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SAR studies of o-hydroxychalcones and their cyclized analogs and study them as novel inhibitors of cathepsin B and cathepsin H

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ABSTRACT

Cathepsins have emerged as a potential target for anti-cancer drug development. In the present study, we have synthesized three structurally related series of flavanoids i.e., 2′-hydroxychalcones, flavanones and flavones and assayed *in vitro* to study their inhibitory potency against cathepsin B and H, promising drug candidate for cancer therapy. Enzyme kinetics studies were carried out in presence of these compounds after preliminary proteolytic studies on endogenous protein substrates. SAR studies suggested that open chain flavanoids were better inhibitors as compared to their cyclized analogs. The most potent inhibitors among the three series were nitro substituted compounds 1g, 2g and 3g with K_i values of $\sim 6.18 \times 10^{-8}$ M, 4.8×10^{-7} M and 7.85×10^{-7} M for cathepsin B and K_i values of $\sim 2.8 \times 10^{-7}$ M, 31.8×10^{-6} M and 33.7×10^{-6} M for cathepsin H, respectively. The relationship between chalcone, flavanones and flavone structures interpreted by docking studies on cathepsin B and H also provided useful insights.

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1. Introduction

Recently, several studies have reported close association between cancer extension and cathepsin expression (Gocheva and Joyce, 2007; Jedeszko and Sloane, 2004; Turk et al., 2004). Protease inhibitors have considerable potential utility for therapeutic intervention in a variety of disease states such as inflammation, osteoporosis, microbial infections and cancer cell invasion (Kerrow and James, 1996; Mort and Buttle, 1997; Frlan and Gobec, 2006; Jedinak and Maliar, 2005; Lim et al., 2004). Lysosomal cysteine proteinases are involved in intracellular protein degradation and inflammatory conditions. Increased levels of cathepsin B [EC 3.4.22.1] and cathepsin H [EC 3.4.22.16] with simultaneously diminished amount of cysteine protease inhibitors have been verified in a variety of tumors and therefore, approve their contribution towards invasion and metastasis. The cathepsin family of lysosomal enzymes is also of special interest because of their involvement in bone resorption, joint inflammation, destruction in rheumatoid arthritis and extracellular matrix degradation. Based on the aforementioned characteristic features, cathepsin B and H has become the focus of intensive investigation in hope to understand its precise role in severe diseases like cancer, angiogenesis, rheumatoid arthritis and especially its potential for use as a target for chemoprevention and anticancer therapy.

The work on the identification of small molecular weight compounds as inhibitors of cysteine proteases in the past decade has been in focus. Recently, different chalcones have been identified as cathepsin B inhibitors (Kim et al., 2013; Caracelli et al., 2012). Chalcones are known as precursors for flavonoids, and show varied biological activities, such as anticancer (Wyns et al., 2012; Zhang et al., 2012), antioxidant (Pan et al., 2012; Sahu et al., 2012), anti-inflammatory (Shin et al., 2013) and antimicrobial properties (Tran et al., 2012a,b). The chalcone has been studied for its inhibition of prostaglandin E2 production, inducing the biosynthesis of glutathione and antibacterial activities against methicillin-resistant staphylococcus aureus (Tran et al., 2009, 2012a,b; Kachadourian et al., 2012). Flavanones and flavones have been reported to have significant potential to cure, treat and prevent tumor, senescence and cancer (Ramos, 2007). Flavanones have been a potential source in the search for lead compounds and biologically active components and have been the focus of much researches and development in the last 30 years (Halsteen, 1983; Silberberg et al., 2006). 2-Hydroxyflavanone markedly inhibited the invasion, motility and cell-matrix adhesion of A549 cells (Yung et al., 2007). Lavandulyl flavanones showed potent β-secretase inhibitory activity which was strongly implicated in the cause of Alzheimer's disease (Shi et al., 2010). As such, the identification of inhibitors of cathepsin B and H would provide valuable tools to explore them as potential drug-candidates in treatment of cancer, arthritis, inflammation and other tissue degeneration processes. We have identified different classes of compounds as inhibitors to endogenous proteolysis at

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a pH where cysteine proteases are active (Raghav et al., 2010a,b, 2011, 2012; Raghav and Singh, 2013). Recently we have reported bischalcones, their derivatives (Raghav and Singh, 2014a) and acylhydrazides, triazoles (Raghav and Singh, 2014b) as novel inhibitors of cathepsin B & H. To further achieve this endeavor, we here present the synthesis of some 2′-hydroxychalcones and their corresponding flavanone and flavone, followed by their evaluation as cysteine protease inhibitors and inhibitory studies on cathepsin B and cathepsin H.

2. Materials and methods

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

2.1. General procedure

Melting points were determined in open capillary tubes and are uncorrected. All the chemicals and solvents used were of laboratory grade. IR spectra (KBr, cm^{-1}) were recorded on a PerkinElmer spectrometer. ^1H NMR spectra was recorded on Bruker 300 MHz NMR spectrometer (chemical shifts in d 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. Refrigerated ultracentrifuge Remi C-24BL was used for centrifugation purpose.

2.2. Proteolytic studies

2.2.1. Preparation of liver homogenate

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

2.2.2. Assay for proteolytic activity

The proteolytic activity was estimated at pH 5.0, 37 °C using 0.1 M acetate buffer as the incubation medium (Kamboj et al., 1992). The homogenate prepared above was incubated with the buffer at 37 °C for 3 h and 24 h, separately. The reaction was stopped by the addition of TCA and the resulting solution was centrifuged to precipitate proteins. The acid soluble proteins were quantitated in the supernatant using Bradford method (Bradford, 1976). The experiments were conducted in triplicate and the results are presented in Table 1 (Fig. 1).

2.2.3. Purification of goat liver cathepsin B and cathepsin H

All the purification steps were carried out at 4 °C. Cathepsin B and H were isolated, separated and purified from goat liver using the well established procedure (Kamboj et al., 1990; Raghav et al., 1995) including 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 sulfate fractionation, molecular sieve chromatography on Sephadex G-100 column chromatography and finally ion-exchange chromatographies on CM-Sephadex C-50 and DEAE Sephadex A-50 column. The specific activities of the cathepsin B and cathepsin H were ~11.15 nmol/min/mg and ~22.91 nmol/min/mg, respectively. The specific activity was calculated by the following equation.

Specific activity = total activity units/total protein

2.2.4. Effect of 2-hydroxychalcones, flavanones and flavones on the activity of cathepsin B and cathepsin H

The activities of cathepsin B were estimated at varying concentrations of synthesized 2′-hydroxychalcones, flavanones and flavones (Fig. 2a–c), 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 Fig. 2a–f, separately. After incubation time of 30 min residual enzyme activity was estimated by the usual enzyme assay (Kamboj et al., 1990) at pH 6.0 using a-N-benzoyl-D,L-arginine-2-naphthylamide (BANA) as substrate. The experiments were performed in triplicate for each concentration and averaged before further calculations. The % activity in each case has been calculated with respect to the control where no compound was added but an equivalent amount of DMSO (20 μl) was present. The results are presented in Table 1. Similar experiments were designed to evaluate the effect of varying concentrations of synthesized 2-hydroxychalcones, flavanones and flavones separately on cathepsin H using LeuβNA as substrate at pH 7.0 (Fig. 2d–f) (Raghav et al., 1995).

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

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

2.3. Drug modeling studies

All docking studies were performed using iGemdock. For these studies, small molecular weight ligands and enzyme active site structure is required. The structure of cathepsin B was retrieved from Protein Data Bank (http://www.rcsb.org/) as cav2IPP B_PYS.pdb (Huber et al., 2013). 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 B along with the substrate BANA and different compounds are shown in Fig. 4.

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