Anti-Tubercular and Anti-Inflammatory Activities of Azetidin-2-One Derivatives and Their Effects on the Activity of Phospholipase A_2

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Abstract: The title compounds have been synthesized and tested for structure activity relationship for Phospholipase A_2 (PLA_2) [E.C. 3.1.1.4] enzyme inhibition. The *in vitro* anti-tubercular, PLA_2 enzyme inhibitory activities of azetidin-2-one derivatives and *in vivo* anti-inflammatory studies using mice are highlighted. The analogues of azetidin-2-one were prepared based on the initial activity against *Mycobacterium tuberculosis* (Mtb). Certain azetidin-2-one analogues described herein showed moderate to good anti-tubercular activity. In particular, two compounds (**4_f**) and (**4g**) exhibited MIC values of 1.56 and 0.78 μg/mL respectively against the Mtb $H_{37}Rv$ strain. Chloro substitution on aryloxy acid apparently enhanced the antimycobacterial activity and also PLA_2 inhibition in the azetidin-2-one series described herein. The ability of azetidin-2-one analogues as anti-inflammatory agents has also been determined. The results show some correlation between anti-inflammatory, anti-tubercular activity and expression of PLA_2 enzyme.

Key Words: Phospholipase A_2 (PLA_2), anti-inflammatory, antitubercular, azetidin-2-one derivatives, mycobacterium tuberculosis (Mtb).

INTRODUCTION

PLA_2 is a growing family of distinct enzymes that exhibits different substrate specificities, cofactor requirements, subcellular localization and cellular functions [1]. The PLA_2 class of enzymes catalyze hydrolysis of the 2-acyl ester of 3-Sn phosphoglycerides to yield arachidonic acid (metabolized to eicosanoids by cycloxygenase and lipoxygenase) and lysophospholipid, which is a rate limiting step in the production of proinflammatory lipid mediators such as prostaglandins, leukotrienes, lipoxins and platelet activating factor [2-5]. In many inflammatory diseases and synovial fluid high levels of PLA_2 enzymes are detected and are believed to be responsible for part of the inflammatory reactions. Injection of purified PLA_2 enzyme from synovial fluid and from snake venom into animal joints confirmed the development of an acute inflammatory response with edema, swelling of synovial cells and hyperplasia [6,7]. Pulmonary inflammation and edema is induced by PLA_2 [8]. Azetidin-2-one derivatives were found to have anti-inflammatory and analgesic activities with low toxicity [9].

Azetidin-2-one derivatives having different substituted aryl acids have been incorporated in their β-lactam ring. The Schiff base as such was not showing any PLA_2 enzyme inhibition but the incorporation of the different substituted aryl acids increased the binding to the PLA_2 enzyme and also enhanced the anti-inflammatory activity. The same is the case with azetidin-2-one derivatives having electron withdrawing groups. But presence of electron donating groups like CH_3 decreased the anti-tubercular and anti-inflammatory activities and also binding to PLA_2 enzyme. Clinical results with cylcoxygenase and lipoxygenase inhibitions demonstrate that inhibition of PLA_2 enzyme results in reduction of both lipid mediators; as a result these PLA_2 inhibitors can be used as anti-inflammatory drugs. In this present study we have compared the relative PLA_2 targeting activity of eight different azetidin-2-ones compounds (**4_a-4_h**) (Scheme **1**). Here the author has tried to correlate the expression of PLA_2 in inflammation and tuberculosis.

CHEMISTRY

Literature review showed azetidin-2-one derivatives posses a variety of therapeutic activities. Azetidin-2-one (β-lactam), a four membered cyclic amide is a part structure of many biologically important antibiotics. The unique structural feature and chemotherapeutic properties of β-lactam antibiotics continue to attract the attention of the synthetic chemists, as they provide a variety of synthetic challenges. Although the first synthesis of β-lactam ring was reported [10] way back in 1907, β-lactams as a class acquired immense importance only after the discovery of penicillin by Fleming [11] in 1928 and its structural confirmation by X-ray crystallography [12], which unambiguously confirmed the presence of 4-membered amide ring (β-lactam). The azetidin-2-one ring was identified as the key structural unit responsible for the antibiotic activity. Discovery of new active β-lactam compounds such as thrombin [13], prostate specific antigen [14], human cytomegalovirus protease [15], cholesterol absorption inhibitor [16] and Cysteine Proteases Inhibitors [17] has renewed interest in the field of azetidin-2-ones. This prompted the author to test these azetidin-2-one derivatives as PLA_2 inhibitors.

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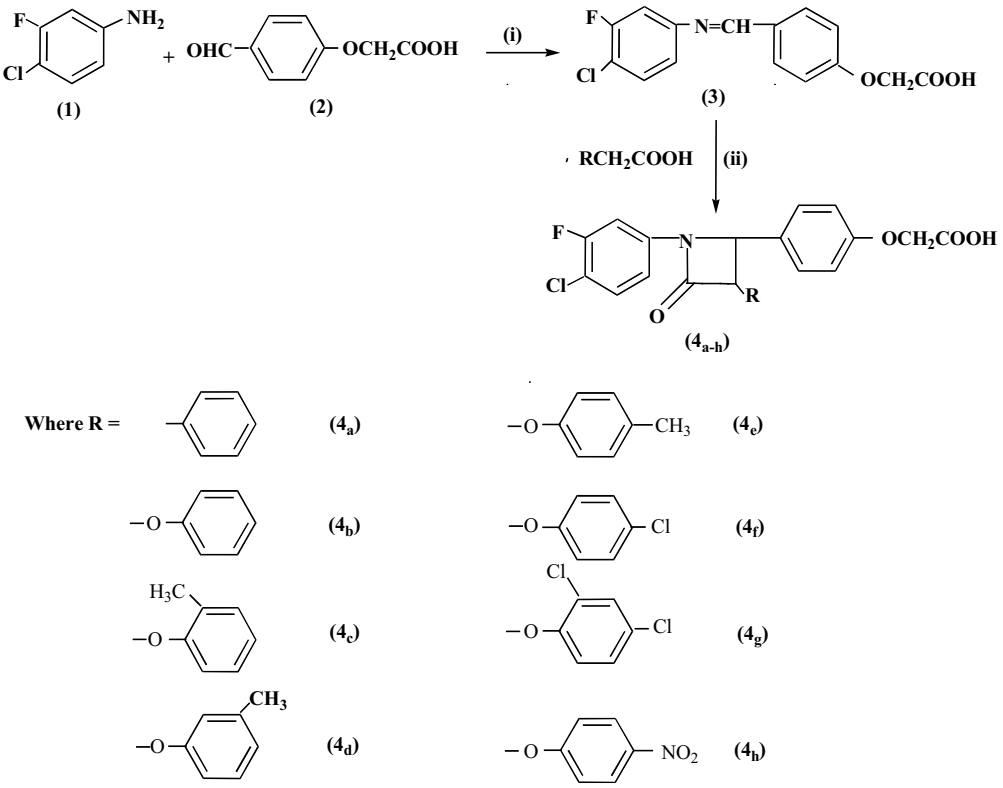

Scheme 1.

The reactivity of β-lactam moiety is greatly influenced by substituents or fused rings. 4-Hydroxy benzaldehyde was converted into an arlyoxy acid derivative to obtain 4-carboxy-methyleneoxy banzaldehyde (**2**) using chloro acetic acid. Then treated with 3-fluoro-4-chloro aniline (**1**) to obtain schiff base (**3**) in presence of a pinch of fused zinc chloride for 1 h, at 120°C the yield was about 85%. The schiff base was then recrystallized from alcohol.

Schiff base (**3**) was dissolved in dry benzene and a few drops of triethylamine was added. To this solution acid chlorides of different phenols and substituted phenols (phenols were converted to carboxy acids using chloro acetic acid) was added with stirring. The mixture was then refluxed for 1 h. Triethylamine hydrochloride formed was filtered off and washed several times with dry toluene. The filterate and washings were collected and concentrated under reduced pressure. The residue obtained was washed with petroleum ether to remove unreacted Schiff base finally to give different substituted azetidin-2-ones ($\mathbf{4_{a-h}}$) as envisaged in the Scheme **1**. The solid obtained was crystallized from aqueous ethanol. The purity was checked on a TLC plate (silica gel) using solvent system Benzene and Methanol (1:3). In all the cases the products obtained were purified by column chromatography. The IR and ^{1}H NMR and elemental analyses data of compounds $\mathbf{4_{a–h}}$ are in complete agreement [21].

BIOLOGICAL ACTIVITY

Determination of Edema Inducing Activity

Groups of six mice (24–26 g) were injected in the right footpads of hind limbs with 3mM dose of azetidin-2-one derivative in 20 µL saline. The left footpads received 20 µL of saline, which served as control. After 45 min, mice were sacrificed by cervical dislocation and both legs were removed at the ankle joint and weighed individually. The increase in weight due to edema was calculated as the edema ratio, which equals the weight of edematous leg x 100/weight of the normal leg. Minimum edema dose is defined as the microgram of protein causing an edema ratio of 120%. Injecting a fixed dose of protein into mice footpads and sacrificing them at regular period of time obtained time course curve of edema inducing activity. Edema ratio was calculated as defined [18].

PLA_2 Activity

Assayed with [^{14}C]oleate-labelled autoclaved *E. coli* as the substrate [19]. The reaction mixture, 350 µL contained100mM Tris/HCl, pH 8.0, 5mM Ca^{2+} and 3.15 x10^9 autoclaved *E. coli* cells (corresponding to 10,000 cpm and 60 nmol of lipid phosphorous). The amount of enzyme protein was chosen such that 10–15% hydrolysis of substrate was obtained when incubated at 37°C for 60 min. The reaction components were mixed in the following order: buffer, calcium, water and azetidin-2-one derivative. Adding labelled *E. coli* substrate started the reaction. The reaction was terminated by adding 100 µL 2.0 M HCl and 100 µL of fatty acid free BSA (100 mg/mL). The tubes were vortex mixed and centrifuged at 20,000g for 5 min. Aliquot (140 µL) of the supernatant containing released [^{14}C] oleic acid was mixed with scintillation cocktail and counted in a Hewlett Packard liquid Scintillation Analyzer TRI CARB 2100 TR.

Antimycobacterial Activity

All synthesized azetidin-2-one analogues were screened for their antimycobacterial activity against two strains of Mtb $H_{37}Rv$. Azetidin-2-ones analogues were found active. The azetidin-2-one analogues, having more than 50% inhibition against $H_{37}R_v$, are presented in Table **2**.

The primary screen was conducted at either 6.25 or 12.5 μg/mL against Mtb $H_{37}R_v$ (ATCC 27294) in BACTEC 12B medium using the Microplate Alamar Blue Assay (MABA) [21]. Compounds demonstrating at least 90% inhibition at 6.25 or 12.5 μg/mL were retested to determine the MIC_{90}, defined as the lowest concentrating inhibiting growth by 90% or higher.

CONCLUSIONS

Schiff base alone did not cause edema when injected into mice footpads. As shown in Table **1**, $\mathbf{4_f}$, $\mathbf{4_g}$ and $\mathbf{4_h}$ demonstrated very strong *in vitro* PLA_2 enzyme inhibition. The compounds $\mathbf{4_f}$, $\mathbf{4_g}$ and $\mathbf{4_h}$ inhibited the edema inducing activity of four different PLA_2 isoenzymes. Compounds $\mathbf{4_{a-e}}$ were not much effective compared to $\mathbf{4_f}$, $\mathbf{4_g}$ and $\mathbf{4_h}$ in both *in vivo* anti-inflammatory and *in vitro* PLA_2 enzyme inhibition. Certain azetidin-2-one analogues described herein showed moderate to good anti-tubercular activity. In particular, two compounds ($\mathbf{4_f}$) and (**4g**) exhibited MIC values of 1.56 and 0.78 μg/mL respectively against the Mtb $H_{37}Rv$ strain. Chloro substitution on aryloxy acid apparently enhanced the antimycobacterial activity and also PLA_2 inhibition in the azetidin-2-one series described herein. The ability of azetidin-2-one analogues as anti-inflammatory agents has also been determined. The results show some correlation between anti-inflammatory, anti-tubercular activity and expression of PLA_2 enzyme. Further studies are in progress to understand the correlation of the expression of PLA_2 in inflammation and anti-tubercular activity.

Table 2. Antimycobacterial Activity Against Mtb of Azetidin-2-One Compounds

Compound no.	% inhibition Mtb $H_{37}R_v$ 6.25 µg/mL	MIC_{90}[a] Against Mtb $H_{37}R_v$
4_a	77	>6.25
4_b	67	>6.25
4_c	72	>6.25
4_d	56	>6.25
4_e	85	>6.25
4_f	100	1.56
4_g	100	0.78
4_h	89	3.13
INH		

[a] MIC are given in μg/mL.

INH = Isoniazid Hydride

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Table 1. Effect of Compounds $4_{a–h}$ on PLA_2 Enzyme Activity and Edema Inducing Activity of PLA_2

Phospholipase A2	Compounds (inhibitors)[b]																	
	4a		4b		4c		4d		4e		4f		4g		4h		Luffariellin B	
	Inhibitory rate (%)	Edema ratio	Inhibitory rate (%)	Edema ratio	Inhibitory rate (%)	Edema ratio	Inhibitory rate (%)	Edema ratio	Inhibitory rate (%)	Edema ratio	Inhibitory rate (%)	Edema ratio	Inhibitory rate (%)	Edema ratio	Inhibitory rate (%)	Edema ratio	Inhibitory rate (%)	Edema ratio
From V. russelli venom[a]	37.6	170±5	58.6	145±6	62.3	121±4	43.5	160±6	65.6	145±5	92.3	110±5	98.9	102±4	75.6	115±7	99.5	101±5
From N. naja venom	55.6	147±5	37.1	161±1	67.4	115±9	47.4	133±5	55.6	123±2	85.8	104±7	95.3	105±4	79.8	103±3	100	103±4
From T. malabaricus venom	NI	137±5	25.1	131±9	NI	109±4	45.7I	135±5	74.8	105±5	89.7	104±3	92.1	104±5	78.0	101±4	99.7	101±2
From pleural fluid	31.2	171±5	NI	171±3	58.5	118±4	48.7	167±9	69.2	114±3	93.8	101±1	97.9	101±4	79.7	101±4	100	102±1
From ascites fluid	NI	150±6	44.8	148±1	59.9	104±7	44.4	149±6	58.8	103±5	96.9	104±7	98.7	103±7	79.4	106±5	100	102±5

Amount of venom taken for each assay was 1 µg ([a]1.4 µg). NI=No inhibition.

[b] Percent inhibition of azetidin-2-one derivative concentration of 2.67mM edema ratio=weight of edematous leg x 100/weight of normal leg.

The PLA_2 enzyme (1 µg): azetidin-2-one derivatives/Luffariellin B (3mM) mixture was preincubated at 37 °C for 1 h prior to injection into the mice

footpads. Prolonged preincubation time up to 12 h, at 37 °C did not have any additional effect on edema ratio. Values of edema ratio are expressed as mean±SD (n=6), P values <0.05 were considered significant when compared to the control by Student's *t*-test.

Inhibitory rate (%)= nanomoles of labelled substrate released as compared to the control.

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[20] **4a**: mp 63-65 °C; IR (KBr); 1609 cm^{-1} (aromatic C=C), 1667 (C=O), 1059 (-N-C=O), 3299 (-COOH) cm^{-1}; ^1H NMR (DMSO-d_6): δ 6.8- 7.6 (m, 12H, Ar-H), 2.9 (d, H_a, CH), 2.5 (d, H_b, CH), 12.2 (s, COOH), 2.0 (s, CH_2) Anal. Calcd for $C_{23}H_{17}O_4NFCl$ (426): C, 68.48; H, 4.07; N, 3.80. Found: C, 68.38; H, 4.02; N, 3.75.

4b: mp 85-87 °C; IR (KBr); 1620 cm^{-1} (aromatic C=C), 1681 (C=O), 1059 (-N-C=O), 3344 (-COOH) cm^{-1}; ^1H NMR (DMSO-d_6): δ 6.4- 7.7 (m, 12H, Ar-H), 2.8 (d, H_a, CH), 3.5 (d, H_b, CH), 12.1 (s, COOH), 2.2 (s, CH_2).Anal. Calcd for $C_{23}H_{17}O_5NFCl$ (442): C, 65.79; H, 3.91; N, 3.65. Found: C, 65.59; H, 3.86; N, 3.62.

4c: mp 71-73 °C; IR (KBr);1637 cm^{-1} (aromatic C=C), 1651 (C=O), 1031 (-N-C=O), 3234 (-COOH) cm^{-1}; ^1H NMR (DMSO-d_6): δ 6.5- 7.4 (m, 11H, Ar-H), 2.7 (d, H_a, CH), 3.6 (d, H_b, CH), 12.2 (s, COOH), 2.5 (s, 3H, CH_3), 2.1 (s, CH_2). Anal. Calcd for $C_{24}H_{19}O_5NFCl$ (456): C, 66.33; H, 4.27; N, 3.52. Found: C, 66.22; H, 4.10; N, 3.16.

4d: mp 113-115 °C; IR (KBr); 1629 cm^{-1} (aromatic C=C), 1677 (C=O), 1065 (-N-C=O), 3019 (-COOH) cm^{-1}; ^1H NMR (DMSO-d_6): δ 6.6- 7.5 (m, 11H, Ar-H), 2.6 (d, H_a, CH), 3.4 (d, H_b, CH), 12.3 (s, COOH), 2.2 (s, 3H, CH_3), 1.9 (s, CH_2). Anal. Calcd for $C_{24}H_{19}O_5NFCl$ (456): C, 66.33; H, 4.27; N, 3.52. Found: C, 66.31; H, 4.21; N, 3.41.

4e: mp 93-95 °C; IR (KBr); 1612 cm^{-1} (aromatic C=C), 1669 (C=O), 1087 (-N-C=O), 2995 (-COOH) cm^{-1}; ^1H NMR (DMSO-d_6): δ 6.5- 7.3 (m, 11H, Ar-H), 2.5 (d, H_a, CH), 3.5 (d, H_b, CH), 12.8 (s, COOH), 2.1 (s, 3H, CH_3), 1.8 (s, CH_2). Anal. Calcd for $C_{24}H_{19}O_5NFCl$ (456): C, 66.33; H, 4.27; N, 3.52. Found: C, 66.12; H, 4.15; N, 3.35.

4f: mp 82-84 °C; IR (KBr); 1626 cm^{-1} (aromatic C=C), 1679 (C=O), 1040 (-N-C=O), 3010 (-COOH) cm^{-1}; ^1H NMR (DMSO-d_6): δ 6.5- 7.6 (m, 11H, Ar-H), 2.6 (d, H_a, CH), 3.6 (d, H_b, CH), 12.5 (s, COOH), 2.0 (s, 3H, CH_3) 1.7 (s, CH_2). Anal. Calcd for $C_{23}H_{16}O_5NFCl_2$ (477): C, 60.28; H, 3.35; N, 3.35. Found: C, 60.12; H, 3.22; N, 3.27.

4g: mp 73-75 °C; IR (KBr); 1638 cm^{-1} (aromatic C=C), 1676 (C=O), 1065 (-N-C=O), 2988 (-COOH) cm^{-1}; ^1H NMR (DMSO-d_6): δ 6.6- 7.3 (m, 10H, Ar-H), 2.4 (d, H_a, CH), 3.7 (d, H_b, CH), 12.2 (s, COOH), 1.9 (s, CH_2). Anal. Calcd for $C_{23}H_{15}O_5NFCl_3$ (512): C, 55.63; H, 2.87; N, 3.10. Found: C, 55.12; H, 2.95; N, 3.15.

4h: mp 76-78 °C; IR (KBr); 1600 cm^{-1} (aromatic C=C), 1675 (C=O), 1067 (-N-C=O), 2989 (-COOH) cm^{-1}; ^1H NMR (DMSO-d_6): δ 6.5- 7.4 (m, 11H, Ar-H), 2.4 (d, H_a, CH), 3.7 (d, H_b, CH), 11.9 (s, COOH), 2.0 (s, CH_2). Anal. Calcd for $C_{23}H_{16}O_7N_2FCl$ (486): C, 58.74; H, 3.26; N, 6.53. Found: C, 58.32; H, 3.11; N, 6.32.

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