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Research article

N-formylpyrazolines and N-benzoylpyrazolines as potential inhibitors cathepsin L

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Abstract: Elevated levels of cathepsins implicated in cancer, inflammation and number of degenerative diseases emphasize the investigation of potential inhibitors in search for novel chemotherapeutic agents with better efficacy. Along with other cathepsins, cathepsin L has emerged out as a potential drug target in these diseased conditions. In the present study, we have assayed the inhibitory potency of two structurally related series of substituted N-formylpyrazolines and N-benzoylpyrazolines as inhibitors to cathepsin L.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 ~6.4×10^{-10} and 5.7×10^{-9} M for cathepsin L, respectively. The inhibitory potential of the compounds have been found comparative to the specific inhibitor, leupeptin. Docking experiments showing interaction between N-formylpyrazolines, N-benzoylpyrazolines and cathepsin L active site also provided useful insights.

Keywords: N-formylpyrazolines; N-benzoylpyrazolines; cathepsin L inhibitors; endogenous proteolysis.

1. Introduction

Proteolytic enzymes have emerged out as promising targets for novel therapeutic treatment strategies seeking to impede cancer progression and metastasis. Cathepsin L, a lysosomal cysteine protease, composed of disulfide-linked heavy and light chains, produced from a single protein precursor, procathepsin L has been found upregulated in a variety of cancers. The elevated levels of cathepsin L has been widely correlated with metastatic aggressiveness and poor patient prognosis. Cathepsin L has also been implicated to contribute to cancer-associated osteolysis, a debilitating morbidity affecting both life expectancy and the quality of life.

A direct correlation between tumor progression, cathepsins level and decreased inhibitor concentration indicate the significance of identification of novel cathepsin inhibitors. Though a large work has been reported on peptide based inhibitors, recent focus on non-peptidyl inhibitors of cathepsins has brought into light various molecules belonging to different classes as potential inhibitors of cathepsins [1-7]. Most of these compounds including pyrazolines and pyrazoles [8-16] are reported to possess anticancer and anti-inflammatory activities. Pyrazolines have been found to inhibit cathepsins B and H [17].

Literature survey suggests that a large work has been accomplished on peptidyl or peptidyl analogues as inhibitors to cysteine proteases [18,19]. However, these inhibitors are not considered to be viable drug candidates for treating diseases like cancer, apoptosis etc. because of gastric instability or the possibility of immunogenic reactions. Therefore, research on non-peptidyl drugs has become an important aspect in drug research and development [20,21]. Keeping in view the involvement of cathepsin L in different cancerous conditions various compounds such as semicarbazones and hydrazones (I-II), schiff bases and their cyclized derivatives [22] pyrazolines [5,7] (III-IV), chalcones and bischalcones (V-VI) [7,23-25] as non-peptidyl inhibitors are reported as shown in Figure 1.

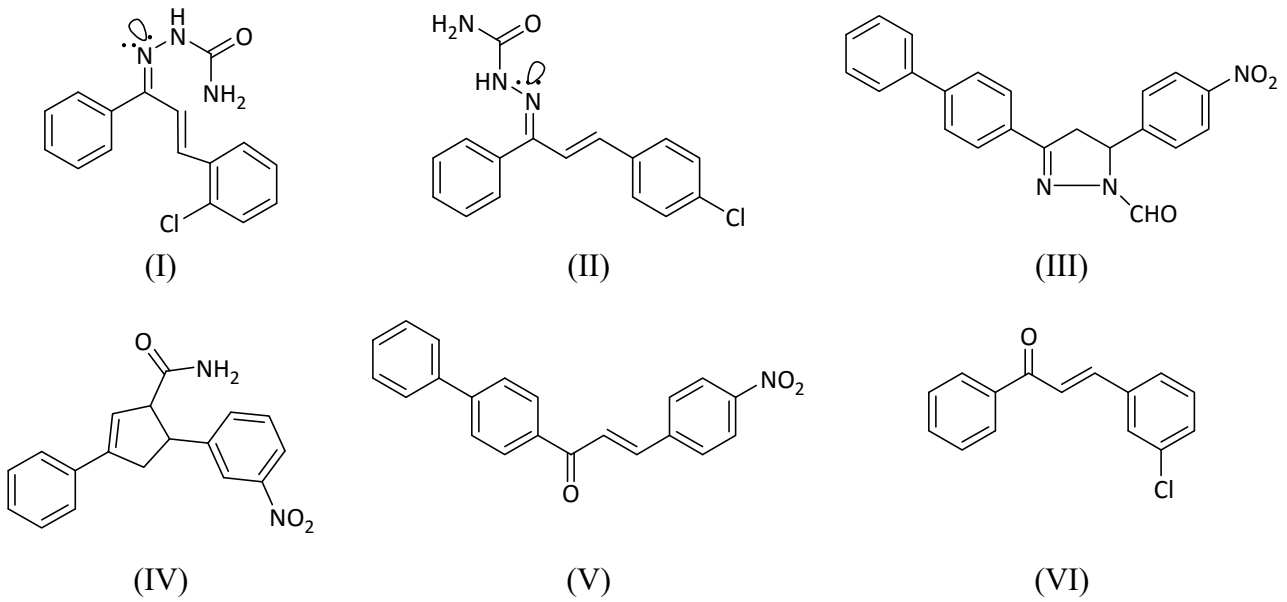


Figure 1. Representative non-peptidyl inhibitors of cathepsin L.

In continuation of our previous work [17], the present work reports the inhibition studies of N-formylpyrazolines and N-benzoylpyrazolines on cathepsin L.

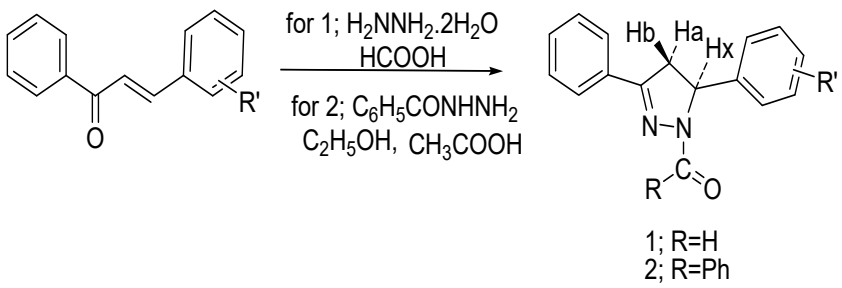
2. Materials and methods

All the chemicals (analytical grade) and biochemicals, Fast Garnet GBC (*o*-aminoazotoluene diazonium salt, substrate Z-Phe-Arg-4mβNA 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.

ELISA plate reader was used for measuring absorbance in the visible range. Refrigerated ultracentrifuge Remi C-24BL was used for centrifugation purpose.

2.1. Synthesis of N-formylpyrazolines and N-benzoylpyrazolines on the activity of cathepsin L

The synthesis (Scheme 1) and characterization of designed compounds, N-formylpyrazolines (1a–1j) and N-benzoylpyrazolines (2a–2j) has been accomplished by the method previously reported [17].



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

Scheme 1. 1; N-formyl-3-subsitutedphenyl-5-phenylpyrazoline,
2; N-benzoyl-3-substitutedphenyl-5-phenylpyrazoline.

2.2. Purification of liver cathepsin L

All the purification steps were carried out at 4 °C. Cathepsin L was isolated, separated and purified from goat liver using the following procedure [26]. The steps included acetone powder preparation, homogenization in cold 0.1 M acetate buffer pH 5.5 containing 0.2 M NaCl and 1 mM EDTA, acid-autolysis at pH 4.0 and 30–80% ammonium sulphate fractionation, molecular sieve chromatography on Sephadex G-100 column chromatography and finally ion-exchange chromatography on CM-Sephadex C-50 column. The specific activity of the cathepsin L was ~16.78 nmol/min/mg.

2.3. Effect of N-formylpyrazolines and N-benzoylpyrazolines on the activity of cathepsin L

The activities of cathepsin L were estimated by the usual enzyme assay [27] at pH 6.0 using Z-Phe-Arg-4mβNA as substrate in presence of reducing agent cysteine. First of all, 100 µL of preactivated enzyme (1.678 units) was equilibrated in 0.855 µL 0.1 M phosphate buffer, pH 6.0 at 37 °C, at varying concentrations of synthesized N-formylpyrazolines and N-benzoylpyrazolines (Figure 2i–ii), separately. The stock solutions of synthesized N-formylpyrazolines and N-benzoylpyrazolines (2a–2j) were prepared in DMSO. Appropriate amount of stock solutions of individual compounds and corresponding amount of DMSO (total 20 µL with a final concentration of DMSO 2%) was added in the reaction mixture to effect different concentrations of each compound as indicated in Figures 2i–ii, separately. After incubation time of 30 min. residual enzyme activity was estimated using 25 µL using Z-Phe-Arg-4mβNA as substrate.

The experiments were performed in triplicate for each concentration and averaged before further calculations. The % activity in each case has been calculated with respect to the control where no compound was added but an equivalent amount of DMSO (20 µL) was present. The results are presented in Table 1.

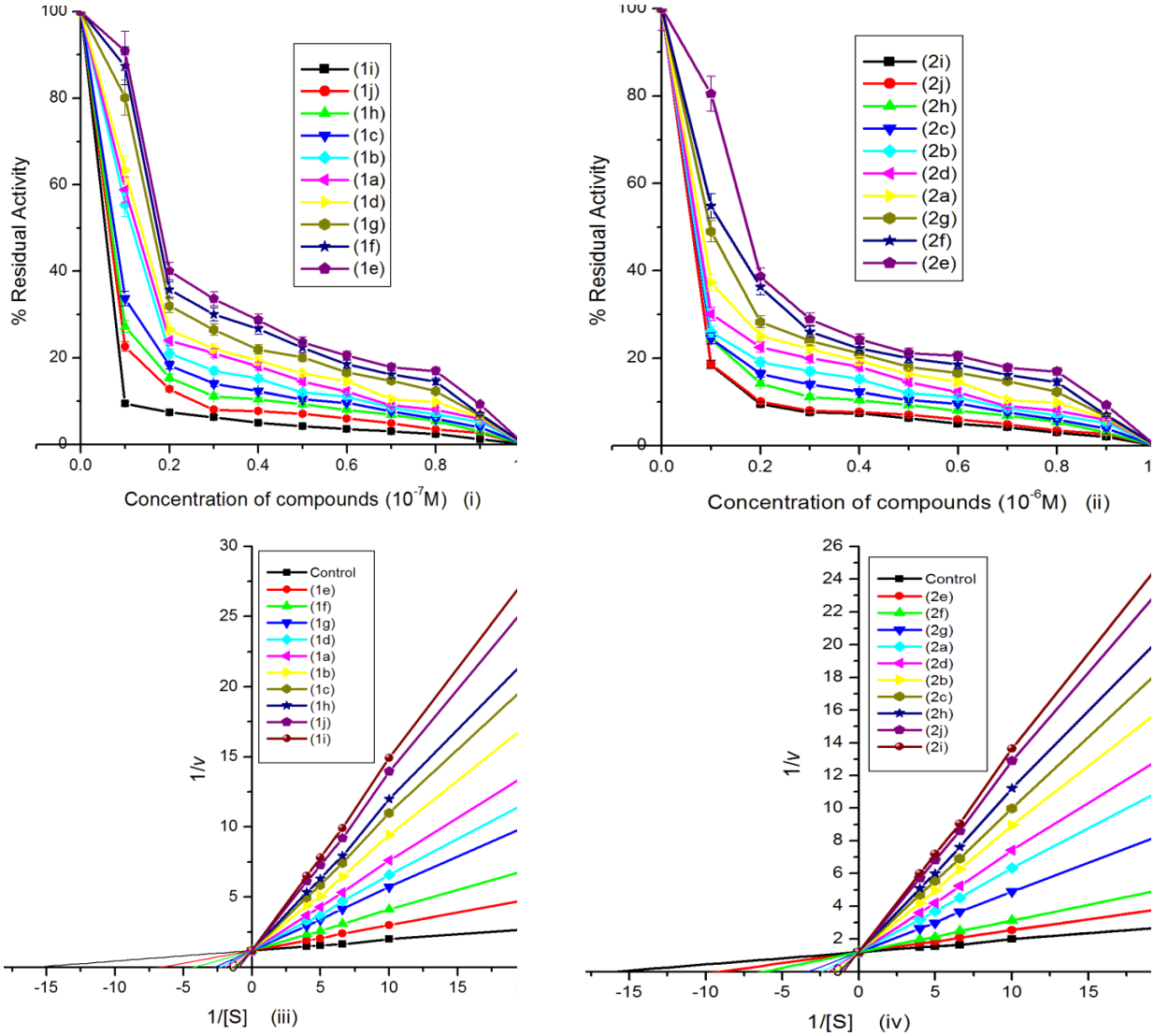


Figure 2. Effect of varying concentration of N-formylpyrazolines (1a–1j) 2(i), N-benzoylpyrazolines (2a–2j) 2(ii) at pH 6.0 on cathepsin L activity. Results are mean of experiment conducted in triplicate. Activities are % of control which contain equivalent amount of solvent. Line: weaver Burk plot for cathepsin L at varying concentrations of Z-Phe-Arg-4mβNA in presence of 1× 10^{-8} M concentration of N-formylpyrazolines (1a–1j) 2(iii) and 1 × 10^{-7} M concentration of N-benzoylpyrazolines (2a–2j) 2(iv), respectively at pH 6.0 using 100 µL of enzyme having specific activity ~16.78 units/mg. The K_m value for control has been found to be 6.024 × 10^{-5} M. The K_i values as calculated from this graph are presented in Table 1.

2.4. Kinetic studies of N-formylpyrazolines and N-benzoylpyrazolines on cathepsin L

After establishing the inhibitory action of compounds on cathepsin L, experiments were designed to evaluate the type of inhibition and to determine the K_i value of these compounds on cathepsin L. 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 L, Figure 2iii–iv. And the K_i values were calculated using the line-weaver burk equation for competitive inhibition $K_{m'} = K_m (1+I/ K_i)$.

Table 1. Cathepsin L inhibition studies in presence of Substituted N-formylpyrazolines and N-benzoylpyrazolines.

S. No.	Compound Name	% residual activity at (Z) × 10^{-7} M concentration of compounds	K_i (10^{-9} M)
	Control	100.0 ± 1.33	
1.	N-formyl-3,5-diphenylpyrazoline (1a)	58.81 ± 5.84 (0.1)	1.32
2.	N-formyl-5-(-2'-chlorophenyl)-3-phenylpyrazoline (1b)	55.35 ± 5.50 (0.1)	1.20
3.	N-formyl-5-(-3'-chlorophenyl)-3-phenylpyrazoline (1c)	33.68 ± 3.33 (0.1)	0.86
4.	N-formyl-5-(-4'-chlorophenyl)-3-phenylpyrazoline (1d)	63.36 ± 6.21 (0.1)	1.52
5.	N-formyl-5-(-2'-methoxyphenyl)-3-phenyl pyrazoline (1e)	90.86 ± 9.05 (0.1)	6.6
6.	N-formyl-5-(-3'-methoxyphenyl)-3-phenyl pyrazoline (1f)	87.40 ± 8.58 (0.1)	3.6
7.	N-formyl-5-(-4'-methoxyphenyl)-3-phenyl pyrazoline (1g)	80.12 ± 7.85 (0.1)	1.77
8.	N-formyl-5-(-2'-nitrophenyl)-3-phenylpyrazoline(1h)	27.20 ± 2.70 (0.1)	0.77
9.	N-formyl-5-(-3'-nitrophenyl)-3-phenylpyrazoline (1i)	9.47 ± 0.93 (0.1)	0.64
10.	N-formyl-5-(-4'-nitrophenyl)-3-phenylpyrazoline (1j)	22.58 ± 2.22 (0.1)	0.70
11.	N-benzoyl-3,5-diphenylpyrazoline(2a)	37.34 ± 3.66	16.65
12.	N-benzoyl-5-(-2'-chloro phenyl)-3-phenylpyrazoline (2b)	26.01 ± 2.57	11.33
13.	N-benzoyl-5-(-3'-chloro phenyl)-3-phenylpyrazoline (2c)	24.49 ± 2.40	10.66
14.	N-benzoyl-5-(-4'-chloro phenyl)-3-phenylpyrazoline (2d)	30.08 ± 2.99	13.69
15.	N-benzoyl-5-(-2'-methoxyphenyl)-3-phenyl pyrazoline (2e)	80.49 ± 8.03	112.00
16.	N-benzoyl-5-(-3'-methoxy phenyl)-3-phenyl pyrazoline (2f)	54.82 ± 5.48	56.60
17.	N-benzoyl-5-(-4'-methoxy phenyl)-3-phenyl pyrazoline (2g)	49.01 ± 4.86	26.71
18.	N-benzoyl-5-(-2'-nitrophenyl)-3-phenylpyrazoline (2h)	24.41 ± 2.40	8.49
19.	N-benzoyl-5-(-3'-nitrophenyl)-3-phenylpyrazoline (2i)	18.46 ± 1.86	5.73
20.	N-benzoyl-5-(-4'-nitrophenyl)-3-phenylpyrazoline (2j)	18.62 ± 1.84	7.09
28.	Leupeptin	90.98 ± 0.89 (0.01)	–

The % residual activity has been calculated with respect to control where no compound was added but an equal amount of solvent was added. The results are SMD of experiment conducted in triplicate. 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.

2.5. Molecular docking studies

All docking studies were performed using iGemdock. For these studies, small molecular weight ligands and enzyme active site structure is required. The structure of cathepsin L was retrieved from Protein Data Bank (http://www.rcsb.org/) as 3BC3 [28]. The structures were prepared in Marvin

sketch minimized and were saved as MDL Mol File. The prepared ligands and the binding site were loaded in the iGemdock software and docking was started by setting the GA- parameters at drug screening setting. The results presented in Table 2 pertain to the interaction data. Fitness is the total energy of a predicted pose in the binding site. The empirical scoring function of iGemdock is the sum total of Van der Waal, H-bonding and electrostatic energy. The docked poses of substrate Z-Phe-Arg-4mβNA, standard inhibitor leupeptin, most inhibitory compounds of each series **1i** and **2i** in the active site of cathepsin L are shown in Figure 3.

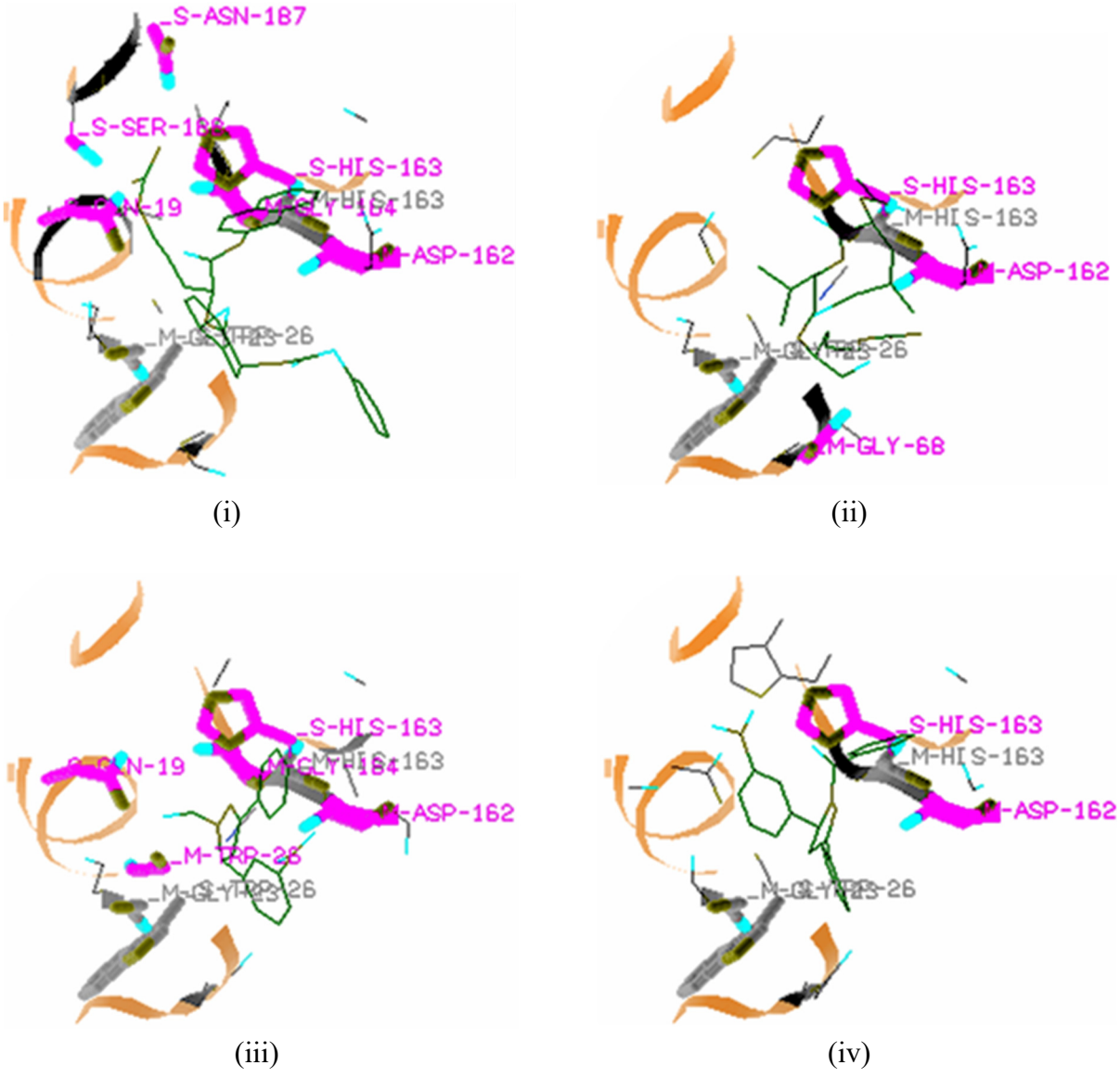


Figure 3. Docking results showing the alignment of most inhibitory in the active site of cathepsin L (3BC3.pdb). Here Figure 3i–iv show alignment of Z-Phe-Arg-4mβNA, Leupeptin, N-formyl-5-(-3'-nitrophenyl)-3-phenylpyrazoline (1i) and N-benzoyl-5-(-3'-nitrophenyl)-3-phenylpyrazoline (2i), in the active site of cathepsin L (3BC3.pdb), respectively.

Table 2. Docking studies energies of cathepsin L in presence of different compounds.

Compound	Total Energy (Kcal/mol)	VDW	H Bond	Electronic
Leupeptin	−114.04	−98.44	−15.59	0
Z-Phe-Arg-4mβNA	−137.75	−99.32	−40.23	1.80
1a	−86.23	−72.56	−13.68	0
1b	−90.94	−77.23	−13.70	0
1c	−86.28	−75.44	−10.83	0
1d	−88.92	−74.94	−13.98	0
1e	−99.29	−86.32	−12.97	0
1f	−95.39	−85.81	−9.58	0
1g	−90.60	−81.28	−9.32	0
1h	−113.75	−84.09	−30.07	0.41
1i	−98.43	−80.56	−17.86	0
1j	−92.95	−84.83	−8.12	0
2a	−92.78	−85.78	−7	0.84
2b	−98.39	−84.85	−13.54	0
2c	−94.99	−87.99	−7	0.80
2d	−97.96	−90.39	−7.57	0
2e	−100.31	−87.50	−12.08	0
2f	−99.97	−81.46	−18.51	0
2g	−93.36	−86.36	−7	0
2h	−114.33	−95.19	−19.94	0
2i	−100.56	−79.12	−21.43	0
2j	−111.17	−92.93	−19.08	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, H and L retrieved from Protein Data Bank (http://www.rcsb.org/) as 3BC3.

3. Results and Discussion

The biological activities exhibited by N-formylpyrazolines and N-benzoylpyrazolines like anticancer and anti-inflammatory where role of cathepsins has been established motivated us to screen the effect of some N-formylpyrazolines (1a–1j) and N-benzoylpyrazolines (2a–2j) on cathepsins. Their inhibitory effect on cathepsins B and H has already been reported in our previous published data [17] where we found that cathepsins B and H were inhibited appreciably and exhibited K_i values of the order of 10^{-8} M. Cathepsin L another significant related enzyme also contributes significantly to the above mentioned diseased conditions and therefore in the present work we have explored the inhibitory effect of title compounds on cathepsin L. The results obtained are discussed below.

3.1. Effect of N-formylpyrazolines and N-benzoylpyrazolines on the activity of cathepsin L

Table 1 shows the % residual activities of cathepsin L in presence of different compounds

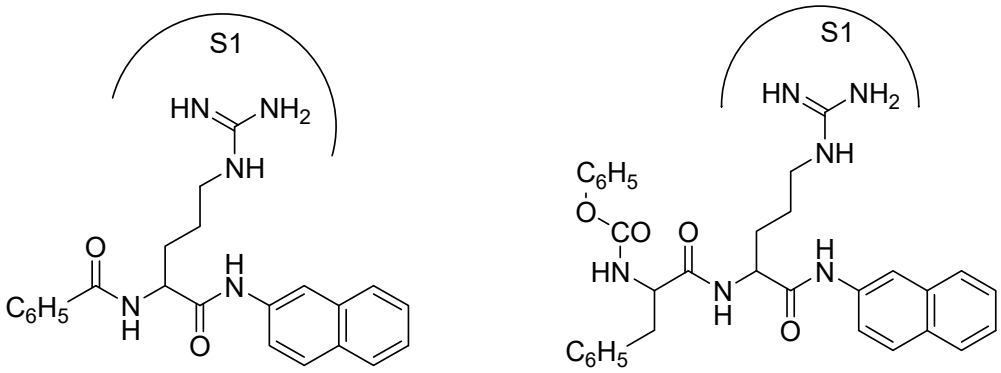
(1a–1j and 2a–2j). Various trends in inhibitory potential of compounds can be visualized. First is that N-formylpyrazolines are significantly better inhibitors than N-benzoylpyrazolines. In general the $–NO_2$ substituted compounds were more potent inhibitors than –Cl and unsubstituted followed by $–OCH_3$ in that order. The results clearly indicate that enzyme activity is greatly reduced in presence of electron withdrawing group than electron releasing group. It can further be observed that in each similarly substituted compounds m-positioned substitution exhibited more inhibition than o- or p-position.

Figure 1i–ii depict the activity of cathepsin L at varying concentrations of title compounds. The plots of % residual activities versus the concentrations of different compounds gave a relationship where increased compound concentration caused more inhibition. Among the various compounds tested, N-formyl-5-(-3'-nitrophenyl)-3-phenylpyrazoline **(1i)** was found to be most inhibitory to cathepsin L activity. Similar trend was observed in N-benzoylpyrazolines. The compounds have been found to be most inhibitory to cathepsin B and H also. However the inhibitory potential for cathepsin L has been found of the order of 10^{-9} M as compared to 10^{-8} M for cathepsins B and H. The results illustrated the significance of present study that the title compounds are better inhibitors to cathepsin L than cathepsin B and H.

3.2. Enzyme kinetic studies

After establishing the inhibitory action of synthesized compounds on cathepsin L, experiments were designed to evaluate the type of inhibition and to determine the K_i value of these compounds on cathepsin L. For that, enzyme activities were evaluated at different substrate concentrations in presence and absence of a fixed concentration of different compounds. The enzyme concentration was kept constant in all the experiments. Line-weaver Burk plots were drawn in 1/S and 1/V in presence and absence of inhibitor for cathepsin L, Figure 1iii–iv. It was found that the plots of 1/V and 1/S were straight lines intersecting at the Y-axis and showed that value of V remained constant in all the compounds whereas the value of K changes with each compound. These studies suggested a competitive type of inhibition exhibited by these compounds [29]. Using the Line-weaver Burk equation for competitive inhibition the K_i values were calculated, which has been presented in Table 1. The K_i value of most inhibiting compound cathepsin L in the corresponding series i.e. N-formylpyrazolines and N-benzoylpyrazolines has been evaluated ~6.4×10^{-10} and 5.7×10^{-9} M for **1i** and **2i**, respectively. The compound, **1i,** has been evaluated as an effective inhibitor of cathepsin L with inhibitory potency approaching that of specific peptidyl inhibitor leupeptin, having the K_i value of 1.45×10^{-9} M for goat brain cathepsin L [27]. The K_i values for cathepsins B have been reported as ~1.1×10^{-9} 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 ~5.19×10^{-8} and 9.8×10^{-7} M respectively [17]. This has been interesting to find out that the title compounds are better inhibitors of cathepsin L in comparison to cathepsin B and H. The inhibitory studies of chalcones and corresponding flavonoids also emphasize a similar trend on these enzymes [23,25]. Similar inhibitory potential of **1i** and **2i** to cathepsins B [17] and L (present study) can be explained on the basis of the binding sites. The binding sites in proteases are designated as **S1**, **S2**, …. **Sn** corresponding to enzyme site designated as **P1**, **P**2, …. **Pn**. These binding sites account for the reaction specificities of proteases [30]. The substrate specificity of catehpsins B and L corresponding to **S1** binding site is similar as both these enzymes show a good acceptability of arginine amino acid (Figure 4). Therefore both these enzymes are

usually inhibited by similarly substituted compounds [7,22,23,25,31]. The similar behavior of cathepsins B and L toward potential inhibitors **1i** and **2i**, can be due to similar binding site **S1.**



BANA Z-Phe-Arg-4mβNA

Figure 4. Interaction of substrates BANA and Z-Phe-Arg-4mβNA to the S1 site.

3.3. Molecular docking experiment

On the basis of the interaction data of docking experiments that include total energy and individual energy terms, an indicative of the fitness of a predicted pose in the binding site, it is suggested that the level of interaction is highest for N-formyl-5-(-3'-nitrophenyl)-3-phenylpyrazoline **(1i)** followed by N-benzoyl-5-(-3'-nitrophenyl)-3-phenylpyrazoline **(2i)** within the active site of cathepsin L (Table 2). Leupeptin, the peptidyl inhibitor of cathepsin L, interact with the enzyme and showed higher interaction energy than all the compounds. Decrease in total energy for leupeptin-cathepsin L has come out be −114.04 which is nearest to substrate BANA, −137.75 when compared to the maximum inhibitory compound in each series. As compared to this the binding energy of title compounds are less (Table 2). N-formylpyrazolines show a higher interaction than N-benzoylpyrazolines (Table 2) in that order. The *in-silico* predictable behavior of enzyme-ligand interaction can give an idea about the interaction. From the Figure 3i it is clear that out of the six amino acids, Glu-19, Asp-162, His-163, Glu-164, Asn-187 and Ser-188 that bind to the substrate Z-Phe-Arg-4mβNA through H-bonding only two i.e. Asp-162 and His-163 binds to leupeptin, a potential peptidyl inhibitor of cathepsin L, Figure 3ii. However, binding pattern of **1i** (Figure 3iii) suggests that in addition to Asp-162 and His-163, Gln-19 and Asp-162 are also common amino acids that bind to substrate as well as **1i**. The energy of binding for leupeptin-cathepsin L has been found to be more but the effective binding through H-bonds is more in case of **1i**, suggesting a better inhibition by the compound **1i** as compared to leupeptin. The *in-silico* results are justified by the *in-vitro* inhibition studies. Having four common amino acids in the binding of **1i** and Z-Phe-Arg-4mβNA also support the enzyme kinetic study where we have evaluated that the compound **1i** is a competitive inhibitor to the cathepsin L. Molecular docking results of **2i** also support the in-vitro study. The compound 2i is comparatively less inhibitory than **1i**. We can see from Figure 3iv that only two amino acids, Asp-162 and His-163 of total six substrate binding amino acids bind to the compound **2i.** Here again we observed that the binding energy of **2i** is more than **1i.** From these results we can conclude that the occupancy of active site by the number of common amino acids for substrate and the inhibitor through H-bonds can be one of the important deciding factors to determine the extent of inhibition.

4. Conclusion

Cysteine proteases have been reported as valuable targets for the development of various antiparasitic agents. A direct co-relation between various diseased conditions such as inflammation, cancer etc. with enhanced level of cathepsin L encouraged us to look for various inhibitors of this enzyme. Although a large number of peptidyl inhibitors to thiol enzymes are well reported in literature, but due to some stability and immunogenic problem related with peptidyl inhibitors; in the recent past, non-peptidyl inhibitors of these enzymes are being searched. The present study adds to the existing knowledge of non-peptidyl inhibitors of cathepsins L, where we have reported that N-formylpyrazolines and N-benzoylpyrazolines act as inhibitors of these cysteine proteases. All the compounds were evaluated as competitive inhibitors of enzymes and the results are well documented with *in silico* experiments.

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Conflict of interest

The authors have declared no conflict of interest.

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