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Research

2'-Hydroxychalcones and their Cyclized Derivatives as Cathepsin L Inhibitors

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Background

Elevated levels of various cathepsins in cancer and inflammation necessitate exploring their potential inhibitors. Cathepsin L has been a potential drug target in these diseased conditions. In search for novel chemotherapeutic agents with better efficacy, potential inhibitors of target molecules need to be identified. In the present study, we have assayed the inhibitory potency of three structurally related series of flavanoids i.e., 2'-hydroxychalcones, flavanones and flavones against cathepsin L.

Methods

Enzyme assays have been carried out to screen the inhibitory effect of title compounds on cathepsin L. The results are compared with control. Enzyme kinetics studies have been carried out using Line-weaver Burk plot to evaluate the type of inhibition and K_i values. For carrying out molecular docking experiments software iGemDock has been used.

Results

The compounds have been evaluated as potential inhibitors to cathepsin L. SAR studies suggested that 2'-hydroxychalcones were better inhibitors as compared to their cyclized derivatives. The most potent inhibitors among the three series were nitro substituted compounds 1g, 2g and 3g with K_i values of ~3.75×10^{-9}M, ~57.32×10^{-9}M and ~70.96×10^{-9}M, respectively. The results are well correlated with docking studies.

Conclusion

Some of the compounds inhibited the enzyme to an appreciable extent at nanomolar concentrations. One of the compound, 1g inhibited cathepsin L with almost same potency as has been reported for specific peptidyl inhibitor, leupeptin.

Keywords: 2'-hydroxychalcone; Flavanone; Flavones; Cathepsin L inhibitors; Anti-cancer agents.

Introduction

Cathepsins have emerged as potential drug targets for the cancer treatment because of their role in tumor invasion and metastasis. Anti-cathepsin therapy has been considered as one of the options in cancer treatment. Of various ubiquitous cathepsins, cathepsin L has a special place and plays a major role in antigen processing, tumor invasion and metastasis, bone resorption, and turnover of intracellular and secreted proteins involved in growth regulation [1-3] and degradation of extracellular proteins [4-6]. Cathepsin L may promote tumor cell invasion and metastasis by catalyzing degradation of the interstitial matrix and basement membranes, thus allowing cancer cells to invade locally and metastasize to distant sites. Several tumor-forming cell lines are known to over-produce cathepsin L [7]. The mRNA level of cathepsin L is related to the In-Vivo metastatic potential of malignantly transformed cells [8]. Antisense RNA inhibition of cathepsin L expression reduced tumorigenicity in two malignant cell lines, suggesting that cathepsin L is a critical factor in tumor growth [9].

Cat L gene is activated by a variety of growth factors (PDGF and EGF), tumor promoters (including v-ras, v-src and v-mos), and second messengers [1, 10-13] (cAMP). In addition to natural inhibitors like Cystatins [14,15], and Stefin B [16,17] expression of cathepsin L is inhibited [18] by Squamous cell carcinoma antigen [19] and human c-Haras p2. All these findings clearly indicate the role of cathepsin L in cancer invasion and progression. As such, the identification of inhibitors of cathepsin L would provide valuable tools to explore them as potential drug-candidates in treatment of cancer. 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 [20-26]. Most of these compounds including chalcones [27-35] and flavanoids [36-39] are reported to possess anticancer and antiinflammatory activities. In continuation of our previous work [40], the present work reports the inhibition studies of 2'-hydroxychalcones and respective flavanones on cathepsin L.

Details

Materials and Methods

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

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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.

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

Purification of Goat Brain 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 [41] including acetone powder preparation, homogenization in cold 0.1 M acetate buffer pH 5.5 containing 0.2 M NaCl and 1mM 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 nmoles/min/mg.

Effect of 2-hydroxychalcones, Flavanones and Flavones on the Activity of Cathepsin L: The activities of cathepsin L were estimated at varying concentrations of synthesized 2'-hydroxychalcones, flavanones and flavones (figure 1(i-iii))**,** separately. First of all, enzyme was equilibrated in 0.1 M phosphate buffer of pH 6.0 at 37°C. The stock solutions of compounds were prepared in DMSO. Appropriate amount of stock solutions of individual compounds and corresponding amount of DMSO (in total 20 µl) was added in the reaction mixture to effect different concentrations of each compound as indicated in figures 1(i-1iii), separately. After incubation time of 30 min. residual enzyme activity was estimated by the usual enzyme assay [41] at pH 6.0 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.

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 Ki 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 Ki values were calculated using Line-weaver Burk equations for competitive inhibition.

Kinetic Studies of 2-hydroxychalcones, Flavanones and Flavones 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 (Raghav, figure 1d-1f). And the K_i values were calculated using the line-weaver burk equation for competitive inhibition Km'= Km(1+I/ K i).

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 cav3BC3L_CSW [42]. 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 the ligands in the active site of cathepsin L along with the substrate Z-Phe-Arg-4mβNA and most inhibitory compounds from each series are shown in (Raghav, figure 2).

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 cav3BC3L_CSW.

Results and Discussion

Chalcones are known as precursors for flavonoids, and show varied biological activities, such as anticancer [36,38], antioxidant [34], antiinflammatory [37] and antimicrobial properties [43]. The chalcone has been studied for its inhibition of prostaglandin E2 production [44]. Flavanones and flavones have been reported to have potential to cure, treat and prevent tumor, senescence and cancer [39]. The biological activities exhibited by chalcones and corresponding flavanoids like anticancer and anti-inflammatory where role of cathepsins has been established motivated us to screen the effect of some 2'-hydroxychalcones, flavanones and flavones on cathepsins B and H [40]. In addition to these two cathepsins, cathepsin L also contribute significantly to these diseased conditions and therefore need to be explored for the inhibitory effect of title compounds. The results obtained are discussed below.

Effect of 2'-hydroxychalcones, Flavanones, Flavones on the Activity of Cathepsin L

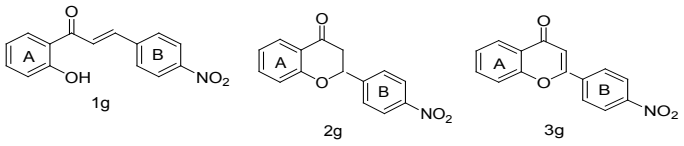
The activity of cathepsin L was estimated at varying concentrations of title compounds, (Raghav, figure 1(i-iii)), respectively. The figures show the relationship between the enzyme activity and concentration of different flavanoids. The plots of % residual activities versus the concentrations of different compounds gave a relationship where increased compound concentration caused more inhibition. Among the various compounds tested, 1-(-2hydroxyphenyl)-3-(-4-nitrophenyl) prop-2-en-1-one (1g) was found to be most inhibitory to cathepsin L activity.

Table-1: Cathepsin L inhibition studies in presence of Substituted 2'-hydroxychalcones, flavanones and flavones

S. No.	Compound Name	% residual activity at (Z) x10^{-7}M Concentration of compounds	K_i (10^{-9}M)
	Control	100.0±1.33	
1.	1-(-2-hydroxyphenyl)-3-phenylprop-2-en-1-one (1a)	30.480±3.00	8.70
2.	1-(-2-hydroxyphenyl)-3-(-4-methylphenyl)prop-2-en-1-one (1b)	59.850±5.92	33.65
3.	1-(-2-hydroxyphenyl)-3-(-4-methoxyphenyl)prop-2-en-1-one (1c)	44.664±4.42	26.42
4.	1-(-2-hydroxyphenyl)-3-(-3-chlorophenyl)prop-2-en-1-one (1d)	19.501±1.89	6.41
5.	1-(-2-hydroxyphenyl)-3-(-4-chlorophenyl)prop-2-en-1-one (1e)	18.026±1.73	5.39
6.	1-(-2-hydroxyphenyl)-3-(-3-nitrophenyl)prop-2-en-1-one (1f)	12.25±1.16	4.40
7.	1-(-2-hydroxyphenyl)-3-(-4-nitrophenyl)prop-2-en-1-one (1g)	11.31±1.11	3.75
8.	1-(-2-hydroxyphenyl)-3-(-2-methoxyphenyl)prop-2-en-1-one (1h)	32.26±3.17	11.40
9.	1-(-2-hydroxyphenyl)-3-(-3-methoxyphenyl)prop-2-en-1-one (1i)	36.07±3.55	14.08
10.	2,3-dihydro-2-phenylchromen-4-one (2a)	42.32±4.21	132
11.	2,3-dihydro-2-(-4-methylphenyl)chromen-4-one (2b)	84.34±8.37	749
12.	2,3-dihydro-2-(-4-methoxyphenyl)chromen-4-one (2c)	73.94±7.32	366
13.	2,3-dihydro-2-(-3-chlorophenyl)chromen-4-one (2d)	34.07±3.33	106
14.	2,3-dihydro-2-(-4-chlorophenyl)chromen-4-one (2e)	23.51±2.28	84.96
15.	2,3-dihydro-2-(-3-nitrophenyl)chromen-4-one (2f)	19.86±1.89	70.94
16.	2,3-dihydro-2-(-4-nitrophenyl)chromen-4-one (2g)	14.40±1.38	57.32
17.	2,3-dihydro-2-(-2-methoxyphenyl)chromen-4-one (2h)	44.66±4.44	183
18.	2,3-dihydro-2-(-3-methoxyphenyl)chromen-4-one (2i)	54.53±5.44	238
19.	2-phenyl-4H-chromen-4-one (3a)	37.89±3.73	225.09
20.	2-(-4-methylphenyl)-4H-chromen-4-one (3b)	62.33±6.21	2062
21.	2-(-4-methoxyphenyl)-4H-chromen-4-one (3c)	52.093±5.17	749.21
22.	2-(-3-chlorophenyl)-4H-chromen-4-one (3d)	30.790±3.02	166.55
23.	2-(-4-chlorophenyl)-4H-chromen-4-one (3e)	29.35±2.91	136.99
24.	2-(-3-nitroyphenyl)-4H-chromen-4-one (3f)	19.50±1.91	99.37
25.	2-(-4-nitroyphenyl)-4H-chromen-4-one (3g)	15.86±0.75	70.96
26.	2-(-2-methoxyphenyl)-4H-chromen-4-one (3h)	47.72±4.75	288.82
27.	2-(-3-methoxyphenyl)-4H-chromen-4-one (3i)	48.11±4.79	360.66
28.	Leupeptin	90.98±0.89(0.01)	-

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

Compound	Total Energy (Kcal/mol)	VDW	H Bond	Electronic
Leupeptin	120.59	87.62	-31.93	-1.72
Z-Phe-Arg-4mβNA	-136.04	-107.26	-31.06	2.29
1g	-92.37	-75.80	-16.56	0
2g	-88.42	-80.02	-8.41	0
3g	-84.94	-71.69	-14.06	0.81



Most inhibitory compounds among 1a-1i, 2a-2i and 3a-3i

Similarly among flavanones and flavones nitro substituted compounds 2g and 3g have been found to be most inhibitory. It has been found that cathepsin L activity is inhibited and is affected by the substituent present in B ring. The compounds have been found to be most inhibitory to cathepsin B also. However the inhibitory potential

for cathepsin L has been found in the order of 10^{-7}M as compared to 10^{-5}M and 10^{-4}M for cathepsins B and H. The results further illustrate the significance of present study that the title compounds are better inhibitors to cathepsin L than cathepsin B and H.

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, [Raghav, figure 1(iv-vi)].

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. Using the Line-weaver Burk equation for competitive inhibition the K_i values were calculated, which has been presented in table 1. The Ki value of most inhibiting compound cathepsin L in the corresponding series i.e. chalcone, flavanone and flavone has been evaluated ~$3.75x10^{-9}$M, ~$57.32x10^{-9}$M and ~$70.96x10^{-9}$M for 1g, 2g and 3g, respectively. The compound, 1g, 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 $1.45x10^{-9}$ for goat brain cathepsin L [45]. The K_i values for cathepsins B have been reported as ~$6.18x10^{-8}$M, $4.8x10^{-7}$M and 7.85 $x10^{-7}$M, respectively and for cathepsin H, the K_i values are ~2.8 $x10^{-7}$M, 31.8 $x10^{-6}$M and 33.7 $x10^{-6}$M respectively [40]. This has been interesting to find out that the title compounds have been selective inhibitors to cathepsin L when compared to cathepsin B and H. Moreover the inhibitory studies of chalcones and corresponding flavanoids further detail the pharmacological potential of these molecules.

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 1-(-2-hydroxyphenyl)-3-(-4-nitrophenyl)prop-2-en-1-one, 1g followed by 2,3-dihydro-2-(-4-nitrophenyl) chromen-4-one, 2g and 2-(-4-nitroyphenyl)-4H-chromen-4-one, 3g within the active site of cathepsin L (table 2). Leupeptin, the peptidyl inhibitor of cathepsin L, interact with the enzyme and showed a higher interaction energy than all the compounds. Decrease in total energy for leupeptin-cathepsin L has come out to be -120.59 which is nearest to substrate BANA, --136.04 when compared to the most inhibitory compound in each series. The contribution of van der Waal interactions are more with a score of -107.26 as compared to H-bonds with a score of -31.06. This is due to peptide protein interaction. Leupeptin is peptidyl in nature and therefore binds effectively with the enzyme active site resulting in higher binding energy. As compared to this the binding energy of title compounds are less (table 2). When compared with in the designed series to evaluate the interaction with cathepsin L, the results clearly support the *In-Vitro* inhibition studies. 2'-hydroxychalcones show a higher interaction than flavanones and flavones (table 1) in that order. The *in-silico* predictable behavior of enzyme-ligand interaction can give an idea about the interaction. 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. It is clear in (Raghav, figure 2(i-iii)) showing the docked view of 1-(-2-hydroxyphenyl)-3-(-4-nitrophenyl)prop-2-en-1-one, 2,3-dihydro-2-(-4-nitro-phenyl)chromen-4-one and 2-(-4-nitroyphenyl)-4H-chromen-4-one in the active site of cathepsin L, where active site groups Trp-26, Asp-162, His-163 have been found to interact with the compounds as well as with substrate Z-Phe-Arg-4mβNA thus indicating a competitive type of inhibition. The results endorse the in vitro studies where the Lineweaver Burk plots also represented a competitive type of inhibition. The results are in consensus with those for cathepsin B and H [40], where also in-silico and in-vitro studies are in accordance with each other.

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 2'-hydroxychalcones, flavanone and flavones act as inhibitors of these cysteine proteases. The compounds 1g, 2g and 3g inhibited cathepsin B activity maximally with K_i values of ~$3.75x10^{-9}$M, ~$57.32x10^{-9}$M and ~$70.96x10^{-9}$M, respectively. All the compounds were evaluated as competitive inhibitors of enzymes and the results are well documented with *in- silico* experiments.

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