

IN-VITRO INHIBITION STUDIES ON ENDOGENOUS PROTEOLYSIS OF LIVER HOMOGENATE IN PRESENCE OF SYNTHESIZED PYRAZOLES

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ABSTRACT

Objective: Pyrazoles have wide application in medicinal chemistry because of diverse pharmacological activities. As a part of our ongoing research to identify potential inhibitors of cysteine proteases, these observations were guiding initiatives in the present work for the synthesis of some pyrazoles with a desire to see their inhibitory effect on endogenous proteolysis.

Methods: We have synthesized a series of substituted pyrazoles by hypervalent iodine oxidation of 1, 3, 5-triphenyl pyrazolines which were obtained from the reaction of the corresponding chalcones with phenyl hydrazines. The structure of the compounds was confirmed on the basis of IR & ¹H-NMR spectrum. The acid soluble proteins were quantitated in the supernatant using Bradford method.

Results: In the present work, we have demonstrated *in-vitro* effects of substituted pyrazoles on endogenous proteolysis in liver homogenate. It was observed that 5-(4'-methylphenyl)-1,3-diphenyl-2-pyrazole (1g) in 3 hr reaction and 5-(4'-fluorophenyl)-1,3-diphenyl-2-pyrazole (1e) in 24 hr reaction showed more inhibition while unsubstituted 1, 3, 5- triphenyl-2-pyrazole (1a) showed high inhibition both is 3 and 24 hr incubation.

Conclusion: These compounds act as inhibitors to proteases active at pH 5.0, and inhibit endogenous protein hydrolysis significantly. In general, some compounds demonstrated ~100% inhibition at 1mM concentration.

Keywords: Cysteine proteases, Inhibition studies, Substituted pyrazoles, Endogenous proteolysis.

INTRODUCTION

1,3,5-trisubstituted pyrazoles constitute a class of important compounds because heterocycles containing pyrazole moieties often demonstrates valuable biological and medicinal activities, such as antimicrobial[1], analgesic[2], anti-inflammatory[3-4], antihyperglycaemic[5], anticancer[6], antioxidant[7], cytotoxic [8], antidepressant and anticonvulsant[9], antitumoral [10] and antidiabetic[11] and antimalarial [12]. They also act as inhibitors of cyclooxygenase-2 and soluble epoxide hydrolase[13], cannabinoid Type 1 receptor antagonists[14], androgen receptor antagonists[15], B-Raf kinase[16], angiotensin converting enzyme [17], PDE-4[18], γ -secretase[19], non-purine xanthine oxidase[20], type II topoisomerases[21], cathepsin S[22] and Monoamine Oxidase[23]. Proteases essential part of the proteolytic machinery are becoming important because of their involvement in oncology, inflammatory conditions, blood rheology control and immune regulation [24] etc. Cysteine proteases have traditionally been viewed as lysosomal mediators of terminal protein degradation. These observations were guiding initiatives in the present work for the synthesis of some pyrazoles with a desire to see their inhibitory effect on endogenous proteolysis. Thus, by substituting different groups in the pyrazole moiety, there occurs gradual changes in the physicochemical properties and hence the biological activity of the compounds. The development of inhibitors of specific cysteine proteases promises to provide new drugs for modifying immunity, osteoporosis, and chronic inflammation etc. Our present work was an effort to synthesize pyrazole derivatives and to see their *in-vitro* effects on endogenous proteolysis of mammalian origin i.e., goat liver.

Experimental Section

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. ¹H NMR spectra was recorded on Bruker 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 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 under cold conditions.

Preparation of liver homogenate

Goat liver was purchased freshly from the local slaughter house. The fresh goat liver was first washed with cold isotonic saline solution. The tissue was then homogenized in 0.1M sodium acetate buffer pH 5.5 containing 0.25% Triton X-100 and 0.2M NaCl in a mixer-cum-blender to obtain 10% (w/v) homogenate [25]. It was then stored at 4°C.

Protein estimation

The acid soluble proteins were quantitated in the supernatant using Bradford method [26].

Table 1: Effect of Substituted pyrazoles on Endogenous Protein Hydrolysis in Liver Homogenate

S. No.	Compound Name	3h		24 h	
		M.D.±S.M.D.	% Residual Activity	M.D.±S.M.D.	% Residual Activity
	Control	0.047 ±0.0125	100	0.076±0.0142	100
1.	1, 3, 5- triphenyl-2-pyrazole (1a)	0.002 ±0.0005	4.25 ±1.06	0.008±0.0022	10.52 ±2.89
2.	5-(4'-chlorophenyl)-1,3-diphenyl-2-pyrazole (1b)	0.018 ±0.0004	38.29±0.851	0.072±0.0003	94.73 ±0.39
3.	5-(4'-methoxy phenyl)-1,3-diphenyl-2-pyrazole (1c)	0.022 ±0.0006	46.80±1.27	0.074±0.0014	97.36 ±1.84
4.	5-(4'-nitrophenyl)-1,3-diphenyl-2-pyrazole (1d)	0.040 ±0.0012	85.10 ±2.55	0.070±0.0012	92.10 ±1.57
5.	5-(4'-fluorophenyl)-1,3-diphenyl-2-pyrazole (1e)	0.024 ±0.0008	51.06 ±1.70	0.004±0.0017	5.26 ±2.23
6.	5-(4'-bromophenyl)-1,3-diphenyl-2-pyrazole (1f)	0.036 ±0.0011	76.59 ±2.34	0.042±0.0002	55.26 ±0.26
7.	5-(4'-methylphenyl)-1,3-diphenyl-2-pyrazole (1g)	0.001 ±0.0001	2.13 ±0.21	0.056±0.0015	73.68 ±1.97

The TCA soluble peptides were estimated at 630 nm using Bradford method and the results are the mean and S.M.D. of the experiment conducted in triplicate and is calculated as protease activity in 0.1% liver homogenate. The % residual activity is calculated w.r.t. control where no compound was added but an equivalent amount of solvent was present.

Assay for proteolytic activity

The proteolysis was carried out at pH 5.0 at 37°C using 0.1 M acetate buffer as the incubation medium. The homogenate mixed with the buffer at this pH was incubated at 37°C for 3 h and 24 h. 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. The experiment was conducted in triplicate and the results are presented in table 1.

General Procedure for the synthesis of 1, 3, 5-triphenylpyrazole: Pyrazoles were synthesized by the addition of IBD (1.5 mmol) to a stirred solution of substituted pyrazolines (1.0 mmol) in dichloromethane (30 ml) at room temperature. The reaction mixture was stirred for 5-6 hrs. Excess of dichloromethane was distilled off on a water bath and the residual mass was recrystallized from an appropriate solvent.

1, 3, 5-triphenyl pyrazole (1a): Yield 76.87%; m.p. 138-139°C; IR (KBr, cm⁻¹): 3059 (=CH stretching of aromatic ring), 1594(-C=N stretching), 1496(-C=C- stretching of aromatic ring); ¹H-NMR(300 MHz, CDCl₃, δ ppm): 6.84 (1H, s), 7.26-7.44(13H, m), 7.94 (2H, d, J=6.8Hz); ¹³C NMR (CDCl₃, 300 MHz, δ ppm): 152.7, 144.4, 139.8, 133.2, 130.4, 129.4, 127.7, 128.5, 127.9, 127.6, 128.4, 122.7, 120.1, 105.8

5-(4'-chlorophenyl)-1, 3-diphenyl pyrazole (1b): Yield 72.67%; m.p. 113-114 °C; IR (KBr, cm⁻¹): 3050(=CH stretching of aromatic ring), 1597(-C=N stretching), 1489(-C=C- stretching of aromatic ring), 774 (-C-Cl stretching); ¹H-NMR (300 MHz, CDCl₃, δ ppm): 6.82 (1H, s), 7.21-7.50(12H, m), 7.92 (2H, d, J=6.0Hz); ¹³C NMR (CDCl₃, 300 MHz, δ ppm): 152.2, 144.2, 139.7, 133.3, 132.6, 129.8, 129.3, 128.7, 128.6, 128.1, 127.5, 126.8, 125.3, 120.4, 106.2

5-(4'-methoxyphenyl)-1, 3-diphenyl pyrazole (1c): Yield 81.23%; m.p. 78-79 °C; IR (KBr, cm⁻¹): 3020 (=CH stretching of aromatic ring), 1597 (-C=N stretching), 1458 (-C=C- stretching of

aromatic ring), 1126(-C-OCH₃); ¹H-NMR (300 MHz, CDCl₃, δ ppm): 3.85(3H, s), 6.65 (1H, s), 6.88 (2H, d, J=9.0Hz), 7.20-7.46(10H, m), 8.03 (2H, d, J=6.0Hz); ¹³C NMR (CDCl₃, 300 MHz, δ ppm): 160.5, 153.1, 143.1, 139.4, 133.8, 131.7, 130.2, 129.4, 128.8, 128.5, 127.1, 126.6, 125.6, 120.3, 114.6, 106.2, 55.8

5-(4'-nitrophenyl)-1, 3-diphenyl pyrazole (1d): Yield 83.40%; m.p. 141-142°C; IR (KBr, cm⁻¹): 3063(=CH stretching of aromatic ring), 1566(-C=N stretching), 1497(-C=C- stretching of aromatic ring), 1350,1528(-C-NO₂ stretching); 6.97 (1H, s), 7.15 (2H, d, J=9.0Hz), 7.28-7.74 (10H, m), 8.10 (2H, d, J=6.0Hz); ¹³C NMR (CDCl₃, 300 MHz, δ ppm): 153.7, 148.5, 144.7, 139.7, 133.8, 131.7, 129.4, 128.6, 128.1, 127.5, 126.7, 124.3, 121.6, 106.5

5-(4'-fluorophenyl)-1, 3-diphenyl pyrazole (1e): Yield 70.67%; m.p. 118-120°C; IR (KBr, cm⁻¹): 3054(=CH stretching of aromatic ring), 1597(-C=N stretching), 1489(-C=C- stretching of aromatic ring), 1034 (-C-F stretching); ¹H-NMR (300 MHz, CDCl₃, δ ppm): 6.88 (1H, s), 7.14 (2H, d, J=6.0Hz), 7.27-7.54 (10H, m), 7.88 (2H, d, J=12.0Hz); ¹³C NMR (CDCl₃, 300 MHz, δ ppm): 162.8, 153.7, 144.1 139.3, 130.8, 130.2, 129.4, 129.2, 128.6, 128.4, 127.4 126.5, 124.3, 122.6, 105.6

5-(4'-bromophenyl)-1, 3-diphenyl pyrazole (1f): Yield 78.54%; m.p. 127-128°C; IR (KBr, cm⁻¹): 3050(=CH stretching of aromatic ring), 1597(-C=N stretching), 1489(-C=C- stretching of aromatic ring), 670 (-C-Br stretching); ¹H-NMR (300 MHz, CDCl₃, δ ppm): 6.85 (1H, s), 7.17-7.60 (12H, m), 7.97 (2H, d, J=9.0Hz); ¹³C NMR (CDCl₃, 300 MHz, δ ppm): 152.1, 143.1

139.9, 132.8, 131.7, 130.2, 129.4, 129.0, 128.6, 128.1, 127.6, 125.7, 125.3, 122.6, 105.2

5-(4'-methylphenyl)-1, 3-diphenyl pyrazole (1g): Yield 93.20%; m.p. 115-116°C; IR (KBr, cm⁻¹): 3040(=CH stretching of aromatic ring), 1593 (-C=N stretching), 1494 (-C=C- stretching of aromatic ring); ¹H-NMR (300 MHz, CDCl₃, δ ppm): 2.41(3H, s), 6.81 (1H, s), 7.15 (2H, d, J=6.0Hz), 7.27-7.66(10H, m), 7.98 (2H, d, J=9.0Hz); ¹³C NMR (CDCl₃, 300 MHz, δ ppm): 152.1, 143.1, 139.9, 132.8, 131.7, 130.2, 129.4, 129.0, 128.6, 128.1, 127.6, 125.7, 125.3, 122.6, 106.2, 23.5

RESULTS AND DISCUSSION

¹H NMR and IR data of synthesized pyrazoles were in full agreement with the proposed structures.

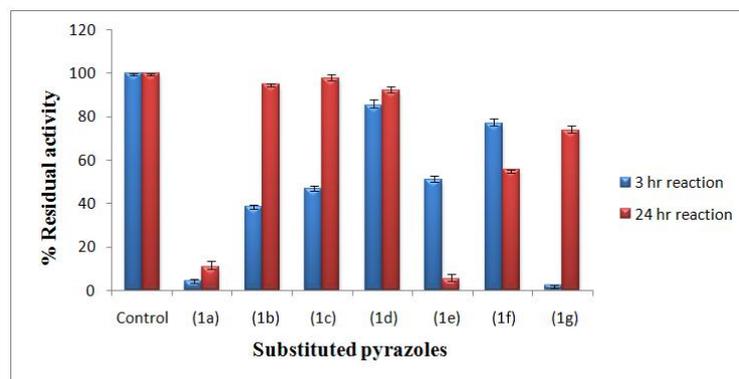
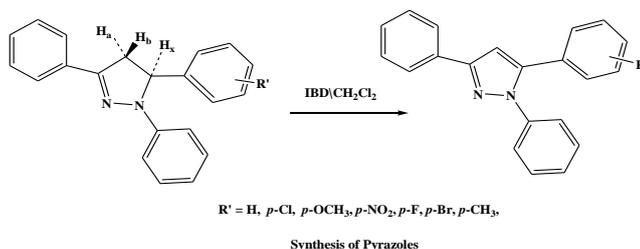


Fig. 1: Effect of 1,3,5-triphenyl pyrazoles on the endogenous proteolytic activity.



The IR spectra of the compounds showed aromatic stretching (1445-1602cm⁻¹), C=N (1580-1620) and C-H stretching (3018-3070 cm⁻¹) bands. ¹H NMR spectra of these compounds displayed one singlet peak in the region of δ 6.65-6.90 and doublet in the region of δ 7.92-8.01. Aromatic protons are observed in the region of δ 7.14-7.60 in the form of multiplets respectively.

The significance of proteases lies in the fact that they are involved in apoptosis, inflammation, and abnormal immune responses in human disorders; therefore, development of inhibitors of specific cysteine proteases may promise to improve the pathogenesis of different disorders [27-29]. These play an important role in regulation of peptide hormones. These are also being used as therapeutic agents. Their involvement in oncology, inflammatory conditions, control of blood reology and immune regulation adds to the significance of a detailed study on the inhibitory mechanisms as this may deliver prospects for the treatment of diverse disorders resulting from defective control of proteolytic processes [30-35]. At present focus on proteases as pharmaceutical targets are increasing and various small molecular weight compounds acting as inhibitors to proteases are being developed. We are also working in the area of synthesis of small molecular weight compounds and their evaluation as protease inhibitors and have evaluated the effect of semicarbazones [36], thiosemicarbazones [37], hydrazones [38], pyrazolines [39] on the endogenous proteolytic activities. In continuation of our previous work we report the effect of pyrazoles on endogenous proteolytic activity in liver. The pyrazoles are the oxidized products of pyrazolines and similar to the effect of pyrazolines, pyrazoles also exerted an inhibitory effect on protease activity at pH 5.0.

At this pH we are concentrating our studies to cysteine proteases only as our previous work suggests that at this pH mainly cysteine proteases are active. Cysteine proteases belonging to papain superfamily having of a catalytic triad i.e. histidine, aspartic acid and cysteine are the key factors in the pathogenesis of cancer invasion, osteoporosis, arthritis etc. The studies were conducted at two different incubation time i.e. at 3h and at 24 h. The experiments were conducted in triplicate and the results are presented in table 1. Different pyrazoles have been found to inhibit endogenous proteolytic activity. We have observed that inhibition decreased in longer reaction time i.e. at 24 h. The inhibition to proteolytic activity was more at 3 h except for halogen substituted pyrazoles. This pattern clearly indicate that pyrazoles either inhibit the active enzymes reversibly or activation of some enzymes in the reaction mixture with time which are less sensitive towards pyrazoles inhibition. Similar trends have earlier been observed in case of pyrazolines. A time dependent inhibition was observed in case of halogen substituted pyrazoles on endogenous proteolytic activity. The results support the anti-inflammatory properties of different pyrazoles and pyrazolines as reported in literature. An important observation was that the reversal in the level of inhibition was less in case of nitrosubstituted pyrazoles also reported previously for pyrazolines from our laboratory. Among different pyrazoles, maximum inhibition was observed in 1, 3, 5- triphenyl-2-pyrazoline (1a) and 5-(4'-methylphenyl)-1,3-diphenyl-2-pyrazoline (1g) at 3 hr reaction. In halogen substituted pyrazoles reverse trend was observed i. e. the inhibition caused by pyrazoles decreased with increase in time. In case of 5-(4'-fluorophenyl)-1,3-diphenyl-2-pyrazoline (1e) the % residual activity decreased from **51.06 ± 1.70** to **5.26 ± 2.23** at 24 hr reaction. It has been noticed that, due to attachment of the methyl group at the 5-position of pyrazole moiety in 3 hr reaction i.e. electron donating group substituted (1g) showed more inhibition and attachment of the fluorine group at the 5-position of pyrazole heterocycle in 24 hr reaction i.e. electron withdrawing group substituted (1e) showed more inhibition while unsubstituted pyrazoles showed high inhibition both is 3 and 24 hr incubation. These compounds act as inhibitors to proteases active at pH 5.0, and inhibit endogenous protein hydrolysis significantly. It was observed that in most of the cases, inhibition was more at 3.0 hr and less at 24.0 hr. The reason may be that binding of proteases with pyrazoles might have been reversed with time. In the initial stages, binding of compounds is more and results in high inhibition and with time activity is again regenerated resulting in lesser or no inhibition after 24.0 hr reaction time.

In general, some compounds demonstrated ~100% inhibition at 1mM concentration. The data reported in this paper may prove to be a helpful guide in studying their effect on different enzymes and provides a case history for relating the enzyme inhibition studies with pyrazoles for various pharmacological purposes. Further studies to acquire more information about structure-activity relationships are in progress in our laboratory.

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