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SAR studies of some acetophenone phenylhydrazone based pyrazole derivatives as anticathepsin agents

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ABSTRACT

Cathepsins have emerged as promising molecular targets in a number of diseases such as Alzeimer's, inflammation and cancer. Elevated cathepsin's levels and decreased cellular inhibitor concentrations have emphasized the search for novel inhibitors of cathepsins. The present work is focused on the design and synthesis of some acetophenone phenylhydrazone based pyrazole derivatives as novel non peptidyl inhibitors of cathepsins B, H and L. The synthesized compounds after characterization have been explored for their inhibitory potency against cathepsins B, H and L. The results show that some of the synthesized compounds exhibit anti-catheptic activity with K_i value of the order of 10^{-10} M. Differential inhibitory effects have been observed for cathepsins B, H and L. Cathepsin L is inhibited more pronounced than cathepsin B and cathepsin H in that order.

1. Introduction

Over expression of cathepsins in different cancerous processes has gained attention of researchers to understand their precise role in tumor cell proliferation and metastasis [1], angiogenesis and invasion [2]. In addition, role of cathepsins in the degradation of extracellular matrix has been an important factor in establishing these as important targets in various conditions related to Alzeimer's disease, inflammation etc. Elevated levels of cathepsins B, H and L [3–5] signify the contribution of inhibitors in control of these diseased states [6–7] to an extent that anti-cathepsin activities are now the focus for the development of novel therapeutic possibilities. Based on the peptidyl nature of cellular catheptic inhibitors initial research was focused on peptide based warheads [8–12], where peptidyl backbone contributed toward specificity and active group resulted in inhibition generating diverse variety of inhibitors usually irreversible in nature. Gastric instability and immunological problems associated with these inhibitors, envisaged the importance of low molecular weight inhibitors which can be easily synthesized.

With this background we explored simple compounds like semicarbazones and thiosemicarbazones carbonyl compounds and chalcones, derivatives of chalcones as inhibitors of cathepsins [13–16]. Some benzofuran derivatives [17], acylhydrazides and triazolesi [18] have also been found to be potential inhibitors of cathepsins. Identification of semicarbazones and thiosemicarbazones [13] and pyrazolines [14] as potential inhibitors of cathepsins B, H and L motivated us to explore the designed molecules having two different pharmacophores i.e., pyrazole and different side chain azomethine groups as a novel class of cathepsins B, H and L inhibitors which may provide new therapeutic opportunities in diseased states caused by imbalanced activities of these cathepsins.

In the present work, we synthesized differently functionalized pyrazole-4-carbaldehydes from hydrazones of aryl methyl ketones. Semicarbazones, thiosemicarbazones and phenyl hydrazones of pyrazole-4-carbaldehydes were also synthesized. All the synthesized compounds were evaluated as inhibitors to cathepsin B, H and L. Detailed analysis of effectiveness of these derivatives may provide a platform for development of the potent enzyme inhibitors. When these analogs were screened against cathepsin B, H and L in enzyme assays, two promising inhibitors were identified. The results are compared with *in silico* studies which support the postulation that synthesized compounds may act as enzyme inhibitors.

2. Experimental protocols

2.1. Materials

All the chemicals were of analytical grade. Fast Garnet GBC (*o*-aminoazotoluene diazonium salt), Various substrates used i.e. α-*N*-benzoyl-D, l-arginine-β-naphthylamide (BANA) for cathepsin

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B, Z-phenylalanylarginyl-β-naphthylamide (Z-Phe-Arg-βNA) for cathespsin L and Leucyl-β-naphthylamide (Leu-βNA) for cathepsin H 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. Purification of cathepsins B, H and L

All the purification steps were carried out at 4 °C. Cathepsin B and H were isolated, separated and purified from goat liver by established procedure [19]. The specific activities of the cathepsin B, H and L thus obtained, were ~10.38, ~22.56 and ~17.48 nmol/min/mg respectively.

2.2.2. Enzyme assays

Stock solutions of the compounds (5 mM) were prepared in DMSO. The purified cathepsin B, H and L were first activated in presence of thiol activators at pH 6.0, 7.0 and 6.0, respectively. Then, 100 μl of the enzyme solution was mixed with 855 μl of 0.1 M 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. 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 [19–21]. In control experiments, an equivalent amount of DMSO was added and percent residual activities were calculated with reference to control.

The compounds exhibiting complete inhibition at 1×10^{-4} M concentration were further studied for their inhibitory effect at their lower concentrations i.e. 10^{-5} M, 10^{-6} M, 10^{-7} M and so on till some activity was observed for cathepsins B, H and L. Table 1 displays the effect of individual compounds on cathepsin B, H and L activities at a particular concentration ($\times 10^{-5}$ M as shown in parenthesis in respective positions). Once the inhibitory potential was established experiments were conducted to study the effect of individual compound at varying concentration. The results are shown in Figs. 1, 2 and 3 (I, II, III, IV, V, VI).

2.2.3. Enzyme kinetic studies

After establishing the inhibitory action of synthesized compounds on cathepsins B, H and L, Lineweaver-Burk plots were drawn to evaluate the type of inhibition and to determine their K_i values. For that, enzyme activity was evaluated at 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 particular concentration of inhibitor (the concentration of each inhibitor at which the experiment was conducted is given in parenthesis in Table 1 as $Z \times 10^{-5}$ M concentration). The enzyme concentration was kept constant in all the experiments as detailed previously. The values represent Mean ± S.M.D. of at least three individual experiments. The K_i values of compounds were calculated using the Lineweaver-Burk equation $K_m = K_m (1 + [I]/K_i)$ for competitive inhibition and $V_{max} = V_{max} (1 + [I]/K_i)$ for non-competitive inhibition. The mode of inhibition was investigated and K_i values obtained are tabulated in Table 2. The K_m values for cathepsin B, H and L were found to be 4.0×10^{-4} M, 5.0×10^{-4} M and 7.6×10^{-5} M, respectively, whereas, the $1/V_{max}$ values were found to be 0.120, 0.170 and 0.130, respectively.

2.2.4. Drug modeling studies

All docking studies were performed using iGemdock. For these studies, the structures of cathepsin B and cathepsin H were retrieved from Protein Data Bank (http://www.rcsb.org/) as 2IPP B [22], 8PCH H [23] and 3BC3L [24], respectively. The bound ligands PYS, NAG and CSW were removed prior to docking. The cavity structure selected was 8 Å. Structures of the ligands were prepared in Marvin sketch and were minimized before saving as MDL Mol File. The ligand structures and enzyme active sites were loaded and docking was run where GA parameters were defined according to drug screening setting for population size, number of generation and solutions. The stabilization energies calculated as E_{total} as a consequence of enzyme ligand interaction for cathepsin B, H and L and are presented in Tables 3, 4 and 5, respectively. The docked poses of most inhibitory compounds, **4d** and **4e** for cathepsin B and H are revealed in Figs. 4 and 5, respectively. The most inhibitory compounds **1b** and **2b** for cathepsin L are depicted in Fig. 6.

2.2.5. General procedure for synthesis

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. ^1H 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 aluminium plates percolated 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.

2.2.6. General procedure for the synthesis of 1H-pyrazole-4-carbaldehydes (**2**)

At 0 °C $POCl_3$ (0.98 ml) was added dropwise with stirring to ice cold DMF solution over a period of 30 min. Then, *p*-bromoacetophenone phenylhydrazone **1a** (1.0 g, 0.0036 mol) in N,N-dimethylformamide (3 ml) was added drop wise. Stirring was continued under ice cold condition for another half an hour. The reaction mixture was brought to room temperature and refluxed at 60–70 °C for 4–5 h. The reaction mixture was then cooled and poured into crushed ice with stirring and neutralized with aq. $NaHCO_3$ solution. The solid obtained was filtered and recrystallized from ethanol. The structure elucidation of compound, 3-(4-bromophenyl)-1-phenyl-1*H*-pyrazole-4-carbaldehyde **2a** was based on the spectral data (IR, ^1H NMR & ^{13}C NMR).

*2.2.6.1. 3-(4-Bromophenyl)-1-phenyl-1H-pyrazole-4-carbaldehyde (**2a**).* Yield 67.85%; m.p.160–162 °C[25]; IR (KBr, cm^{-1}): 1674 (—C=O str), 1597(—C=N str), 1450, 1566(—C=C—str), 825(—C—Br str); ^1H NMR (300 MHz, $CDCl_3$, δ ppm): 7.38–7.40 (3H, m, Ar—H), 7.64(2H, d, *J* = 8.1 Hz, Ar—H), 7.77–7.82(4H, m, Ar—H), 9.03 (1H, s, —C_5H), 10.06 (1H, s, CHO); ^{13}C NMR (75 MHz, $CDCl_3$, δ ppm): 184.52, 152.93, 139.72, 133.08, 132.95, 131.84, 129.99, 129.38, 126.11, 122.44, 120.45, 110.72.

Following exactly the same procedure as detailed above for compound **2a**, the other substituted (1*H*)-pyrazole-4-carbaldehydes (**2b**–**2f**) were prepared from the corresponding acetophenone and substituted benzaldehydes. The physical and spectral data of synthesized compounds are given below.

*2.2.6.2. 3-(4-Nitrophenyl)-1-phenyl-1H-pyrazole-4-carbaldehyde (**2b**).* Yield 75.63%; m.p.165-167 °C[25]; IR (KBr, cm^{-1}): 1682 (—C=O str), 1597(—C=N str), 1450, 1585(—C=C—str), 1342, 1520 (—NO_2 str); ^1H NMR (300 MHz, $CDCl_3$, δ ppm): 7.44–7.60 (3 H, m, Ar—H), 8.02(2H, d, Ar—H), 8.26–8.37(4 H, m, Ar—H), 9.43 (1H, s,