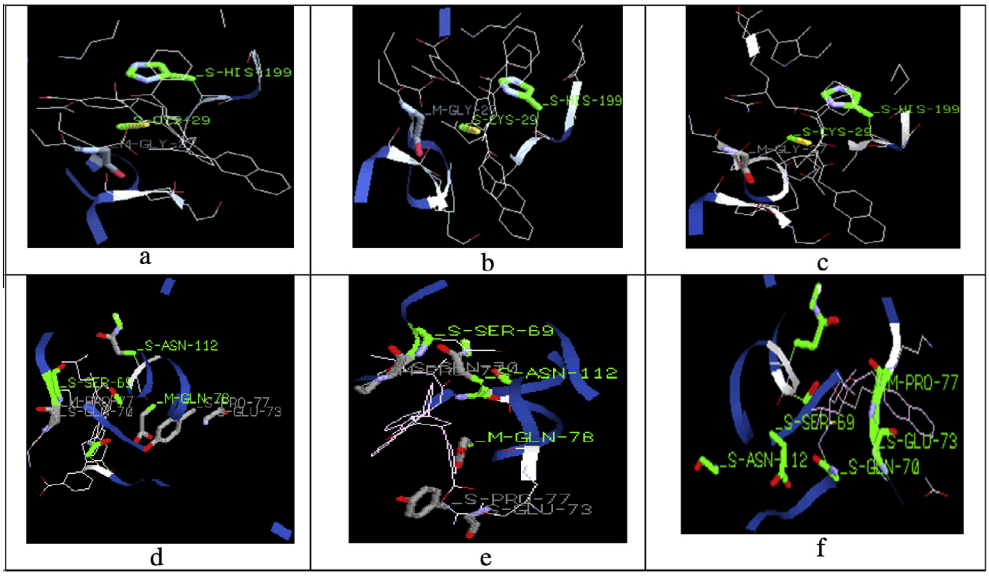
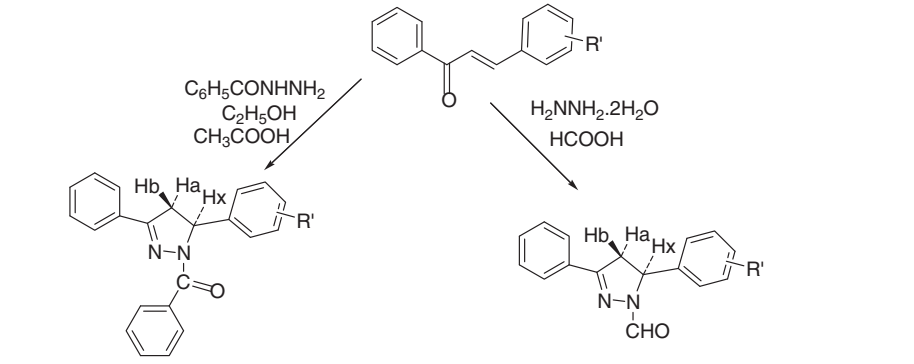


Fig. 4. Docking results showing the alignment of most inhibitory compounds along with the substrate in the active site of cathepsin B (cav2IPP B_PYS.pdb) and LeußNA in the active site of cathepsin H (cav8PCH H_NAG.pdb). Here (a and b) show alignment of 1i and 2i, along with BANA in the active site of cathepsin B (cav2IPP B_PYS.pdb), respectively; and (c and d) show the alignment of 1i and 2i along with Leu ßNA in the active site of cathepsin H (cav8PCH H_NAG.pdb), respectively.



Scheme 1.

compounds with methyl or methoxy group at the same position indicating that activity decreases in presence of electron withdrawing groups whereas it is less affected by the presence of electron donating groups. A similar trend has previously been reported for semicarbazones [49], phenylhydrazones [50] and arylhydrazones [51]. Concrete conclusions about the structure activity relationship and the potency of compounds cannot be drawn at this stage because at this pH a large number of proteases are present in the homogenate which are responsible for hydrolysis of proteins. At pH 5.0, where most of the proteolytic activity is attributed to cysteine proteases it was thought proper to study the effect of synthesized compounds on purified cathepsin B and cathepsin H.

3.2. Effect of synthesized compounds on the activities of cathepsin B and cathepsin H

The activities of cathepsin B and cathepsin H were estimated at varying concentrations of title compounds (Fig. 2a–d), respectively. The figures show the relationship between the enzyme activity and concentration of different compounds. It can be observed that at 10^{-4} M concentration of each compound, where proteolytic activity was not completely blocked, cathepsins B and H were completely inhibited emphasizing the importance of study on individual enzymes. The results also imply that in addition to cathepsins B and H other cysteine proteases are also active at pH 5.0 and are responsible for the proteolytic activities which are insensitive toward the designed compounds signifying that the compounds act as specific inhibitors of cathepsins B and H. Among the various compounds tested, **N-formyl-5-(-3′-nitrophenyl)-3-phenylpyrazoline (1i)** was found to be most inhibitory to cathepsin B and cathepsin H activity in series of N-formylpyrazolines. Similar trend was observed in case of N-benzoylpyrazolines where 2i was evaluated as most inhibitory. It has been found that cathepsin B and cathepsin H activity is maximally inhibited by the nitro substituent in both the series.

3.3. Enzyme kinetic studies

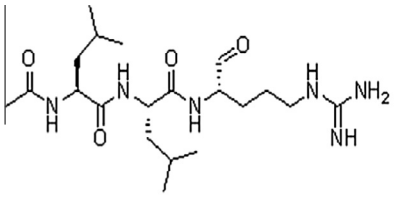
Line-weaver Burk plots drawn in presence and absence of inhibitors for cathepsin B (Fig. 3a and b) and for cathepsin H (Fig. 3c and d) separately show that the plots of 1/V vs 1/S were straight lines intersecting at the *Y*-axis with constant V_{max} values in all the compounds whereas the value of K_m changed in presence of each compound. These studies suggested a competitive type of inhibition exhibited by these compounds. Using the Line-weaver Burk equation for competitive inhibition the K_i values were calculated, which

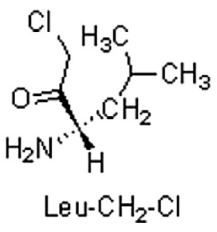
Table 2
Docking studies showing decrease in different energies of cathepsin B & H in presence of substituted N-formylpyrazolines and N-benzoylpyrazolines.

Compound	Enzyme							
	Cathepsin B				Cathepsin H			
	Total energy, kcal/mol	VDW, kcal/mol	HBond, kcal/mol	Electronic, kcal/mol	Total energy, kcal/mol	VDW, kcal/mol	HBond, kcal/mol	Electronic, kcal/mol
BANA	−128.61	−87.34	−38.19	−3.08	–	–	–	–
Leupeptin	−113.44	−88.45	−25.00	0	–	–	–	–
Leu-βNA	–	–	–	–	−77.01	−61.81	−15.21	0
LeuCH$_2$Cl	–	–	–	–	−59.12	−46.12	−13	0
1a	−80.16	−63.99	−16.18	0	−77.06	−70.16	−6.89	0
1b	−83.90	−68.50	−15.40	0	−77.60	−68.09	−9.5	0
1c	−82.27	−65.47	−16.80	0	−80.82	−73.84	−6.97	0
1d	−81.27	−64.33	−16.94	0	−79.40	−69.18	−10.22	0
1e	−84.11	−66.30	−17.82	0	−79.63	−69.13	−10.50	0
1f	−82.33	−71.85	−10.49	0	−83.50	−76.50	−7.00	0
1g	−83.66	−66.27	−17.39	0	−84.89	−72.98	−11.92	0
1h	−93.63	−65.00	−28.63	0	−94.42	−68.37	−25.66	−0.38
1i	−89.40	−59.53	−30.36	0.49	−93.28	−70.78	−22.50	0
1j	−84.79	−67.80	−17.28	0.29	−90.38	−71.08	−18.66	−0.64
2a	−91.38	−77.80	−13.57	0	−82.09	−71.16	−10.92	0
2b	−99.23	−84.80	−14.43	0	−84.34	−73.93	−10.41	0
2c	−96.42	−82.94	−13.48	0	−82.04	−71.028	−11.02	0
2d	−96.36	−82.24	−14.12	0	−84.54	−74.94	−9.60	0
2e	−97.53	−83.87	−13.66	0	−88.21	−76.21	−12.00	0
2f	−95.52	−78.43	−17.09	0	−87.09	−76.59	−10.50	0
2g	−96.89	−82.44	−14.44	0	−88.37	−74.59	−13.78	0
2h	−102.48	−73.86	−29.07	0.46	−93.00	−76.90	−15.70	−0.39
2i	−110.71	−81.43	−29.28	0	−99.53	−68.57	−30.43	−0.52
2j	−106.98	−78.58	−28.10	−0.30	−95.23	−76.90	−18.34	0

The results are one of the docking experiments run using iGemdock under drug screening settings. The ligands were loaded as MDL mol file. The active site was extracted from the structure of cathepsin B and cathepsin H were retrieved from Protein Data Bank (http://www.rcsb.org/) as cav2IPP B_PYS.pdb [48], and (cav8PCH H_NAG.pdb) [49], respectively.

have been compiled in Table 1. The K_i value of most inhibiting compound for cathepsin B in the corresponding series i.e. N-formylpyrazolines and N-benzoylpyrazolines has been evaluated as $\sim 1.1 \times 10^{-9}$ M and 19.5×10^{-8} M for compounds 1i and 2i, respectively; similarly for cathepsin H, these compound showed maximum inhibition with K_i values $\sim 5.19 \times 10^{-8}$ M and 9.8×10^{-7} M respectively. We have demonstrated here that in general, these compounds showed more inhibition on cathepsin B activity in comparison to cathepsin H. Analysis of the effect of these compounds on cathepsin B and cathepsin H by the combination of serendipitous biological selectivity with target-directed chemical specificity suggests an optimistic future for their use as cysteine protease inhibitors as therapy in a number of disease processes. In order to ascertain inhibition ability of the studied compounds, results were compared with potential inhibitors of cathepsin B e.g. Leupeptin and cathepsin H e.g. Leu-CH$_2$—Cl, respectively. As reported in literature, Leupeptin being a potential peptide inhibitor of cathepsin B [65], inhibited the goat brain cathepsin B [61] competitively with K_i value of 12.5×10^{-9} M whereas K_i value for human liver cathepsin B [66] was reported to be 7.0×10^{-9} M. In contrast, K_i value for human liver cathepsin [67] H was reported to be 9.2×10^{-6} M. Inhibition by leupeptin has been attributed to the exact complementarily of peptidyl side chain and the active site directed formyl group that is susceptible to nucleophilic attack by the thiol group present at the active site of both these enzymes.





It can be observed that leupeptin showed ~98.8% inhibition at 10^{-6} M concentration for cathepsin B whereas it showed ~51.8% inhibition at 10^{-5} M concentration for cathepsin H which is in accordance with the previously reported results. Similarly, Leu-CH$_2$—Cl showed ~9.3% inhibition at 10^{-5} M concentration for cathepsin B whereas it showed ~93.5% inhibition at 10^{-5} M concentration for cathepsin H. The results obtained are comparable with earlier results reported for brain cathepsin B [68] and cathepsin H [62]. In the present context, we observed that the inhibition of cathepsin B caused by the compound, 1i, is quite comparable to leupeptin where the K_i value for the title compound has been found to be 1.1×10^{-9} M. Inhibition caused by 1i is quite significant where there is no complementary binding site in the molecule as is present in leupeptin. N-formylated pyrazolines show more inhibition due to better electrophilic center as compared to N-benzoylated pyrazolines and therefore are better inhibitors for these enzymes. In addition, the significance of benzoylated pyrazolines can be well correlated with indomethacin which is *p*-chloro benzoyl derivatives of indole acetic acid, the compound act as a non steroid anti-inflammatory drug and is known to inhibit cyclooxygenase enzyme responsible for inflammation. It may be worth mentioning here that lysosomes have long been reported as agents of inflammation in polyarthritis, bacterial endotoxicity and rheumatoid arthritis [69–71]. The anti-inflammatory activity of indomethacin can be assigned due to cyclooxygenase inhibition as well as inhibition of cathepsins [72]. The synthesized pyrazoline derivatives have been found to inhibit cathepsin B and H up to sub nanomolar range indicating the possible use of title compounds as anti-inflammatory compounds.

3.4. Molecular docking experiment

Docking methods can provide valuable insight into the binding mode between the ligand and the enzyme active site thereby have an important role in the understanding of ligand–enzyme interactions. On the basis of the interaction data of docking experiments

that include total energy and individual energy terms, an indicative of the fitness of a predicted pose in the binding site, it is suggested that the level of interaction is highest for N-benzoylpyrazolines (2a–2j) followed by N-formylpyrazolines (1a–1j) with the active site of cathepsin B and the same order is found in case of cathepsin H (Table 2). In cathepsin B, all the compounds showed a lesser interaction than the peptidyl inhibitor, leupeptin. Decrease in total energy (kcal/mol) for leupeptin–cathepsin B has come out be −113.44 which is quite close to substrate BANA-128.61 in comparison to the compounds under consideration. This is due to peptide protein interaction. Leupeptin is peptidyl in nature and flexibility in the peptide molecule provide better interactions and therefore binds effectively with the enzyme active site resulting in higher binding energy. The synthesized compounds are smaller in structure and provide lesser interaction area. This is further explained while discussing the docking results of cathepsin H. From molecular docking experiments, it can be interpreted that the compounds should inhibit cathepsin B to a lesser extent in comparison to leupeptin. We have found that the most inhibitory compound, 1i, inhibited the enzyme with the K_i value of 1×10^{-9} M which is equivalent to leupeptin (detailed before). Thus, the present study justifies its importance that mere computational data may not be helpful in designing the enzyme inhibitors that eventually can evolve as therapeutic agent. An experimental *in vitro* study is equally or more informative.

Fig. 4a and b shows the docked view of most inhibitory compounds, 1i and 2i along with the substrate BANA in the active site of cathepsin B. The docked view of standard inhibitor leupeptin along with BANA has been displayed in Fig. 4c for comparison. The amino acid of the active site involved in the H-bonding with the compound is detailed as green, the structure of the compound is shown as thin lines. It can be observed that active site amino acids Cys-29, His-199 interact with the most inhibitory compound as well as with the leupeptin. Therefore, we can observe that interaction of designed compounds with the active site of the enzyme is similar to that of leupeptin. Literature reports that leupeptin is a competitive inhibitor of the enzyme cathepsin B therefore we expect that the compounds should also inhibit the enzyme in a competitive manner. Docking experiments supports the *in vitro* studies. It can further be distinguished from these figures that the compounds and leupeptin are in alignment with substrate BANA and bind at the same site of enzyme. On comparing the docking energies of the most inhibitory formyl and benzoyl pyrazolines, 1i and 2i, with the most inhibitory previously reported [57] most inhibitory 1,3,5-triphenylpyrazolines and 3,5-diphenylpyrazolines we found that for cathepsin B compounds, 2d and 4d, which are also nitro substituted show a decrease in total energy of interaction approx 90–86 kcal/mol. It can further be observed that functionalization of pyrazolines resulted in significant inhibition of cathepsin B thus validating the significance of present study. The most inhibitory 1,3,5-triphenylpyrazolines and 3,5-diphenylpyrazolines, 2d and 4d, previously reported from our work exhibited K_i values for cathepsin B in 42 nM and 53 nM, respectively however in the present study we have found that formylated pyrazoline 1i, exhibited a K_i value in 1 nM, equivalent to leupeptin, is also a formylated peptidyl inhibitor.

In cathepsin H, the decrease in total energy for the reference inhibitor Leu-CH_2Cl was less as compared to all the designed compounds (Table 2). Here, it can be seen that though Leu-CH_2Cl is specific inhibitor for cathepsin H [71], but possess only one amino acid residue as compared to leupeptin–cathepsin B complex. Therefore, the leu-CH_2Cl-cathepsin H interaction causes a lesser decrease in energy of −59.12 kcal/mol only. The interaction energy data suggest the compounds are better inhibitors than the standard leu-CH_2Cl. The docked view of most inhibitory compounds, 1i and 2i along with leu-βNA in the active site of cathepsin H are presented in Fig. 4d and e. For comparison Fig. 4f, detailing the interaction of leu-CH_2Cl along with leu-βNA is also provided here. All these compounds interact with amino acyl acceptor site of the enzyme. Amino acids Ser-69, Gln-78 and Asn-112 interact with the 1i, 2i and leu-CH_2Cl as well as leu-βNA, therefore indicating a competitive inhibition caused by these compounds, which is confirmed by enzyme kinetic studies. The results reported here are slightly different from previously reported 1,3,5-triphenylpyrazolines and 3,5-diphenylpyrazolines, where 2b and 4b which are chloro-substituted were the most inhibitory compounds with the K_i values of 1.17 and 1.56 μM, respectively. However the corresponding nitro substituted compounds were also found to exhibit significant K_i values of 2.3 and 3.3 μM. Overall we can conclude that electron withdrawing groups affected the inhibitory potential of the compounds.

In the present work we can see that although the *in vitro* inhibition and *in silico* interaction data do not correlate well but type of inhibition exhibited by these compounds can be visualized from the docked poses and interacting amino acids. In both the enzymes, the enzyme–ligand interaction data is more for benzoylated pyrazolines than formylated pyrazolines. A plausible explanation can be proposed on the basis of more interaction surface area provided by phenyl group in computational studies and a prone electrophilic center of formyl group responsible for more inhibitory potential of formyl group. In these two series of compounds, benzoyl group can provide more interaction sites due to the larger surface area as compared to H-present in formylated compounds, therefore the decrease in energy as found in computational study is more as compared to formyl group. However in formylpyrazolines, the electrophilic center i.e. H—C=O is more susceptible to —SH nucleophilic attack when compared with C_6H_5—C=O site present in benzoylpyrazolines. The *in vitro* studies show the actual inhibitory potency of the compounds therefore evaluation of formylated pyrazolines as more potent inhibitors of these thiol enzymes, cathepsin B and cathepsin H is properly justified.

In order to explore nonpeptidyl novel inhibitors to cathepsin B & H, we here for the first time report the comparative study of these compounds on clinically significant enzymes cathepsin B & H. Such studies are important where the structurally related compounds showing potential biological activities are evaluated for their inhibitory effect on physiologically significant enzyme cathepsin B and cathepsin H.

Conflict of interest

The authors have declared no conflict of interest.

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Appendix A. Supplementary material

The data regarding the characterization of compounds can be found in the supplementary file. Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bioorg.2014.07.012.

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