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NOVEL INHIBITOR OF PHOSPHOLIPASE A_2 WITH TOPICAL ANTI-INFLAMMATORY ACTIVITY

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Activation of a phospholipase A_2 (PLA_2) is a key step in the production of precursors for the biosynthesis of lipid mediators of inflammation. Inhibition of this enzyme could result in the suppression of three important classes of inflammatory lipids, prostaglandins, leukotrienes and platelet activating factor (PAF), and offers an attractive therapeutic approach to design novel agents for the treatment of inflammation and tissue injury. In this report we describe a novel compound, BMS-181162 4(3'-carboxyphenyl)-3,7-dimethyl-9(2",6"6"-trimethyl-1"-cyclohexenyl),2Z,4E,6E,8E-nonatetraenoic acid which specifically inhibits a 14 kD human PLA_2 and effectively blocks phorbol ester induced skin inflammation in mice. BMS-181162 is the first reported specific inhibitor of PLA_2 and its specificity may make it a useful tool in the dissection of the role of PLA_2 in the inflammatory process.

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Activation of phospholipase A_2 leads to the release of arachidonic acid from the sn-2 position of membrane phospholipids (1). Arachidonic acid (AA) is the precursor for the biosynthesis of leukotrienes and prostaglandins and inhibition of AA release could suppress the formation of these lipid mediators of inflammation (2). The release of AA from 1-O-alkyl phosphatidylcholine by PLA_2 also yields an equimolar amount of lysophosphatidylcholine which can be converted by an acetyl transferase to platelet activating factor (PAF) (3). Therefore, a blockade of PLA_2 could result in the suppression of three important classes of lipid mediators and offers an attractive therapeutic approach to the design of novel agents for the treatment of inflammation and tissue injury.

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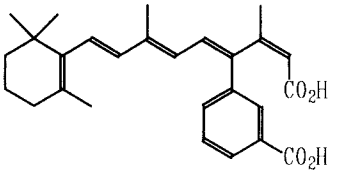

Figure 1. Structure of BMS-181162.

Phospholipase enzymes are widely distributed and both secretory and intracellular forms have been identified (4,5). Several intracellular PLA_2's associated with secretory granules in inflammatory cells have been identified and shown to be structurally related to the venom and pancreatic enzymes. These enzymes of molecular weight about 14 kDa, contain seven disulfide bonds, and do not appear to be selective in cleaving AA from the sn-2 position of phospholipids (6). These enzymes are activated upon secretion into the extracellular space and contribute to the inflammatory reaction. Indeed, PLA_2 activity has been shown to be elevated in inflamed tissues such as rheumatoid arthritic joints (7), psoriatic skin (8), serum from patients with pancreatitis (9), and gram negative septic shock (10). The origin of the PLA_2 in these exudates and tissues is unclear at this time. However, it is possible that the source is inflammatory cells since the enzyme found in human synovial fluid from rheumatic joints is identical in amino acid sequence to that secreted by human platelets (11,12). Regardless of the source, it is clear that secreted PLA_2s, when injected into joints or skin, can cause acute inflammatory reactions and tissue destruction (13,14).

Evidence for the involvement of a PLA_2 in inflammation would be supported by specific inhibitors which can produce an anti-inflammatory effect *in vivo*. A number of compounds have been described which have effects on the arachidonic acid cascade. Lonapalene, a 5-lipoxygenase inhibitor, has been reported to be active in treating the skin inflammatory disease psoriasis (15). An inhibitor of PLA_2 would also be expected to diminish tissue levels of 5-lipoxygenase products. Manoalide, a marine natural product which inhibits PLA_2, has anti-inflammatory activity in models of acute skin inflammation (16). These results suggest that interference with enzymes involved in arachidonic acid release and metabolism may be useful in treating inflammatory conditions. In this report we describe a novel compound, BMS-181162 (see Figure 1), which inhibits a 14 kDa human PLA_2 and blocks phorbol ester induced inflammation in mice.

MATERIALS AND METHODS

Chemistry

The synthesis of 13-*cis*-12-(3'-carboxyphenyl)retinoic acid (BMS-181162) was accomplished as shown in Chart 1. Reaction of methyl 3-bromomethylbenzoate (1) with

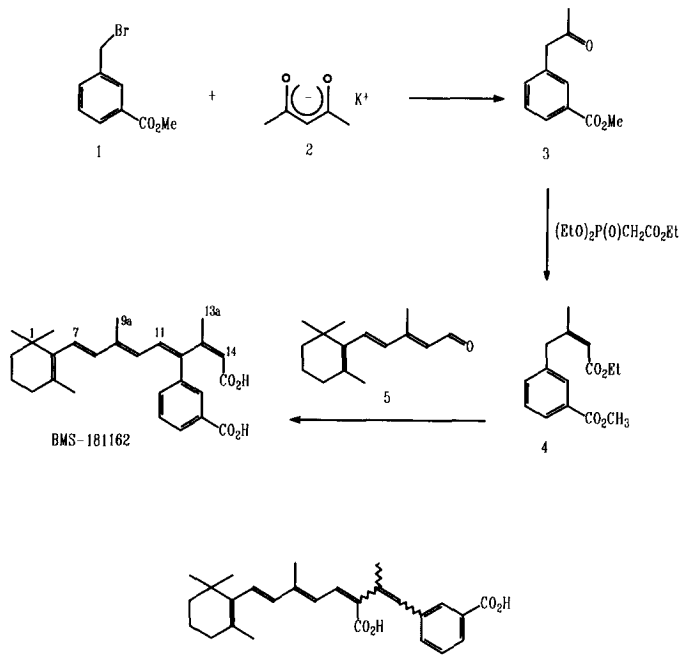

Chart 1. Synthesis of BMS-181162.

potassium acetylacetonate (2) afforded m-carbomethoxyphenylacetone (3) in 58% yield. Wittig-Horner condensation with triethylphosphonoacetate produced ethyl 4-(3'-carbomethoxyphenyl)-3-methyl-2-butenoate (4) in 80% yield and base-promoted condensation with *trans*-ß-ionylideneacetaldehydye (5) gave BMS-181162 in 68% yield, after crystallization. Since the substrate 4 could provide an ambident anion, giving rise to product of the general structure 6, the reaction was repeated using carbon-13 labeled Wittig reagent derived from [1,2-^{13}C]acetic acid. The non-^{1}H-decoupled ^{13}C NMR spectrum showed a doublet of doublets (J_{C-H} = 153.7 Hz, J_{C-C} = 73.6 Hz) at δ 119.9 ppm, consistent with a 12-aryl substituted structure. The geometry was determined from the ^{1}H NMR. The vinylic coupling constant confirmed the *trans* geometry of the double bond at position 7. The 9a methyl group was identified by its NOE with the proton at C-7; its NOE with the C-11 proton and the NOEs of the remaining methyl group (13a) with both the proton at C-11 and the proton at C-14, established the geometry as *trans* for the double bonds at 9 and 11, and as *cis* at the 13 position. Analysis for BMS-181162, M.P. 117-118°C; $C_{27}H_{32}O_4$ m/z: Calcd. 420.2300; found 420.2302.

Phospholipase Assays

Phospholipase A_2 was purified 3,000 fold from human platelets as previously described (17). PLA_2 activity was measured using a modification of the method of Franson et al.(18). The substrate used consisted of [1-^{14}C] oleic acid labeled, autoclaved Escherichia coli as substrate. Various concentrations of test compounds were preincubated for 7 min at 37° with PLA_2 (7.5 µg/ml) in a buffer consisting of 25 mM HEPES (pH 7), 150 mM NaCl, 5.0 mM $CaCl_2$ and 10% DMSO (test compound solvent). The reaction mixture totaled 0.1 ml. To start the reaction, the E. coli membrane substrate was added (0.1 mM phospholipid, 0.5 µCi/µmol) and incubated for 30 min at 37°. The reaction was stopped by addition of 1.9 ml of tetrahydrofuran (THF) and the entire solution was applied to a solid-phase extraction column (aminopropyl resin, Analytichem). The column was rinsed with 1

ml of THF. The free oleic acid was then eluted from the column with 1.0 ml of 2% (v/v) acetic acid in THF and quantitated by liquid scintillation counting. The data is presented as percent inhibition relative to the vehicle control. IC_{50} values were determined by interpolation of a linear regression plot of percent inhibition versus log concentration. Phosphatidyl inositol-specific phospholipase C (PI-PLC) was assayed similar to that reported (19). A 25,000 x g supernatant of rat brain homogenate is combined with phosphatidyl [^{3}H] inositol (80 μM) in 100 mM acetate buffer, pH 5.0, with 5 mM $CaCl_2$. Enzyme activity is monitored by release of ^{3}H-inositol. Acid sphingomyelinase assay was performed essentially as described by Quintern and Sandhoff (20). A 25,000 x g supernatant from rat brain homogenate was mixed with 100 μM N-methyl-^{14}C-sphingomyelin, mixture was incubated at 37°C and reaction progress monitored by release of ^{14}C-phosphorylcholine. Phospholipase D (PLD) was assayed by combining 0.3 U of PLD from Streptomyces chromofuscus (Sigma Chemical Co.) with 400 μM ^{14}C-phosphatidylcholine, 0.2% (v/v) Triton X-100, 10 mM $CaCl_2$, and 40 mM Tris-HCl, pH 8.0. The mixture was incubated at 37°C and reaction progress monitored by extracting the reaction mixture according to the procedure of Bligh and Dyer (21), separating the ^{14}C-phosphatidic acid (PA) product from the substrate by TLC, and quantitating the percent conversion to PA.

Phorbol Ester-Induced Mouse Ear Inflammation

12-O-tetradecanoylphorbol 13-acetate (TPA, 2μg) in 20 μl of acetone/water (99:1) was applied to the inner and outer surfaces of mouse ears. Immediately after TPA application, 20 μl of test compound in the same vehicle was applied in the same way. Six hours later the ears were removed and punch biopsies (5/16") were taken and weighed. Inhibition of edema was assessed by a comparison of the biopsy weights obtained in the presence and absence of the test compound.

RESULTS

BMS-181162 dose-dependently inhibits human platelet PLA_2 with an IC_{50} = 40 μM (Figure 2). For comparison, the inhibition of this enzyme by manoalide and

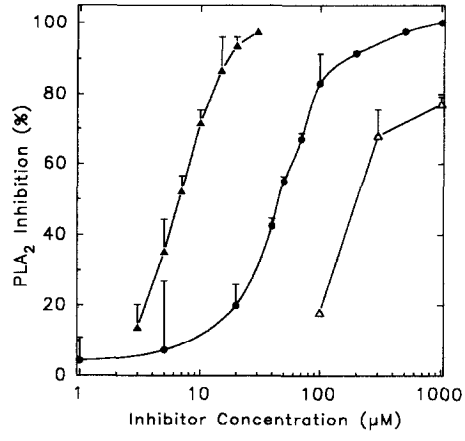

Figure 2. The dose-dependent inhibition of human platelet PLA_2 by BMS-181162 (•), manoalide (▲) and DEDA (△). Each point is the mean of duplicate samples from three separate experiments. The vertical bars are standard deviations.

Table 1
Effect of BMS-181162 on Various Phospholipase Activities[a]

Enzyme	Source	IC_{50} (μM)
PLA_1	Rhizopus	>>100[b]
Platelet PI-specific PLC	Rat (brain)	>>100[c]
Sphingomyelinase	Rat (brain)	>>120[d]
Phospholipase D	Streptomyces	stimulates

[a]Assays were performed as described in methods.
[b]No inhibition at 100 μM.
[c]9% inhibition at 100 μM.
[d]No inhibition at 120 μM.

dimethyleicosadienoic acid (DEDA) are also shown. Manoalide has been reported to block human PLA_2 with an IC_{50} = 0.02 μM (22) and this result is different from the IC_{50} we observed (6 μM). The reported assay used a low substrate concentration (5 μM vs 100 μM) and long preincubation time with the enzyme (30 min vs 7 min). Since manoalide exhibits time-dependent inhibition, this long preincubation would maximize the inhibitory effect. DEDA has also been reported to block a macrophage cell line PLA_2 using a micellar substrate-based assay (23). The reported IC_{50} was 16 μM which is lower than what we observed (200 μM).

Since BMS-181162 is effective in inhibiting human platelet PLA_2 it was important to determine if the effect seen is specific or a result of general inhibition of phospholipases. The effect of BMS-181162 on PI-specific PLC, PLD and sphingomyelinase is shown in Table 1. BMS-181162 did not significantly inhibit PI-PLC while PLD and sphingomyelinase were not affected.

A single topical dose of the phorbol ester tetradecanoylphorbol-13-acetate (TPA) to mouse skin results in an edematous response that reaches a maximum level at 6 h (24). These responses quickly diminish as the lesion resolves. In mouse epidermal cells, PLA_2 is activated following TPA treatment leading to the enhanced production of prostaglandins (25). Indeed, in the single dose TPA mouse ear edema model, inhibitors of prostaglandin and leukotriene biosynthesis have been shown to modulate inflammatory reactions (26). BMS-181162 effectively blocked edema with an ED_{50} of 160 μg/ear (Figure 3). BMS-181162 was more potent than the 5-lipoxygenase inhibitor lonapalene which had an ED_{50} of 800 μg/ear. The PLA_2 inhibitor Manoalide has been reported to be active in this model with an ED_{50} of 100 μg/ear (27).

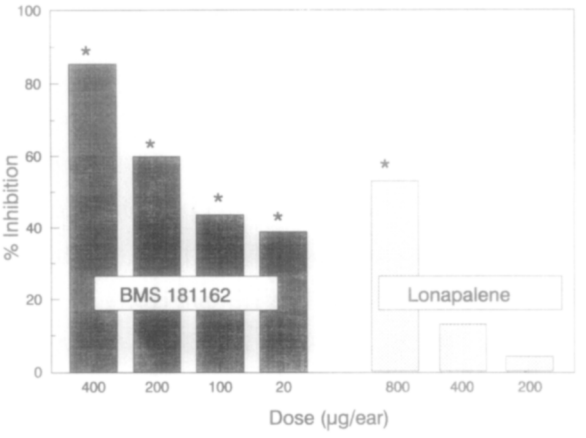

Figure 3. The effect BMS-181162 and lonapalene on TPA-induced mouse ear edema. TPA and test compound were topically co-applied in acetone-water (99:1) to the inner and outer surfaces of each ear (10 µl/surface) of CD-1 mice (n = 10). Control animals received only TPA. After six hours the animals were killed by CO_2 inhalation, and after excision of the ears, 5/16" punch biopsies were taken and weighed. Bars marked with asterisk (*) are significantly different from control ($p < 0.05$).

DISCUSSION

Inhibition of lipid mediator biosynthesis including leukotrienes, prostaglandins and PAF could result in an effective therapy for inflammatory diseases. To that end, a vigorous search for inhibitors of arachidonic acid release is underway.

None of the compounds reported to date are specific PLA_2 inhibitors. The marine natural product manoalide is a potent inhibitor of venom and mammalian PLA_2s. This compound also has analgesic activity in the phenylquinone-induced mouse writhing assay and topical anti-inflammatory activity in the acute phorbol ester-induced mouse skin inflammation assay (27). However, it is difficult to attribute the *in vivo* activity of this compound to PLA_2 inhibition alone, since other biochemical activities are affected by manoalide. Manoalide also blocks PI-PLC in intact cells preventing the formation of inositol 1,4,5-triphosphate and inositol-1-phosphate in norepinephrine-stimulated DDT-MF-2 cells (28). It also was recently reported that manoalide inhibits K^+ depolarization-activated calcium channels and agonist-induced calcium mobilization in several cell types (29). These observed activities, in addition to PLA_2 inactivation, may contribute to the observed anti-inflammatory properties.

BMS-181162 was identified in a screening program targeted at human secreted low molecular weight PLA_2. The biochemical profile shows that this compound is specific for PLA_2 and does not appreciably affect PI-PLC, PLD or sphingomyelinase. A number of

enzymatic pathways have been proposed which could account for the release of free AA in cells: direct release from phospholipids by PLA_2 (30), hydrolysis of phospholipids by PLC to generate diacylglycerol which can then be acted upon by diacylglycerol lipase to generate free AA (31), or phospholipase D mediated hydrolysis of phospholipids which releases phosphatidic acid which then can be hydrolysed by a phosphatidic acid specific PLA_2 (32). Since BMS-181162 does not appreciably inhibit PLC or PLD, the observed anti-inflammatory effect is not likely attributable to blockade of PLC or PLD.

In summary, BMS-181162 is a specific inhibitor of PLA_2 and has in vivo anti-inflammatory activity. The demonstrated specificity of BMS-181162 makes this correlation the strongest argument to date that inhibition of PLA_2 may be sufficient to cause anti-inflammatory effects.

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