Evaluation of Antiinflammatory and Antihyperalgesic Activity of Some Novel Monocyclic β-lactam Compounds in Rats.

R. K. GOEL[1], AMANPREET SINGH, M. P. MAHAJAN AND S. K. KULKARNI*

Pharmacology Division, University Institute of Pharmaceutical Sciences, Panjab University, Chandigarh-160014.
[1]Department of Pharmaceutical Sciences, Guru Nanak Dev University, Amritsar-143005.

Synthesis and evaluation of some novel monocyclic beta-lactam compounds as anti-inflammatory and anti-hyperalgesic agents has been carried out in the present study. Such type of compounds has been reported in literature as human leukocyte elastase (HLE) inhibitors. The compounds were administered orally (15 mg/kg) to rats 30 min before injecting carageenan in the planter aponeurosis. The compounds showed a marked effect on paw edema and associated inflammatory pain. Compound 1 showed antiinflammatory effect even better than indomethacin (10 mg/kg) used as reference standard. These results suggest the need of carrying out further *in vitro* studies on the mechanism of action, which may be HLE inhibition.

Serine proteases are involved in numerous biological activities such as regulation of hemostasis and fibrinolysis (thrombin, plasmin, factor Xa), digestion (chymotrypsin, trypsin, pancreatic elastase) and phagocytosis (leucocyte elastase, cathepsin G). But human leucocyte elastase (HLE) has been the subject of extensive studies, having its biological role in numerous diseases such as pulmonary emphysema[1], chronic bronchitis[2], adult respiratory distress syndrome[3], rheumatoid arthritis[4], atherosclerosis[5], cystic fibrosis[6], chronic bowel disease[7] and other inflammatory disorders. Study of human leucocyte elastase also has its role in the development of suitable inhibitors to be used as potential therapeutic agents. This interest has led over the past fifteen years to the synthesis of a wide variety of inhibitors. Several strategies have been pursued for the development of HLE inhibition and various types of inhibitors have recently been reported[8-16]. The use of β-lactam structure to provide time dependent mechanism based inhibition of HLE has been reported to control inflammatory degeneration[17]. Monocyclic beta-lactam nucleus offered a structure, which could be modified to improve HLE activity, and hydrolytic stability, which may result in systemically

active agents. A thorough analysis of the mechanism of inhibition of HLE and the mechanism of beta-lactam hydrolysis led to the preparation of potent and highly stable HLE inhibitors. This work has led to the development of 4-[(4-carboxyphenyl)-oxy]-3,3-diethyl-1-[(phenyl-methyl) amino] carbonyl]-2-azetidinone (fig. 1a) as the first orally active HLE inhibitor. Compounds with a methoxy or a methyl group at para positions of the benzene ring were found to be very potent HLE inhibitors as well as effective against hamster lung hemorrhage[18]. Further the alkyl substitution on the benzylic urea afforded enhanced HLE inhibition and *in-vivo* efficacy (fig. 1b)[19]. Works of Firestone *et al.* established that the substituted 2-azetidinone rings as minimal structure necessary for effective HLE inhibition[20]. Further modification of these compounds afforded inhibitors that were efficacious in preventing HLE induced lung damage in hamsters. Ultimate objective was a systemically active agents with high oral bioavailability. The effect of varying the C-4 substituent of 3,3-diethyl-1-[(benzylamino)carbonyl]-2-azetidinone on HLE inhibition and in a model of HLE induced lung damage in hamsters was explored. The substituent at this position had a marked effect on *in-vivo* activity. Compounds containing 4-hydroxybenzoic acid and 4-hydroxyphenyl acetic acid ethers at C-4 were among the most active analogs[21]. A series of N-acyloxymethyl and N-

*For correspondence
E-mail: skpu@yahoo.com

aminocarbonyloxymethyl derivatives of 2-azetidinones with different substituent patterns at C-3 and C-4 positions of the beta lactam have been designed as potential mechanism based HLE inhibitors[22]. Functionalised N-aryl-azetidinones had been shown to inactivate HLE and porcine pancreatic elastase by an enzyme mediated process[23]. Disubstituted amino azetidinones have also been found to act as potential anti-inflammatory agents.

We have synthesized some novel substituted monocyclic β-lactam for hypocholestrolemic activity and in collaboration with Specs and BioSpecs Inc. USA., We got our compounds modeled using computer software PASS (Prediction of Activity Spectra for Substances)[24] to explore the possibilities of these compounds for different pharmacodynamic activities. Computer system PASS predicts simultaneously several hundreds of biological activities depending upon the chemical structures of compounds. This software illustrates the predicted activity spectrum of a compound as probable activity (P_a) and probable inactivity (P_i). Prediction of this spectrum by PASS is based on SAR analysis of the training set containing more than 35 000 compounds, which have more than 500 kinds of biological activity. Compounds 1, 2 and 3 were selected with potential for antiinflammatory activity based on these PASS predictions.

MATERIALS AND METHODS

Chemical methods:

All the chemicals used in the present study were procured from local commercial suppliers . Melting points were determined using open glass capillaries and are
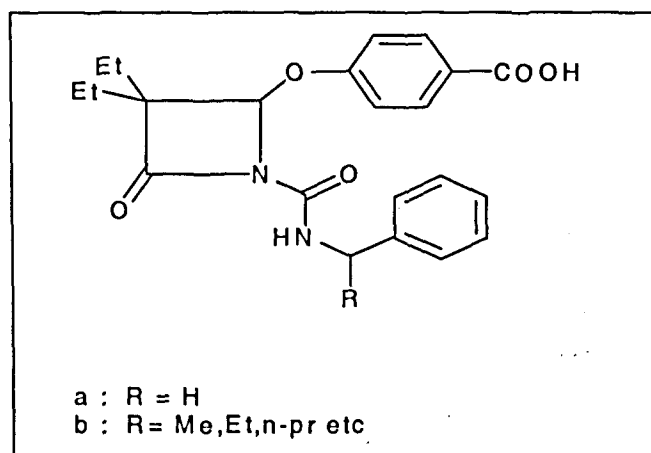

a : R = H
b : R = Me,Et,n-pr etc

Fig. 1: Azetidinones reported as first orally active HLE Inhibitors.

uncorrected. NMR spectra were determined on a Brucker 200 MHz and 300 MHz. The [1]H-chemical shifts are expressed in ppm relative to tetramethyl silane (Me_4Si). IR spectra were obtained on a Shimadzu FT-IR. Microanalysis for C, H, and N were performed on Perkin Elmer CHN analyzer. Substituted 2-azetidinones were prepared via [2+2] cycloaddition of imines and ketenes also known as Staudinger reaction[25] (fig. 2). Imines were prepared by treating primary amine with equivalent aldehyde proportion in dichloromethane in the presence of anhydrous magnesium sulphate.

Preparation of 1-p-methoxyphenyl-3-(1,3-butadienyl)-4-phenyl-azetidin-2-one (1)[26,27]:

To a stirred solution of benzylidine anisidine (10 mmol) and triethylamine (15 mmol) in dry methylene chloride (20 ml) in ice bath was added drop wise a solution of sorbyl chloride (12 mmol) in dry methylene chloride (10 ml). After completion of reaction (tlc), the resulting mixture was washed with saturated sodium bicarbonate solution (2x20 ml) and then with water (2x30 ml) and dried over anhydrous sodium sulphate. Solvent removed under reduced pressure and the crude product was purified by silica gel column chromatography. (eluent: ethylacetate/hexane in 1:9 ratio). Yield : 69%, mp: 80-82°, IR (KBr): 1729 cm-1 (C=O).^{1}H NMR: (300 MHz, $CDCl_3$) δ: 3.69 (dd, J = 4.7 Hz and 13.1 Hz, 1H, H-3) , 3.74 (s, 3H, OCH_3),. 4.75 (d, J=2.6Hz,1H,H-4) 5.12 (d, J = 9.7 Hz , 1H, H-4'), 5.22 (d, J = 8.8Hz, 1H, H-1'), 5.84 (dd, J = 8.2 Hz and 14.0 Hz, 1H, H-5'), 6.26-6.38 (m, 2H, , H-2',H-3'), 6.78 (dd, 2H, J=3.1Hz and 8.7 Hz ArH), 7.20-
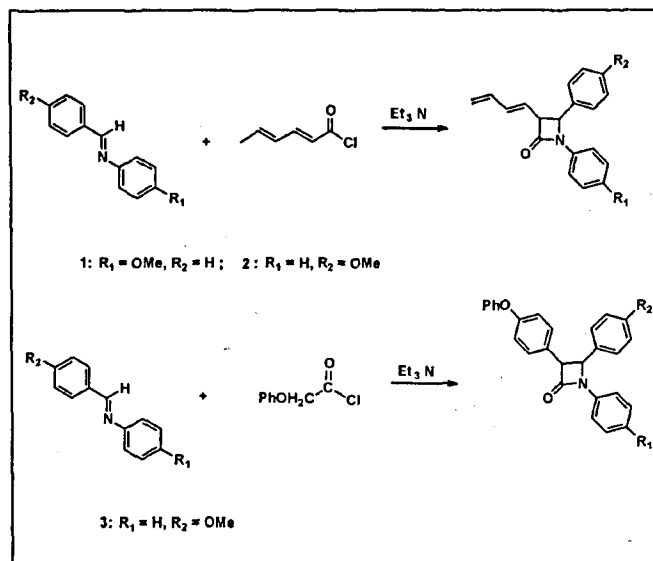

1: R_1 = OMe, R_2 = H ;   2: R_1 = H, R_2 = OMe

3: R_1 = H, R_2 = OMe

Fig. 2: Synthetic scheme.

7.25 (m, 2H, ArH), 7.27-7.39 (m, 5H, ArH). Anal.Calc.($C_{20}H_{19}NO_2$): C, 78.66; H, 6.27; N, 4.59 found: C, 78.36; H, 6.09; N, 4.45.

Preparation of 1-phenyl-3-(1,3-butadienyl)-4-p-methoxyphenyl azetidin-2-one (2)[26,27]:

To a stirred solution of p-methoxybenzylidineaniline (10 mmol) and triethylamine (15 mmol) in dry methylene chloride (20 ml) in ice bath was added drop wise a solution of sorbyl chloride (12 mmol) in dry methylene chloride (10 ml). After completion of the reaction (tlc), the resulting mixture was washed with saturated sodium bicarbonate solution (2x20 ml) and then with water (2x30 ml) and dried over anhydrous sodium sulphate. Solvent removed under reduced pressure and the crude product so obtained was purified by silica gel column chromatography. (eluent:ethylacetate/hexane in 1:9 ratio). Yield: 70%, mp: 104-105°, IR (KBr) : 1747 cm^{-1} (C=O). ^{1}H NMR: (300 MHz, CDCl$_3$) δ: 3.74 (dd, J = 2.4 Hz and 8.1 Hz, 1H, *H*-3) , 3.80 (s, 3H, OC*H*$_3$), 4.75 (d, *J*=2.4Hz,1H,*H*-4) 5.13 (d, J = 9.6 Hz , 1H, H-4'), 5.22 (d, J = 9.93 Hz, 1H, , H-5'), 5.86 (dd, J = 8.0Hz and 14.0 Hz, 1H, , H-1'), 6.26-6.38 (m, 2H, , H-2', H-3'), 6.90 (dd, 2H, J = 3.3 Hz and 8.4Hz ArH), 7.00-7.05 (m, 1H, ArH),7.17-7.35(m, 6H, ArH). Anal. calc. ($C_{20}H_{19}NO_2$): C, 78.66; H, 6.27; N, 4.59 found: C, 78.69; H, 6.27; N, 4.67.

Preparation of 1-phenyl-3-phenoxy-4-(4¢-methoxyphenyl)- 2-Azetidinone(3):

To a stirred solution of 4-methoxybenzylidineaniline (10 mmol) and triethylamine (15 mmol) in dry methylene chloride (20 ml) in ice bath was added drop wise a solution of phenoxyacetyl chloride (12 mmol) in dry methylene chloride (10 ml). After completion of the reaction (tlc), the resulting mixture was washed with saturated sodium bicarbonate solution (2x20 ml) and then with water (2x30 ml) and dried over anhydrous sodium sulphate. Solvent removed under reduced pressure and the crude product so obtained was purified by column chromatography over silica gel. (eluent:ethylacetate/hexane in 1:9 ratio). Yield: 43%, mp: 75-78°, IR (KBr): 1716 cm^{-1}(C=O) ^{1}HNMR (CDCl$_3$, 200 MHz) δ: 3.71 (s, 3H, OCH$_3$), 5.32 (d, 1H, *J=4.70 Hz* H-4), 5.50 (d, 1H, *J=4.78 Hz*, H-3), 6.75-6.79 (m, 4H, ArH), 7.09 -7.14 (m, 5H, ArH), 7.22 - 7.36 (m, 5H, ArH), Anal. calc. ($C_{21}H_{17}NO_2$): C,80.06; H,5.39; N,4.41 found: C,79.81; H,5.42; N,4.38.

Evaluation of anti-inflammatory activity:

Wistar rats of either sex (20-30g), (150-200 g) bred in Central Animal House facility of the Panjab University were used. The animals were housed under standard laboratory conditions, maintained on a natural light and dark cycle and had free access to food and water. Animals were acclimatized to laboratory conditions before the test. Each animal was used only once. All experiments were carried out between 0900 and 1700 h. The experimental protocols were approved by the Institutional Animals Ethics Committee and conducted according to the Indian National Science Academy guidelines for the use and care of experimental animals. Acute edema was induced in the right hind paw of rats by injecting 0.1 ml of freshly prepared 1% aqueous solution of carrageenan (Sigma, USA) in the plantar region of the right hand paw. The left paw received 0.1 ml of saline, which served as control and the volume of hind paws were measured using a plethysmometer (UGO Basile, Italy) at 30, 60, 120, 180, 240 and 300 min after carrageenan challenge. Indomethacin and test compounds (1, 2 and 3) were suspended in 0.5% carboxymethyl cellulose and administered per orally (p.o.) 30 min. prior to carrageenan injection. Control animals received corresponding amount of vehicle (0.5% carboxymethyl cellulose). Inflammation was expressed as the percentage change in paw volume[28].

Induction and assessment of carrageenan-induced hyperalgesia:

Acute inflammation was induced in the right hind paw by injecting 0.1 ml of freshly prepared solution of 1 % carrageenan. The left paw received 0.1 ml of saline, which served as control. The response to inflammatory pain was determined by measuring paw withdrawal latency of carrageenan-injected paw when dipped in water bath maintained at 47 ± 0.5°. Baseline latency to paw withdrawal from thermal source was established thrice, at 5 min intervals apart and averaged. A cut-off time of 15 s was imposed to avoid any injury to the paw. The paw withdrawal latency for left and right paw was observed at 30, 60, 120, 180 and 240 min after drug administration[29].

Statistical analysis:

Results were expressed as mean±S.E.M. The significance of the difference in the responses of treatment groups in comparison to the control was determined by one-way analysis of variance (ANOVA) followed by Dunnet's test and $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Carrageenan (1% w/v) produced significant paw edema in control group, indicating inflammatory response. Test compounds 1 and 3 (15 mg/kg, p.o) produced a significant
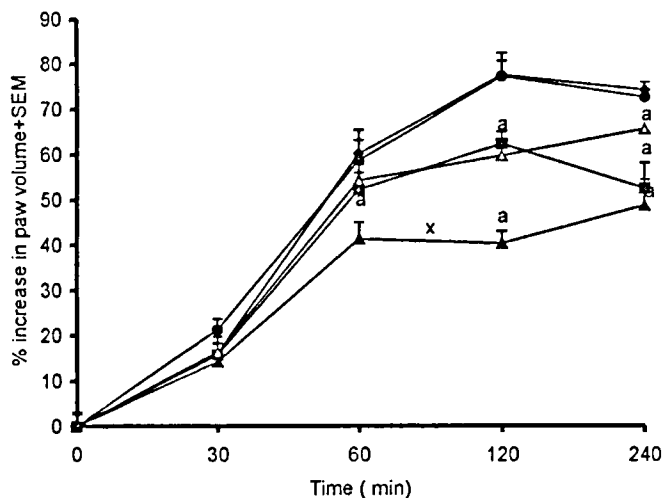
Fig. 3: Effect of compounds 1, 2 and 3 on carrageenan-induced paw edema.

Effect of test compounds on carrageenan-induced rat paw edema. Control is represented by (-□-), indomethacin by (-◙-), compound 1 by (-▲-), compound 2 by (-●-) and compound 3 by (-x-) a=p<0.05 when compared to carrageenan-treated group (control).

(p<0.05)decrease in carrageenan-induced increase in the paw volume as compared to control rats (fig. 3). Test compound 1 exhibited a better antiinflammatory profile as compared to compound 3 and indomethacin (10 mg/kg, p.o). Carrageenan–treated paws showed significant (p<0.05) decrease in paw withdrawal latency (hyperalgesia) in comparison to saline-treated paws. Test compounds 1 and 3 reversed the carrageenan-induced inflammatory hyperalgesia significantly (p<0.05) (fig. 4).

Monocyclic β-lactam compounds have been reported to inhibit the HLE through acylation of serine and histidine residues of enzyme by their carbonyl moiety. Further acylation of enzyme has been reported to be dependent on opening of β-lactam ring[30]. which has been further linked to the presence of optimal N-acyl group[17]and C_4 leaving group[20]. Small alkyl/alkoxy groups are required at C_3 for good oral bioavailability[18]. Our results also supported the above findings as the presence of phenoxy (compound 3) and 1,3-butadienyl (compound 1) at C_3 favors for antiinflammatory activity. N-substitution by 4'-methoxyphenyl (compound 1) offered better activity than phenyl (compound 2). These results indicated that the compounds 1, 2 and 3 may show their antiinflammatory activity through HLE inhibition.
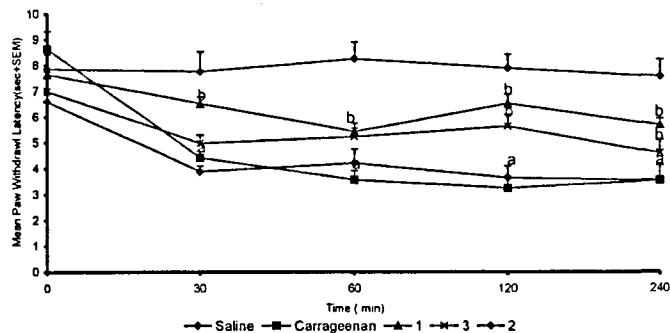

Fig. 4: Effect of compounds 1, 2 and 3 on carrgeenan-induced hyperalgesia.

Effect of test compounds on carrageenan-induced hyperalgesia. Saline treated is represented by (-□-), carrageenan-treated by (-◙-), compound 1 by (-▲-), compound 2 (-●-) by and compound 3 by (-x-) a=p<0.05 when compared to carrageenan-treated group (control), a=p<0.05 when compared to carrageenan treated group (control).

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