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2,3-Dihydroquinazolin-4(*1H*)-one derivatives as potential non-peptidyl inhibitors of cathepsins B and H

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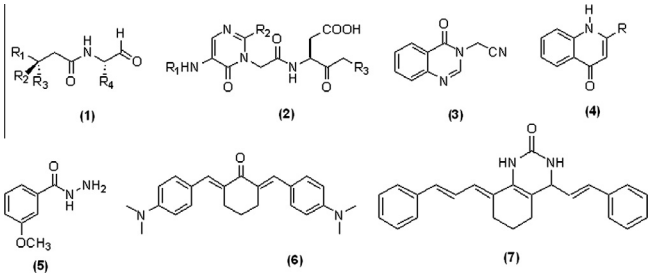
ABSTRACT

A direct correlation between cathepsin expression–cancer progression and elevated levels of cathepsins due to an imbalance in cellular inhibitors-cathepsins ratio in inflammatory diseases necessitates the work on the identification of potential inhibitors to cathepsins. In the present work we report the synthesis of some 2,3-dihydroquinazolin-4(*1H*)-ones followed by their evaluation as cysteine protease inhibitors in general and cathepsin B and cathepsin H inhibitors in particular. 2,3-Dihydroquinazolin-4(*1H*)-ones, synthesized by the condensation of anthranilamide and carbonyl compound in presence of PPA-SiO_2 catalyst, were characterized by spectral analysis. The designed compounds were screened as inhibitors to proteolysis on endogenous protein substrates. Further, a distinct differential pattern of inhibition was obtained for cathepsins B and H. The inhibition was more to cathepsin B with K_i values in nanomolar range. However, cathepsin H was inhibited at micromolar concentration. Maximum inhibition was shown by compounds, **1e** and **1f** for cathepsin B and compounds **1c** and **1f** for cathepsin H. The synthesized compounds were established as reversible inhibitors of cathepsins B and H. The results were also compared with the energy of interaction between enzyme active site and compounds using iGemdock software.

1. Introduction

Cathepsins are challenging therapeutic targets of drug design. Cathepsins B and H have long been associated with cancer progression because of their ability to degrade extracellular matrices facilitating invasion, angiogenesis and metastasis as is evident from numerous clinical reports and experimental models [1–4]. Increased levels of these enzymes in tumor state have been predictive factors for cancer patients [5,6]. In addition, elevated levels of these cysteine proteases have also been reported in various inflammatory conditions [7] such as rheumatoid arthritis and periodontitis. One of the reasons for these elevated levels of the enzymes has been attributed to an imbalance between cellular inhibitors and cathepsins ratio [8,9]. Therefore identification of compounds which act as potent inhibitors to cathepsins is a major thrust area in the drug development and chemotherapy.

Inhibitors of cathepsin B have been found effective in reducing the invasive potential of tumor cells [10]. Deletion of cathepsin H impaired angiogenic switching of the pre-malignant hyperplastic islets significantly and a reduction in the subsequent numbers of tumor has been observed [11]. Inhibitors that target cysteine cathepsins have been used *in vitro* to show that these enzymes play an important role in tumor invasion [12]. In the past decade, various studies have been carried out to identify small molecular weight compounds as inhibitors of cysteine proteinases such as aldehydes **(1),** pyrimidone **(2),** quinazolone **(3)** and 4-quinolinones **(4)** [13–16]. In a recent study, some acyl hydrazides **(5)** [17] and bischalcones and their quinazoline-2(1*H*)-one derivatives **(6–7)** [18] as inhibitors to cathepsin B and H have been reported.



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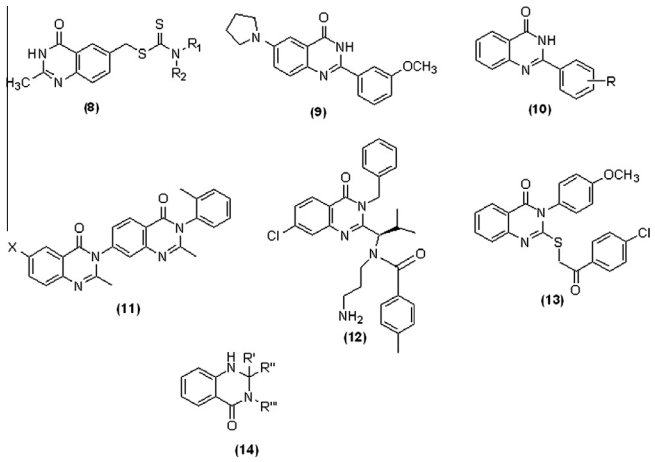
Literature reports that quinazolinone moieties have been explored as a key functional group in a variety of anticancer agents. Many quinazolinones have contributed to the quest for an ultimate antitumor chemotherapeutic agent. 4(3*H*)-Quinazolinone derivatives **(8–13)** [19–24] and 2,3-dihydro quinazolin-4(*1H*)-ones **(14)** [25] showed promising antitumor potency.



It may be worth mentioning here that lysosomes have been reported as agents of inflammation in polyarthritis, bacterial endotoxicity and rheumatoid arthritis [26–28]. Involvement of cathepsin B and H in various inflammatory diseases also emphasizes the significance of designing and development of these inhibitors as anti-inflammatory agents [29]. It is also an established fact that non steroidal anti-inflammatory drugs (NSAIDs) inhibit the proliferation rate and induces apoptosis in colon cancer cell line in a prostaglandin independent pathway [30]. The role of piroxicam, an NSAID is known to exert its pharmacological action by inhibiting lysosomal enzymes [31]. The drug, used as a therapeutic agent in osteoarthritis and rheumatoid arthritis, has been found in the chemoprevention of colon carcinogenesis [32]. These literature reports confirm a direct correlation between lysosomal enzyme inhibition and development of anti-tumor and anti-inflammatory agents.

Keeping in view the presence of quinazolinone moiety in compounds exhibiting anticancer activity it was thought proper to synthesize some compounds with these structural background in order to develop some potential inhibitors to cathepsins B and H.

In continuation of our previous work on low molecular weight compounds as inhibitors to endogenous proteolytic activities of cysteine proteases [33–38], in the present study, we report 2,3-dihydroquinazolin-4(*1H*)-ones as cathepsin B and H inhibitors. SAR, inhibitory potency and type of inhibition exerted by these compounds are reported which may provide new therapeutic opportunities in cancer treatment. The results are compared with *in silico* studies to rationalize our findings.

2. Experimental protocols

2.1. Materials

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

2.2. Methods

2.2.1. Proteolytic studies

2.2.1.1. Preparation of liver homogenate. The fresh goat liver was first washed with cold isotonic saline solution. The tissue was then homogenized in 0.1 M sodium acetate buffer pH 5.5 containing 0.2 M NaCl, 1 mM EDTA and 0.1% Triton X-100 in a mixer-cum-blender to obtain 10% (w/v) homogenate [33–38]. It was then stored at 4 °C.

2.2.1.2. Assay for proteolysis study. The proteolysis was carried out at pH 5.0 at 37 °C using 0.1 M acetate buffer as the incubation medium. Then, 100 μl of homogenate was mixed with 880 μl buffer and 20 μl of compounds at this pH and was incubated at 37 °C for 3 h and 24 h, separately. The % residual activity is calculated w.r.t. control where no compound was added but an equivalent amount of solvent was present. The reaction was stopped by the addition of 400 μl TCA and the resulting solution was centrifuged to precipitate proteins. The acid soluble proteins were quantitated in the 200 μl supernatant using 50 μl Bradford dye according to Bradford method [39]. The experiments were conducted in triplicate and the results are presented in Table 1. Endogenous Protein Hydrolysis is calculated in 0.1% liver homogenate at 10^{-4} M concentration of compounds and is calculated as proteolytic activity in mg/h/ml in enzyme homogenate.

2.2.2. Purification of goat liver cathepsin B and cathepsin H

All the purification steps were carried out at 4 °C. Cathepsins B and H were isolated, separated and purified from goat liver by the already established procedure as reported previously [17,18]. Following the steps of liver acetone powder preparation, homogenization in cold 0.1 M sodium acetate buffer pH 4.76 containing 0.2 M NaCl and 1 mM EDTA, acid-autolysis at pH 4.0 and 30–80% ammonium sulfate fractionation. Further fractionation of proteases was based on molecular weight on Sephadex G-100 column chromatography, cation-exchange chromatography on CM-Sephadex C-50 and DEAE-Sephadex A-50.

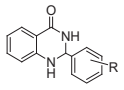
The specific activities of the cathepsin B and cathepsin H were ~10.38 nanomoles/min/mg and ~22.56 nanomoles/min/mg, respectively.

2.2.3. Enzyme assays

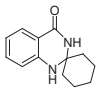
Stock solutions of the compounds (5 mM) were prepared in DMSO. The purified cathepsins B and H were first activated in presence of thiol activators at pH 6.0 and pH 7.0, respectively. Then, 15 μl of the enzyme solution was mixed with 940 μl of 0.1 M sodium phosphate buffer containing 1 mM EDTA separately, for 10 min at 37 °C. Then, 20 μl of stock solution of different compounds under study were added separately to the activated enzyme assay mixtures to effect final drug concentrations as 1×10^{-4} M in 1 ml assay (4.5% DMSO). After 30 min, 25 μl of 100 mM substrate stock solution was added to start the reaction. The released β-naphthylamine was quantitated colorimetrically at 520 nm by the usual assay procedure as adopted previously [17,18]. In control experiments, the equivalent amount of respective solvents was added and percent residual activities were calculated with reference to control.

The compounds which showed 100% inhibition at 1×10^{-4} M concentration were further studied for their inhibitory effect at their lower concentrations (Table 1) by adding appropriate amount of individual compounds in the reaction mixture separately to effect the final concentration of each compound as 0.001×10^{-4} M, 0.005×10^{-4} M, 0.01×10^{-4} M, 0.05×10^{-4} M, 0.1×10^{-4} M, 0.25×10^{-4} M, 0.5×10^{-4} M, 0.75×10^{-4} M and 1.0×10^{-4} M (Figs. 1 and 2).

Table 1
Effect of substituted 2,3-dihydro-2-phenylquinazolin-4(1*H*)-one on hydrolysis of endogenous protein substrates, cathepsin B and cathepsin H activities.



(1a-1h)



(1i)

Code No.	Endogenous Protein Hydrolysis				Cathepsin B activity		Cathepsin H activity	
	3 h		24 h		Mean ± SMD	% Residual activity	Mean ± SMD	% Residual activity
	Mean ± SMD	% Residual Activity	Mean ± SMD	% Residual Activity				
Control	4.78 ± 0.04	100	4.95 ± 0.02	100	5.65 ± 0.06	100	3.70 ± 0.25	100
1a	0 ± 0.00	0	0 ± 0.00	0	5.58 ± 0.10(0.1)	98.76	2.95 ± 0.03(0.1)	79.73
1b	4.73 ± 0.03	98.95	0 ± 0.00	0	2.77 ± 0.008(0.05)	49.02	3.03 ± 0.10(0.05)	81.89
1c	4.39 ± 0.02	91.84	0 ± 0.00	0	5.43 ± 0.10(0.1)	96.11	1.45 ± 0.04(0.1)	39.18
1d	4.55 ± 0.02	95.19	4.84 ± 0.05	97.78	3.34 ± 0.08(0.05)	59.11	3.55 ± 0.08(0.05)	95.94
1e	4.20 ± 0.05	87.86	0 ± 0.00	0	3.11 ± 0.01(0.001)	55.04	3.63 ± 0.03(0.01)	98.11
1f	4.67 ± 0.08	97.69	0 ± 0.00	0	0.89 ± 0.02(0.001)	15.75	3.48 ± 0.02(0.01)	94.05
1g	0 ± 0.00	0	0 ± 0.00	0	5.22 ± 0.01(0.1)	92.38	3.39 ± 0.06(0.1)	91.62
1h	2.98 ± 0.07	62.34	0.53 ± 0.05	10.70	3.92 ± 0.02(0.05)	69.38	3.27 ± 0.05(0.05)	88.37
1i	3.82 ± 0.04	79.92	0 ± 0.00	0	4.95 ± 0.07(0.1)	87.61	3.51 ± 0.02(0.1)	94.86

The results are presented as Mean ± S.M.D. of the experiment conducted in triplicate. The % residual activity is calculated w.r.t. control where no compound was added but an equivalent amount of solvent was present. Endogenous Protein Hydrolysis is calculated as % residual activity in 0.1% liver homogenate at 10–4 M concentration and is calculated as proteolytic activity in mg/h/ml in enzyme homogenate. The TCA soluble peptides were estimated at 630 nm using Bradford method. Cathepsins B and H activities were calculated as % residual activity and measured at 10^{-4} M concentration using BANA and Leu-βNA as substrates in enzyme preparations having specific activity as ~10.38 nmol/min/mg and ~22.56 nmol/min/mg, respectively. Value of Mean ± S.M.D. and % residual activity in presence of leupeptin and Leu-CH_2-Cl taken as positive control for cathepsin B and cathepsin H has been calculated as 0.067 ± 0.0012 and 1.20 (0.001 mM) for cathepsin B and 0.241 ± 0.015 and 6.50 (0.01 mM) for cathepsin H, respectively.

2.2.4. Kinetic measurements

After establishing the inhibitory action of synthesized compounds on cathepsins B and H, experiments were designed to evaluate the type of inhibition and to determine their K_i values. For that, enzyme activity was evaluated at six different substrate concentrations (2.5×10^{-4} M, 2.0×10^{-4} M, 1.5×10^{-4} M, 1.0×10^{-4} M, 0.50×10^{-4} M, 0.30×10^{-4} M, 0.25×10^{-4} M and 0.20×10^{-4} M) in presence and absence of a fixed concentration of inhibitor indicated in Table 2. The enzyme concentration was kept constant in all the experiments as detailed previously (Figs. 3 and 4). The values represent Mean ± S.M.D. of at least three individual experiments.

2.2.5. General procedure

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

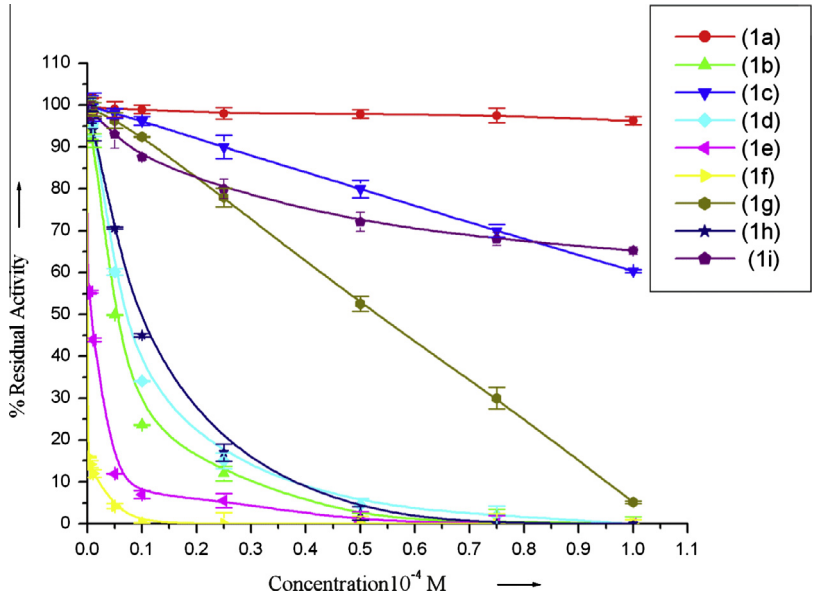


Fig. 1. Effect of varying concentrations of 2,3-dihydroquinazoline-4(*1H*)-one at pH 6.0 on cathepsin B activity in presence of 2.5 mM concentration of BANA. Results are the mean of the experiments conducted in triplicates at respective concentrations of compounds. Activities are expressed as percent of control which contains equivalent amount of solvent.

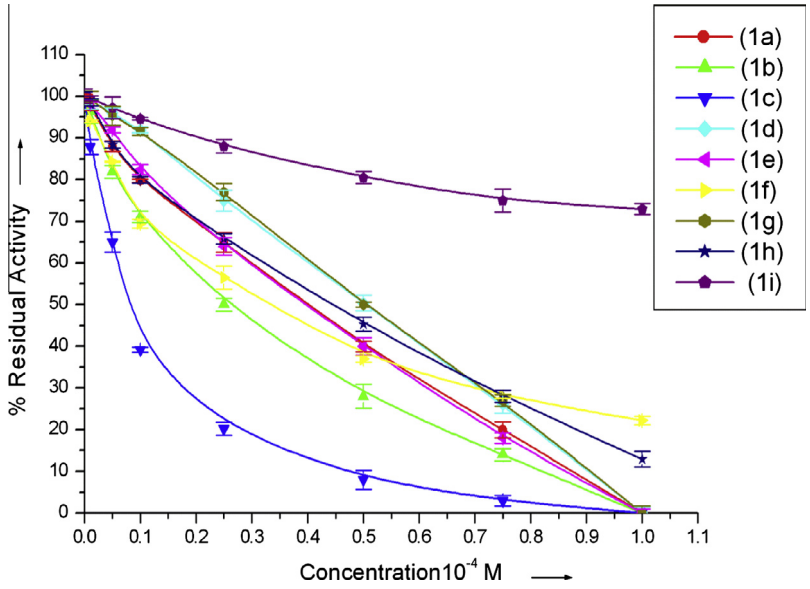


Fig. 2. Effect of varying concentrations of 2,3-dihydroquinazoline-4(*1H*)-one at pH 7.0 on cathepsin H activity in presence of 2.5 mM concentration of Leu-βNA. Results are the mean of the experiments conducted in triplicates at respective concentrations of compounds. Activities are expressed as percent of control which contains equivalent amount of solvent.

Table 2
K_i values and type of Inhibition exerted by 2,3-dihydroquinazoline-4(1*H*)-ones on cathepsins B and H.

S.no.	Compound name	Type of inhibition	K_i values (μM)	
			Cathepsin B	Cathepsin H
1	**1a**	Competitive	111.100	14.410
2	**1b**	Competitive	1.570	10.420
3	**1c**	Competitive	52.630	2.500
4	**1d**	Competitive	2.810	56.820
5	**1e**	Competitive	0.041	23.250
6	**1f**	Competitive	0.0064	8.180
7	**1g**	Competitive	32.520	52.630
8	**1h**	Competitive	10.640	18.580
9	**1i**	Competitive	14.980	90.090

The experiments were conducted in triplicate in presence and absence of a fixed concentration of different compound, separately (experimental value is given in Table 1 in parenthesis). The results were then plotted between 1/*V* and 1/*S* to obtain Lineweaver–Burk plots and then the K_i values were calculated using Lineweaver–Burk equations for competitive and non-competitive inhibition depending upon the results. Enzyme assays were conducted using BANA and Leu-βNA as substrates for cathepsin B and cathepsin H activities having specific activity as ~10.38 nmol/min/mg and ~22.56 nmol/min/mg, respectively.

2.2.6. Synthesis of 2,3-dihydroquinazolin-4-(1H)-ones

Anthranilamide (0.50 g, 0.0037 mol), benzaldehyde (0.10 g, 0.0073 mol) and PPA-SiO_2 (0.0012 mol) were added to alcohol (2 ml) [40]. The mixture was refluxed for the 5–6 h. The progress of the reaction was monitored by TLC. After completion, solvent was evaporated at reduced pressure, and solid was recrystallized from alcohol. The structure elucidations of compounds were based on the spectral data (IR, ^{1}H NMR and ^{13}C NMR).

2-Phenyl-2,3-dihydroquinazolin-4(1*H*)-one (1a): %Yield: 84%, m.p.°C: 216–218 [41]; IR (KBr, cm^{-1}): 3325, 3178 (—NH str), 1651 (—C=O str), 1454–1602 (—C=C— str); ^{1}H NMR ($CDCl_3$, 300 MHz, δ ppm): 5.40 (1H, br s, —NH), 5.76 (1H, br s, —NH), 6.35 (1H, s, —CH), 6.60 (1H, d, *J* = 9.0 Hz, Ar—H), 6.71 (1H, m, Ar—H), 7.17 (1H, m, Ar—H), 7.28–7.31 (5H, m, Ar—H), 7.73 (1H, d, *J* = 9.0 Hz, Ar—H); ^{13}C NMR (75 MHz, $CDCl_3$, δ ppm): 169.84, 147.95, 144.64, 133.92, 128.65, 128.04, 127.68, 126.82, 117.58, 115.80, 113.95, 68.60.

2-(4-Nitrophenyl)-2,3-dihydroquinazolin-4(1*H*)-one (1b): %Yield: 88%, m.p.°C: 205–206 [40]; IR (KBr, cm^{-1}): 3279, 3178 (—NH str), 1643 (—C=O str), 512, 1426 (—C=C— str); ^{1}H NMR ($CDCl_3$, 300 MHz, δ ppm): 5.57 (1H, br s, —NH), 5.90 (1H, br s, —NH), 6.65 (1H, s, —CH), 6.75 (1H, d, *J* = 7.8 Hz, Ar—H), 7.13 (1H, m, Ar—H), 7.24 (1H, m, Ar—H), 7.68 (1H, d, *J* = 7.8 Hz, Ar—H), 7.77 (2H, d, *J* = 9.0 Hz, Ar—H), 8.15 (2H, d, *J* = 9.0 Hz, Ar—H); ^{13}C NMR (75 MHz, $CDCl_3$, δ ppm): 169.30, 152.72, 147.21, 146.76, 133.76, 128.44, 127.93, 120.09, 117.28, 115.25, 113.68, 68.65.

2-(4-Methylphenyl)-2,3-dihydroquinazolin-4(1*H*)-one (1c): %Yield: 78%, m.p.°C: 232–234 [41]; IR (KBr, cm^{-1}): 3310, 3156 (—NH str), 1651 (—C=O str), 1605, 1465 (—C=C— str); ^{1}H NMR ($CDCl_3$, 300 MHz, δ ppm): 2.40 (s, 3H, —CH_3), 5.03 (1H, br s, —NH), 5.78 (1H, s, —NH), 6.09 (1H, s, —CH), 6.59 (2H, d, *J* = 6.0 Hz, Ar—H), 6.77 (1H, m, Ar—H), 7.08 (1H, d, *J* = 9.0 Hz, Ar—H), 7.26 (1H, m, Ar—H), 7.39 (2H, d, *J* = 6.0 Hz, Ar—H), 7.81 (1H, d, *J* = 9.0 Hz, Ar—H); ^{13}C NMR (75 MHz, $CDCl_3$, δ ppm): 165.44, 147.28, 141.94, 136.22, 133.86, 128.27, 128.76, 126.09, 117.62, 115.24, 114.88, 68.72, 23.08.

2-(4-Methoxyphenyl)-2,3-dihydroquinazolin-4(1*H*)-one (1d): %Yield: 92%, m.p.°C: 188–189 [41]; IR (KBr, cm^{-1}): 3299, 3109 (—NH str), 1659 (—C=O str), 1610, 1474 (—C=C— str); ^{1}H NMR ($CDCl_3$, 300 MHz, δ ppm): 3.75 (s, 3H, —OCH_3), 5.05 (1H, br s, —NH), 5.76 (1H, br s, —NH), 6.11 (1H, s, —CH), 6.62 (1H, d, *J* = 9.0 Hz, Ar—H), 6.76 (1H, m, Ar—H), 6.84 (2H, d, *J* = 6.0 Hz, Ar—H), 7.22 (1H, m, Ar—H), 7.42 (2H, d, *J* = 6.0 Hz, Ar—H), 7.82 (1H, d, *J* = 9.0 Hz, Ar—H); ^{13}C NMR (75 MHz, $CDCl_3$, δ ppm): 168.25, 150.44, 143.27, 135.52, 133.58, 130.92, 129.59, 121.28, 117.32, 116.71, 116.46, 114.22, 70.76.

2-(4-Chlorophenyl)-2,3-dihydroquinazolin-4(1*H*)-one (1e): %Yield: 91%, m.p.°C: 200–202 [41]; IR (KBr, cm^{-1}): 3310, 3186 (—NH str), 1651 (—C=O str), 1605, 1465 (—C=C— str); ^{1}H NMR ($CDCl_3$, 300 MHz, δ ppm): 5.32 (1H, br s, —NH), 5.78 (1H, br s, —NH), 6.48 (1H, s, —CH), 6.62 (1H, d, *J* = 6.0 Hz, Ar—H), 6.74 (1H, t, *J* = 9.0 Hz, Ar—H), 7.19 (1H, m, Ar—H), 7.28 (2H, d, *J* = 7.5 Hz, Ar—H), 7.44 (2H, d, *J* = 7.5 Hz, Ar—H), 7.77 (1H, m, Ar—H); ^{13}C NMR (75 MHz, $CDCl_3$, δ ppm): 169.80, 147.15, 142.38, 133.45, 132.67, 128.78, 128.64, 128.16, 117.35, 115.23, 113.40, 71.48.

2-(4-Fluorophenyl)-2,3-dihydroquinazolin-4(1*H*)-one (1f): %Yield: 84%, m.p.°C: 203–204 [41]; IR (KBr, cm^{-1}): 3302, 3178 (—NH str), 1651 (—C=O str), 1605, 1454 (—C=C— str); ^{1}H NMR ($CDCl_3$, 300 MHz, δ ppm): 5.57 (1H, br s, —NH), 6.65 (1H, br s,

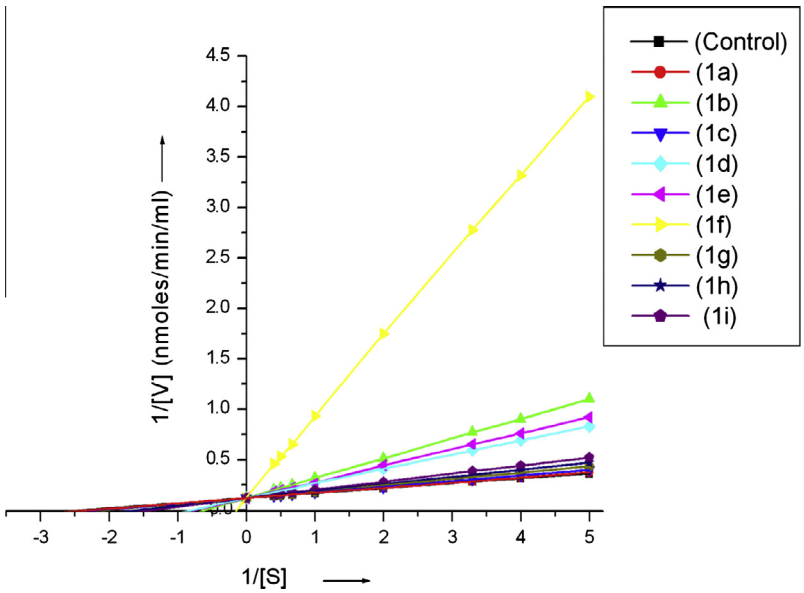


Fig. 3. Lineweaver–Burk plots for cathepsin B activity on varying concentrations of BANA in presence of minimum concentration of 2,3-dihydroquinazoline-4(*1H*)-one as reported in Table 1 at pH 6.0. The K_m and $1\backslash V_{max}$ value for Control have been found to be 4.0×10^{-4} M and 0.120.

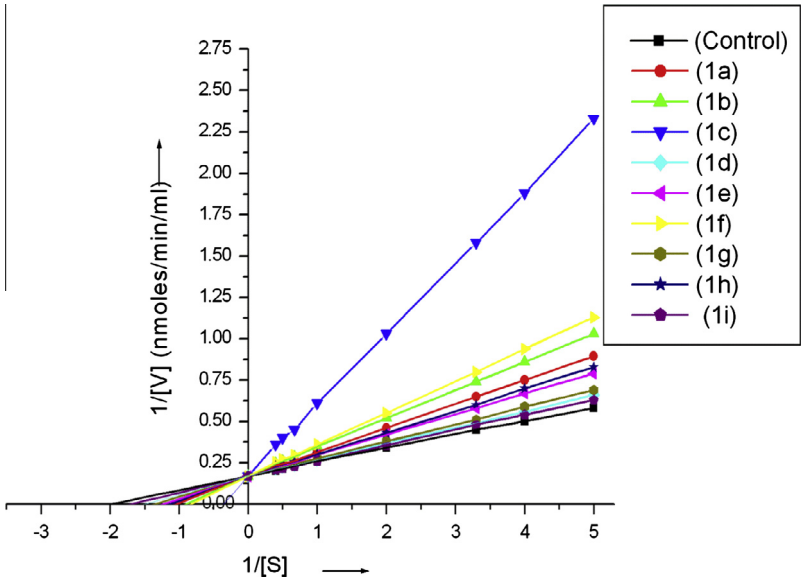


Fig. 4. Lineweaver–Burk plots for cathepsin H activity on varying concentrations of Leu-βNA in presence of minimum concentration of 2,3-dihydroquinazoline-4(*1H*)-one at pH 7.0 as reported in Table 1. The K_m and $1\backslash V_{max}$ value for control have been found to be 5.0×10^{-4} M and 0.170.

—NH), 6.75 (1H, s, —CH), 7.09 (1H, s, Ar—H), 7.19–7.27 (3H, m, Ar—H), 7.51–7.62 (3H, m, Ar—H), 8.28 (1H, s, Ar—H); ^{13}C NMR (75 MHz, $CDCl_3$, δ ppm): 170.23, 160.18, 147.44, 140.70, 133.54, 128.65, 128.25, 117.65, 115.32, 115.22, 113.65, 71.84.

2-(4-Bromophenyl)-2,3-dihydroquinazolin-4(*1H*)-one (1g): %Yield: 82%, m.p.°C: 202–203 [42]; IR (KBr, cm^{-1}): 3310, 3186 (—NH str), 1651 (—C=O str), 1605, 1474 (—C=C— str); ^{1}H NMR ($CDCl_3$, 300 MHz, δ ppm): 5.58 (1H, br s, —NH), 5.69 (1H, br s, —NH), 6.57 (1H, s, —CH), 6.60 (1H, d, J = 6.0 Hz, Ar—H), 6.92 (2H, d, J = 7.5 Hz, Ar—H), 7.14 (1H, m, Ar—H), 7.28 (1H, m, Ar—H), 7.31 (2H, d, J = 7.5 Hz, Ar—H), 7.68 (1H, d, J = 6.0 Hz, Ar—H); ^{13}C NMR (75 MHz, $CDCl_3$, δ ppm): 170.24, 147.66, 143.58, 133.08, 131.85, 129.12, 128.44, 121.46, 117.46, 115.28, 113.78, 70.94.

2-(4-(Dimethylamino) phenyl)-2,3-dihydroquinazolin-4(1*H*)-one (1h): %Yield: 92%, m.p.°C: 224–228 [40]; IR (KBr, cm^{-1}): 3294, 3186 (—NH str), 1651 (—C=O str), 1625, 1454 (—C=C— str); ^{1}H NMR ($CDCl_3$, 300 MHz, δ ppm): 3.01 (6H, s, —$(NCH_3)_2$), 4.34 (1H, br s,—NH), 5.70 (1H, br s, —NH), 5.82 (1H, s, —CH), 6.68 (1H, d, J = 6.0 Hz, Ar—H), 6.90 (1H, m, Ar—H), 6.95 (2H, d, J = 9.0 Hz, Ar—H), 7.28 (1H, m, Ar—H), 7.44 (2H, d, J = 9.0 Hz, Ar—H), 7.95 (1H, d, J = 6.0 Hz, Ar—H); ^{13}C NMR (75 MHz, $CDCl_3$, δ ppm): 168.24, 147.98, 147.72, 133.98, 133.64, 128.45, 127.78, 117.25, 115.21, 114.65, 113.62, 68.76, 41.24, 41.30.

1*H*-Spiro(cyclohexane-1,2-quinazolin)-4(3*H*)-one (1i): %Yield: 82%, m.p.°C: 219–220 [40]; IR (KBr, cm^{-1}): 3362, 3179 (—NH str), 1647 (—C=O str), 1618, 1458 (—C=C— str); ^{1}H NMR ($CDCl_3$, 300 MHz, δ ppm): 1.35 (2H, m, >CH_2), 1.37–1.53 (4H, m, >CH_2), 1.72–1.76 (4H, m, >CH_2), 4.86 (1H, br s, —NH), 6.55 (1H, br s, —NH), 6.59 (1H, d, J = 9.0 Hz, Ar—H), 6.83 (1H, t, J = 7.5 Hz, Ar—H), 7.15 (1H, t, J = 7.5 Hz, Ar—H), 7.72 (1H, d, J = 9.0 Hz, Ar—H); ^{13}C NMR (75 MHz, $CDCl_3$, δ ppm): 167.33, 148.28, 133.25, 128.42, 117.04, 115.25, 113.82, 68.51, 35.77, 28.89, 20.05.

Table 3
Docking studies showing decrease in different energies of cathepsin B in presence of different 2,3-dihydroquinazoline-4(*1H*)-ones.

Ligand	Total energy	VDW	H bond	Elec
1a	−79.7252	−65.7884	−13.9368	0
1b	−82.7275	−63.0387	−19.6888	0
1c	−77.7906	−65.6278	−12.1628	0
1d	−78.518	−63.3019	−15.2161	0
1e	−77.5528	−65.3722	−12.1806	0
1f	−77.922	−65.7997	−12.1223	0
1g	−77.4476	−65.2799	−12.1677	0
1h	−80.9438	−68.3925	−12.5513	0
1i	−71.6854	−58.2401	−13.4453	0
BANA	−124.953	−73.9838	−48.2915	−2.67805
Leupeptin	−105.009	−85.236	−19.7728	0

The results are one of the docking experiments run using iGemdock under standard docking settings. The ligands were prepared in Marvin sketch and saved as MDL mol file. The active site was extracted from the structure of cathepsin B retrieved from protein data bank (http://www.rcsb.org/) as cav2IPP B_PYS.pdb [43].

Table 4
Docking studies showing decrease in different energies of cathepsin H in presence of different 2,3-dihydroquinazoline-4(*1H*)-ones.

Ligand	Total energy	VDW	H bond	Elec
Leu-βNA	−85.5961	−70.7725	−14.8236	0
Leu-CH_2Cl	−59.3601	−42.9381	−16.4221	0
1a	−73.3826	−60.7197	−12.6629	0
1b	−83.2776	−58.847	−24.145	−0.28557
1c	−69.2345	−61.1921	−8.0424	0
1d	−71.2896	−58.6849	−12.6047	0
1e	−69.2709	−61.4746	−7.79627	0
1f	−72.3316	−59.6535	−12.678	0
1g	−71.9699	−59.3417	−12.6282	0
1h	−76.2175	−63.1457	−13.0718	0
1i	−73.121	−62.9103	−10.2107	0

The results are one of the docking experiments run using iGemdock under standard docking settings. The ligands were prepared in Marvin sketch and saved as MDL mol file. The active site was extracted from the structure of cathepsin H retrieved from Protein Data Bank (http://www.rcsb.org/) as (cav8PCH H_NAG.pdb) [44].

2.2.7. Drug modeling studies

All docking studies were performed using iGemDOCK. The structures of ligands were prepared in Marvin sketch minimized and were saved as MDL Mol File. The structure of cathepsins B and H were retrieved from Protein Data Bank (http://www.rcsb.org/) as cav2IPP B_PYS.pdb [43] and cav8PCH H_NAG.pdb [44], respectively. The structures were used for docking purpose assuming that there is not much alteration in amino-acid sequence of different organisms. These enzymes are known to retain the main ordered structures of papain superfamily [45]. It is also reported that overall folding pattern of polypeptide chain is grossly same in these proteases and utilize same catalytic mechanism [46]. After loading the prepared ligands and the binding site, docking was started at Standard Docking Accuracy Settings. Fitness is the total energy of a predicted pose in the binding site which is the sum total of electronic, H-bonding and Van der Waal interactions. The results for cathepsin B and H are presented in Tables 3 and 4, respectively. The docked poses of the ligands in the active site of cathepsin B along with the substrate BANA and the peptide inhibitor, leupeptin are shown in Fig. 5 and the docked poses of the ligands in the amino acyl binding site of cathepsin H along with the substrate Leu-βNA and the peptide inhibitor, Leu-CH_2Cl are shown in Fig. 6.

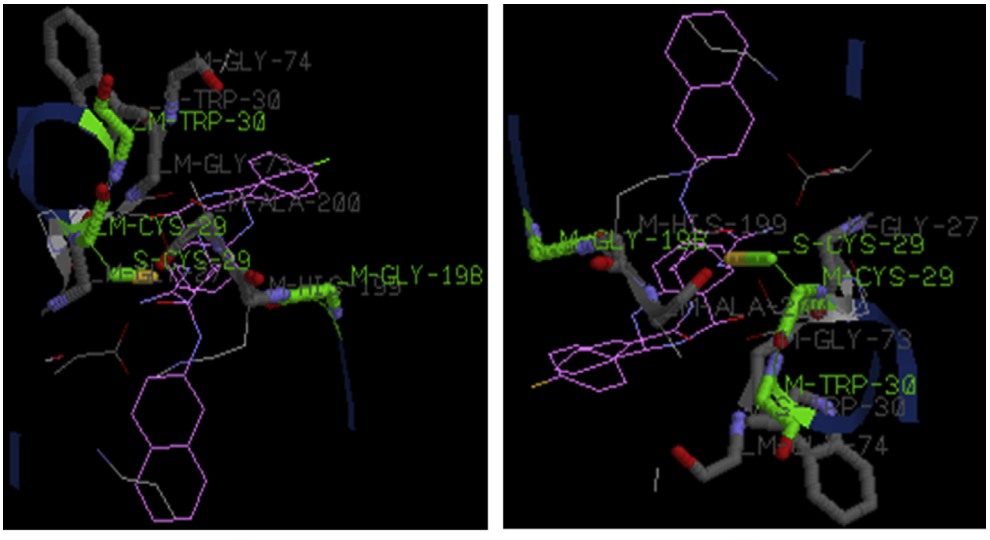
3. Results and discussion

3.1. Chemistry

2,3-Dihydroquinazoline-4(*1H*)-one derivatives were synthesized in excellent yields using silica-supported polyphosphoric acid (PPA-SiO_2) as a heterogeneous and reusable catalyst (Scheme 1). PPA-SiO_2 is safe, easy to handle, environmentally gentle with less disposal problems.

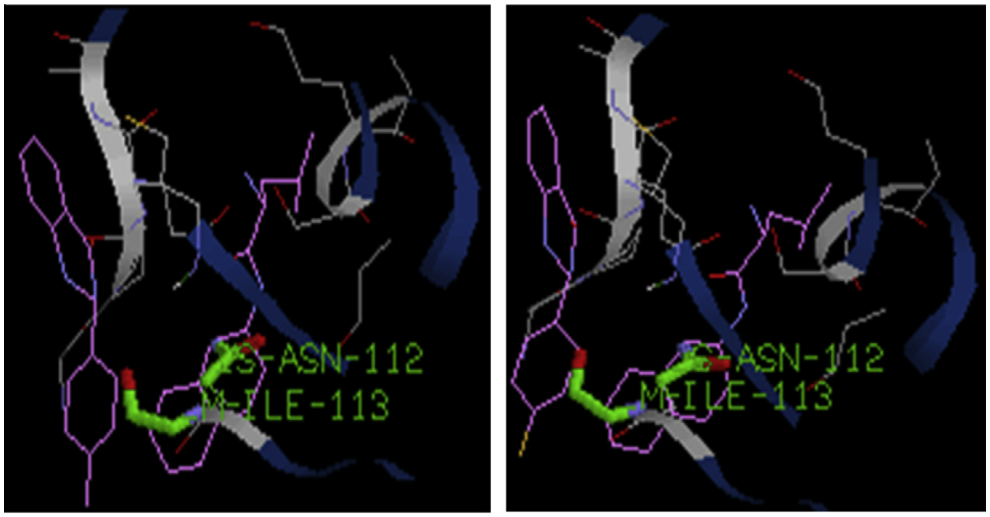
The synthesized compounds were characterized using spectral data (IR and ^{1}H NMR). The IR spectra showed mainly stretching bands at 3300–3100, 3100–3000, 1655–1643 and 1600–1450 cm^{-1} assigned to (N—H), (C—H), (C=O) and aromatic (C=C) functionalities, respectively.

The ^{1}H NMR spectrum of compounds in DMSO-d_6 shows signals at δ 4.30–6.65 assignable to —NH protons. The characteristic —CH protons of tetrahydro-4-quinazolinones were observed as a singlet at δ 5.82–7.13 which is absent in case of spiroquinazolinone Multiplets observed in the δ 6.34–8.45 ppm region are assigned to protons of phenyl rings. The sharp singlet observed at δ 2.38–2.43, 3.01–3.12 and 3.63–3.80 ppm is assigned to methyl, N-methyl and methoxy protons. The above spectral data suggested the successful synthesis of title compounds.



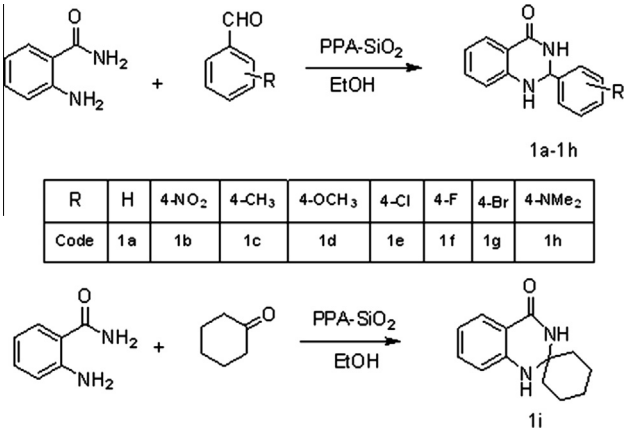
(I) (II)

Fig. 5. Docking results (I) and (II) showing the alignment of most inhibitory compounds **1e** and **1f**, respectively with BANA in the active site of cathepsin B (cav2IPP B_PYS.pdb).



(I) (II)

Fig. 6. Docking results (I) and (II) showing the alignment of most inhibitory compounds **1c** and **1f**, respectively with Leu-βNA in the active site of cathepsin H (cav8PCH H_NAG.pdb).



R	H	4-NO_2	4-CH_3	4-OCH_3	4-Cl	4-F	4-Br	4-NMe_2
Code	1a	1b	1c	1d	1e	1f	1g	1h

Scheme 1. Scheme for synthesis of 2,3-dihydroquinazoline-4(*1H*)-ones.

3.2. Pharmacological evaluation

Elevated levels of cathepsins and low levels of their inhibitors have been observed in tumor cells and suggest the role of cathepsins as biological markers of malignant tumors [47]. The identification and development of low molecular weight compounds as inhibitors of specific cysteine proteases has been an active area of research for cancer therapy. Literature survey suggests that a large work has been accomplished on peptidyl or peptidyl analogs as inhibitors to cysteine proteases [48]. However, these inhibitors are not considered to be viable drug candidates for treating diseases like cancer, apoptosis etc. because of the possibility of immunogenic reactions or gastric instability. Therefore, research on non-peptidyl drugs has become an important aspect in drug research and development. More recently, a variety of benzophenone and thiosemicarbazone analogs [49,50], thiocarbazate [51] and pyrazoles [52] as small molecular weight inhibitors of cathepsin L have been synthesized. Thiosemicarbazones and semicarbazones have also been reported to inhibit cathepsin B [53]. We have also reported some acyl hydrazides [17] and bischalcones, their quinazoline-2(1*H*)-one derivative and quinazoline-2(1*H*)-thione derivatives [18] as inhibitors to cathepsins B and H. In addition, few pyrazoline derivatives [54,55] have also proved to be potential inhibitors to cathepsins B and H. Comparative inhibitory behavior of chalcones and their cyclic derivatives has also been reported on these enzymes [56]. Keeping in view, the potential shown by different quinazolinones as anticarcinogenic agents previously briefed, and role of cathepsins in cancer 2,3-dihydroquinazolin-4(1*H*)-one derivatives were screened *in vitro* for evaluating their inhibitory potency of cysteine proteases on endogenous protein substrates as well as against activity of cathepsins B and H. The potential of cathepsin inhibitors as anti-inflammatory agents can also be explored. We have reported these compounds act as potent inhibitors of cathepsins B and H with effective K_i values.

3.2.1. Effect of 2,3-dihydroquinazoline-4(1H)-one on in vitro endogenous proteolysis in liver homogenate

Table 1 presents the inhibition of endogenous proteolytic activity in presence of different 2,3-dihydroquinazoline-4(*1H*)-one (**1a–1h**) at pH 5.0, where most of the proteolytic activity is attributed to cysteine proteases [33] at 3 h and 24 h reaction time. It can be observed that proteolytic activity is inhibited appreciably in presence of these compounds. In some cases ~100% inhibition is achieved at 1×10^{-4} M concentration. Compounds bearing methoxy **1d** and bromo group **1g** at the 4-position of benzene ring led to a dramatic decrease in proteolytic activity. It can be concluded that nitro **1b**, methyl **1c**, chloro **1e** and fluoro **1f** group at the 4-position of benzene ring and spiro compound **1i** played a crucial role in inhibiting the proteolytic activities at 24 h reaction. However, the unsubstituted compound showed negligible effect on enzyme activity, i.e. enzymatic activities were influenced by the substituents on the compounds under consideration. When we compared the results of 3 h and 24 h reaction, it was found that the inhibition to endogenous proteolysis increases with time in presence of these compounds i.e., inhibition of proteolytic activity was more at 24 h reaction in comparison to 3 h reaction. After establishing the inhibitory potential of designed compounds on cysteine proteases in general, the effect of synthesized compounds on purified cathepsins B and H was studied.

3.2.2. Effect of 2,3-dihydroquinazoline-4(1H)-one on the activity of cathepsin B and H

Table 1 also present the % residual activities of cathepsins B and H at a MIC indicated in parenthesis. It can be observed that **1e** and

1f are most inhibitory to cathepsin B inhibiting 50% and 85% at 0.001 M concentration. The results are quite encouraging in comparison to inhibitory potential of leupeptin run alongwith as a positive control. At the same concentration ~98% inhibition is observed. The results are further confirmed later while conducting enzyme kinetic studies. The activities of cathepsin B were estimated at varying concentrations of designed compounds as detailed in Sections 2.1 and 2.2. Fig. 1 shows the relationship between the enzyme activity and concentration of substituted 2,3-dihydroquinazoline-4(*1H*)-ones.

Among the various compounds tested, 2-(4-fluorophenyl)-2,3-dihydro quinazolin-4(*1H*)-one **1f** was found to be most inhibitory which showed 100% inhibition at 25 μM concentration and ~50% inhibition was achieved at 100 nM concentration.

Similarly, the activities of cathepsin H was estimated at varying concentrations of synthesized compounds at pH 7.0 using Leu-βNA as a substrate. Fig. 2 shows that 2,3-dihydro-2-(4-methylphenyl)quinazolin-4(*1H*)-one **1c** was most inhibitory to cathepsin H exhibiting 100% inhibition at 10 μM concentration and 50% inhibition at 750 μM concentration followed by 2-(4-fluorophenyl)-2,3-dihydro quinazolin-4(*1H*)-one **1f**. The results of inhibition of cathepsins B and H are presented in Table 2. The inhibitory studies were further extended to determine the type of inhibition and K_i values of respective compounds.

In order to discover novel non-peptidyl inhibitors of cathepsins B and H, the present work focused on the synthesis of some 2,3-dihydroquinazoline-4(*1H*)-one derivatives has been accomplished which can lead to the development of new chemotherapeutic agents in cases where these cathepsins are responsible for invasion and metastasis of cancer cells. In addition, the compounds can be useful in the treatment of rheumatoid arthritis and other tissue degenerative disorders.

3.2.3. Mechanism of inhibition

The Lineweaver–Burk double-reciprocal plots (Figs. 3 and 4) show intercepts of all lines converging at the *y*-axis ($1/V_{max}$), whereas the slope (K_m/V_{max}) and *x*-axis intercepts ($1/K_m$) vary with inhibitor concentration. Consequently, the V_{max} values remain constant, whereas the apparent values of K_m increase with increasing inhibitor concentrations, showing a competitive inhibition.

This behavior is consistent with a mutually exclusive binding mode between inhibitor and substrate; therefore, these inhibitors compete with substrate for the free enzyme active site.

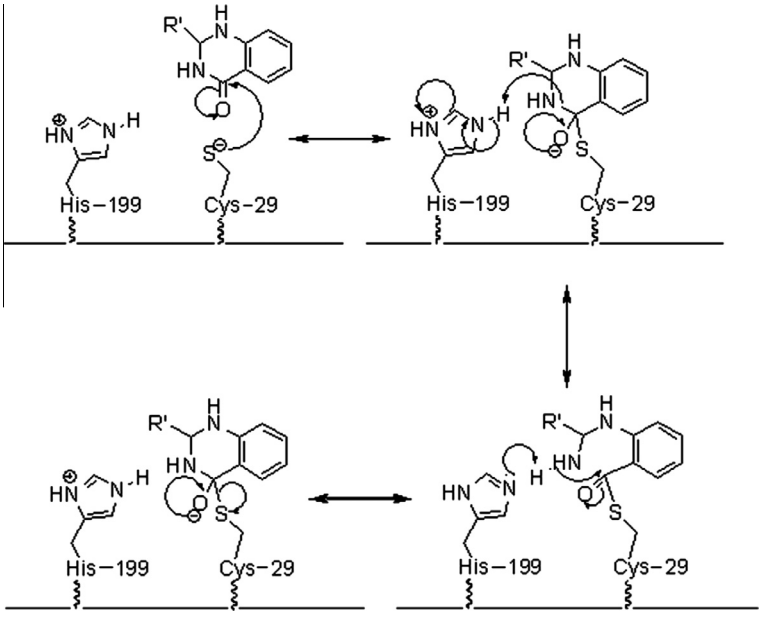
The K_i values of compounds were calculated using the Lineweaver–Burk equation $K_{m'} = K_m\ (1+[I]/K_i)$ for competitive inhibition which are presented in Table 2.

Preliminary inhibition studies of designed compounds on endogenous protein substrates suggested that inhibition increases with increase in time. The inhibition was more at 24 h incubation time when compared with 3 h incubation. It may be worth mentioning here that homogenate consist of mixture of cysteine proteases and all of these may not be susceptible toward the synthesized compounds and therefore initial studies carried out on homogenate need study on purified enzymes. During kinetic studies on purified enzymes, we analyzed that the enzymes are inhibited in a reversible manner indicating the importance of present work. With this background we proposed the mechanism of inhibition which has been shown in Scheme 2. The mechanism is based on the molecular docking experiments (explained later in the text) as well as on *in vitro* endogenous proteolysis studies. The thiolate of cysteine-29 is acylated as shown with the assistance of His-199. A similar mechanism has been proposed for inhibition of inhibition of cathepsin L by benzophenone functionalized thiosemicarbazones [49] where the active site is regenerated.

Here, we have proposed that the active site is acylated as in case of leupeptin. Similar to these compounds, leupeptin is also reported a reversible inhibitor of cathepsin B.

3.2.4. Structure–activity relationship (SAR) studies

It is a well known fact that cysteine protease inhibitors play an important role in several diseases including cancer, metastasis, inflammation and other tissue degenerative processes. Targeting this enzyme family is therefore one of the strategies in the development of new drug molecules for chemotherapy in these diseases. The substitution pattern was carefully altered in the designed compounds to achieve a proper SAR study between the electronic environment of the inhibitor and enzyme active site. In the present study, we found that cathepsin B is inhibited more than cathepsin



Scheme 2. Proposed mechanism for inhibition of cathepsin B by 2,3-dihydroquinazoline-4(*1H*)-ones.

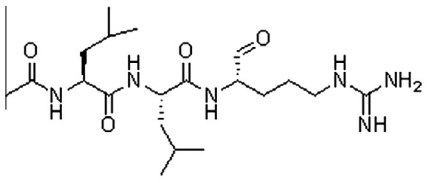
H by 2,3-dihydroquinazoline-4(1*H*)-one derivatives and at the same time when we studied the kinetics of inhibition, it was found that both cathepsins B and H were inhibited in a competitive manner (Table 2).

During SAR studies, we could analyse that the potential of inhibition shown by the synthesized compounds can be related to (i) the electronic effects of the substitutents and (ii) steric factors. It seems that these factors greatly contribute to the binding of inhibitor with the enzyme as there are not much stereochemical possibilities in the ligand itself. The designed molecules possess only one chiral centre and the possible orientation of substituent do not have much possibilities. (Stereochemical view of energy minimzed structures used for molecular docking purpose has been supplied as supplementary data.)

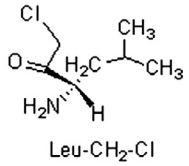
It can be observed from the results that in general for cathepsin B, electron withdrawing substituent inhibited the enzymes more as compared to electron releasing substituent For example —OCH_3, —CH_3, —$N(CH_3)_2$ exhibited lesser inhibitory effect. However, —NO_2, —Cl and —F substituted compounds proved to be potential inhibitors to the enzymes. It can further be observed that within a group, the inhibitory potential is affected by the size as well as electronegativity of the substituent. Bromo affected the enzyme inhibition to a lesser extent as compared to chloro followed by fluoro substituted compounds. The high inhibitory potential of chloro and fluoro compounds, **1e** and **1f** also possessing high log *p* value (in the range of 3.19–3.65), and an indicative of lipophilicity makes them suitable drug candidate for the treatment of diseases in cases where cathepsin B is responsible.

The results indicated that substituent greatly affected the inhibitory potential of the compounds for cathepsin B and cathepsin H.

In order to establish inhibition ability of the studied compounds, results were also 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 is a potential peptidyl inhibitor of cathepsin B [57] and preferentially inhibit cathepsin B in comparison to cathepsin H, K_i value for human liver cathepsin H was reported to be 9.2 μM [58].



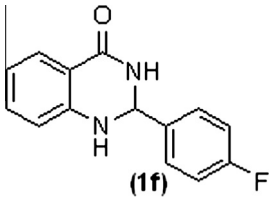
Leupeptin



Leu-CH_2-Cl

2-(4-Fluorophenyl)-2,3-dihydroquinazolin-4(1*H*)-one **1f**, 2-(4-nitrophenyl)-2,3-dihydro quinazolin-4(1*H*)-one **1b** and 2-(4-chlorophenyl)-2,3-dihydroquinazolin-4(1*H*)-one **1e** showed maximum inhibition on cathepsin B activity. Compound **1f** ($K_i \sim 0.0064$ μM) has been found to inhibit cathepsin B to the extent of leupeptin. Here, K_i value of compound is slightly lesser than that of K_i of leupeptin for brain enzyme, 12.5 nM [59] but is comparable to liver cathepsin B and was reported to be 7.0 nM [60]. Another compound **1e** also inhibited the enzyme effectively ($K_i \sim 0.041$ μM). Compound **1b** has been found to inhibit cathepsin B with K_i values of 1.57 μM.

2-(4-Methylphenyl)-2,3-dihydroquinazolin-4(1*H*)-one **1c** showed maximum inhibition on activity of cathepsin H. Spiro compounds did not show much inhibition on both cathepsins B and H activity. Leu-CH_2-Cl, a potential inhibitor to cathepsin H showed ~93.5% inhibition at 10 μM concentration which is in accordance with the previously reported results [61]. When the inhibition pattern of the compounds under consideration is compared with Leu-CH_2-Cl on cathepsin H activity, it was found that the inhibition caused by test compounds is comparable. Compound, **1c** and **1f** have been found to inhibit cathepsin H effectively with K_i values of 2.5 μM and 8.2 μM, respectively. Cathepsins B and H, both are inhibited by fluoro substituted compounds.



(1f)

In the present work, where we have reported the synthetic non-peptidyl novel inhibitors for cathepsin B and H, the enzyme inhibitions achieved are comparable with peptidyl inhibitors and therefore, the work has a great significance. Investigation of the inhibition potential of these compounds on activity of cathepsin B and cathepsin H suggests a positive future for their use as cysteine protease inhibitors as therapeutic agents in a number of disease processes. The compounds under investigation add to the existing knowledge of non peptidyl inhibitors of cathepsins B and H and can be potential candidates for anti-cancer and anti-inflammatory drug development and chemotherapy.

3.3. Molecular docking experiment

The empirical scoring function of iGemDOCK is the estimated sum total of Van der Waal, H-bonding and electrostatic energy. From molecular docking experiments, we observed that all of compounds inhibited the enzyme in a competitive manner as these compete at the binding site of enzyme with substrate.

On the basis of the interaction data of docking experiments (Table 3), it was observed that all the compounds showed a lesser interaction than the reference leupeptin, a peptidyl inhibitor. The maximum interaction is observed for BANA with a score of −124.953. Decrease in total energy for leupeptin–cathepsin B has come out be −105.009 of which the contribution of Van der Waal interactions are more with a score of −85.236 as compared to H-bonds with a score of −19.7728. Leupeptin–cathepsin B binding energy is due to peptide protein interaction. Leupeptin is peptidyl in nature and therefore being a flexible molecule binds effectively with the enzyme active site resulting in higher binding energy. iGemDOCK provide algorithms for flexible docking approach for both ligands and proteins [62] therefore flexible ligands like leupeptin will show a larger decrease in total energy as compared to the molecules under study as these are smaller in structure and possess lesser flexibility compared to leupeptin. Therefore, the binding energy of title compounds is less than leupeptin, but the *in vitro* analysis reveals that the compounds show comparable inhibition to this reference inhibitor. The proposed mechanism is previously shown in Scheme 2. The results clearly indicate the significance of the *in vitro* inhibition studies.

However in cathepsin H, the decrease in total energy for the reference inhibitor Leu-CH_2Cl was less as compared to all the designed compounds. Here, it can be seen that though Leu-CH_2Cl is specific inhibitor for cathepsin H [61,63], but possess only one amino acid residue as compared to leupeptin–cathepsin B. Therefore, the Leu-CH_2Cl–cathepsin H interaction cause a decrease in energy of −59.3601 of which −42.9381 is the Van der Waal interaction and −16.4221 is due to hydrogen bonds. As listed in Table 4, all the designed compounds have been found to show more decrease in ligand–cathepsin H interaction energy than Leu-CH_2Cl–cathepsin H. The compound, **1c**, found to be most inhibitory to cathepsin H show a total decrease in energy as

−69.234 of which −61.1921 is assigned to Van der waal interactions whereas −8.0424 is of H-bond.

Fig. 5 shows the docked view of compounds, **1e** and **1f**, respectively along with the substrate BANA in the active site of cathepsin B. The active site consisting of Cys-29 which interacts with the substrate *cf* Fig. 5 interacts with the designed compounds under consideration. In addition, Trp-30 and Gly-198 amino acids residues of cathepsin B also interact with substrate as well as with the inhibitor. It can be observed that all 2,3-dihydroquinazoline-4(1*H*)-ones are in good alignment with the substrate BANA. Like leupeptin, which has been reported as competitive inhibitor to cathepsin B, the designed compounds also show competitive inhibition. The competitive inhibition of 2,3-dihydroquinazoline-4(*1H*)-ones, as established by *in vitro* studies is also supported by *in silico* studies where the docked poses of the inhibitory compounds are in good alignment with the substrate in the active site.

Fig. 6 shows the docked view of compounds, **1c** and **1f**, respectively alongwith Leu-βNA in the aminoacyl binding site of cathepsin H. Here, the compound seems to interact with the enzyme at a site different than Leu-βNA, which gives an idea about non-competitive type of inhibition. But, the *in vitro* studies show that the enzyme cathepsin H like cathepsin B is also inhibited in a competitive manner by these compounds. The mechanism of inhibition proposed for cathepsin B might hold equally good for cathepsin H, where the —SH group present at the active site of the enzyme is involved in the nucleophilic attack resulting in enzyme inhibition.

In silico studies have been used as a supporting tool for enzyme inhibition studies. The *in silico* predictable behavior of enzyme–ligand interaction can give an idea about the interaction between these two but in-solution studies are equally important.

4. Conclusion

In the past, synthetic inhibitors of cysteine proteases containing a peptidyl fragment which interact with the binding pockets have been synthesized. These peptidyl inhibitors may be more selective and specific but faces some stability and immunological problems. In an attempt to obtain new lead, scaffolds with good efficacy and minimal toxicity, we have reported here synthesis and evaluation of differently substituted 2,3-dihydroquinazoline-4(*1H*)-one as potent inhibitors of cathepsins B and H as these cathepsins are druggable target in cancers, rheumatoid arthritis and other important diseases.

2-(4-Fluorophenyl)-2,3-dihydroquinazolin-4(*1H*)-one **1f** and 2-(4-chlorophenyl)-2,3-dihydro quinazolin-4(*1H*)-one **1e** substituted compounds showed maximum inhibition on cathepsin B. Whereas for cathepsin H, 2,3-dihydro-2-(4-methylphenyl)quinazolin-4(*1H*)-one **1c** and 2-(4-fluorophenyl)-2,3-dihydroquinazolin-4(*1H*)-one **1f** have been found to be the most potent inhibitors. The inhibition on cathepsin B was more pronounced in presence of each compound than cathepsin H. All the designed compounds inhibited both cathepsins B and H activity in a competitive manner. The results presented in this study also signify the importance of *in vitro* solution experiments. The present work on exploration of these compounds as novel potential inhibitors of cathepsins B and H activity may provide a useful insight in drug discovery and their possible role as anticancer chemotherapeutic or anti-inflammatory agent is expected.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bioorg.2015.01.005.

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