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BMS-229724 Is a Tight-Binding Inhibitor of Cytosolic Phospholipase A_2 That Acts at the Lipid/Water Interface and Possesses Anti-Inflammatory Activity in Skin Inflammation Models

JAMES R. BURKE, LYNDA B. DAVERN, PAUL L. STANLEY, KURT R. GREGOR, JACQUES BANVILLE, ROGER REMILLARD, JOHN W. RUSSELL, PATRICK J. BRASSIL, MARK R. WITMER, GRAHAM JOHNSON, JEFFREY A. TREDUP, and KENNETH M. TRAMPOSCH

Dermatology Drug Discovery, Bristol-Myers Squibb Pharmaceutical Research Institute, Buffalo, New York (L.B.D., P.L.S., K.M.T.); and Candiac, Quebec, Canada (J.B., R.R.), Wallingford, Connecticut (P.J.B., G.J., J.W.R.), Princeton, New Jersey (J.R.B., K.R.G., M.R.W., J.A.T.)

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ABSTRACT

Cytosolic phospholipase A_2 (cPLA$_2$) catalyzes the selective release of arachidonic acid from the *sn*-2 position of phospholipids and is believed to play a key cellular role in the generation of arachidonic acid. BMS-229724 (4-[4-[2-[2-[bis(4-chlorophenyl)methoxy]ethyl-sulfonyl]ethoxy]phenyl]-1,1,1-trifluoro-2-butanone) was found to be a selective inhibitor of cPLA$_2$ (IC_{50} = 2.8 μM) in that it did not inhibit secreted phospholipase A_2 in vitro, nor phospholipase C and phospholipase D in cells. The compound was active in inhibiting arachidonate and eicosanoid production in U937 cells, neutrophils, platelets, monocytes, and mast cells. With a synthetic covesicle substrate system, the dose-dependent inhibition could be defined by kinetic equations describing competitive inhibition at the lipid/water interface. The apparent equilibrium dissociation constant for the inhibitor bound to the enzyme at the interface (K_I^{*app}) was determined to be $1 \cdot 10^{-5}$ mol% versus an apparent dissociation constant for the arachidonate-containing phospholipid of 0.35 mol%. The unit of concentration in the interface is mole fraction (or mol%), which is related to the surface concentration of substrate, rather than bulk concentration that has units of molarity. Thus, BMS-229724 represents a novel inhibitor of cPLA$_2$, which partitions into the phospholipid bilayer and competes with phospholipid substrate for the active site. This potent inhibition of the enzyme translated into anti-inflammatory activity when applied topically (5%, w/v) to a phorbol ester-induced chronic inflammation model in mouse ears, inhibiting edema and neutrophil infiltration, as well as prostaglandin and leukotriene levels in the skin. In hairless guinea pigs, BMS-229724 was active orally (10 mg/kg) in a UVB-induced skin erythema model in hairless guinea pigs.

Leukotrienes and prostaglandins are derived from arachidonic acid and are potent lipid mediators of inflammation and pain. The hydrolysis of arachidonoyl-containing phospholipids to produce arachidonate is catalyzed by phospholipase A_2 (PLA$_2$). The 85-kDa group IV cytosolic phospholipase A_2 (cPLA$_2$) appears to be the most likely candidate to catalyze this hydrolysis, since the enzyme is highly selective for arachidonoyl-containing phospholipids and is tightly regulated by receptor-mediated stimulation and intracellular calcium levels (Clark et al., 1995). Because of its putative role in the generation of pro-inflammatory eicosanoids, the enzyme has received considerable medicinal interest.

In addition to the cell biological studies that have been performed to investigate the role of cPLA$_2$ in eicosanoid production, the use of cPLA$_2^{-/-}$ "knockout" mice has provided compelling evidence for the role of cPLA$_2$ in inflammatory disorders. When compared with wild-type mice, the cPLA$_2^{-/-}$ mice showed markedly reduced allergen-induced anaphylactic responses and near complete blockade of leukotriene production and neutrophil infiltration when challenged with LPS/zymosan to the lungs (Uozumi et al., 1997; Nagase et al., 2000). Interestingly, postischemic brain injury was also reduced in these cPLA$_2^{-/-}$ mice, indicating a role of cPLA$_2$ in neuronal injury (Bonventre et al., 1997).

In unstimulated cells, the enzyme is normally located in the cytosol, but translocates to the membrane in response to

ABBREVIATIONS: PLA$_2$, phospholipase A_2; cPLA$_2$, cytosolic PLA$_2$; sPLA$_2$, secreted PLA$_2$; LPS, lipopolysaccharide; DMPM, 1,2-dimyristoyl-*sn*-glycero-3-phosphomethanol; PAPC, 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine; [^{14}C]PAPC, 1-palmitoyl-2-arachidonoyl-[*arachidonoyl*-1-^{14}C]-*sn*-glycero-3-phosphocholine; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; PLC, phospholipase C; PLD, phospholipase D; IL, interleukin; TNFα, tumor necrosis factor-α; LTB$_4$, leukotriene B_4; PGE$_2$, prostaglandin E_2; PAF, platelet-activating factor; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; AACOCF$_3$, arachidonyl trifluoromethyl ketone; NF-κB, nuclear factor-κB; AP-1, activating protein-1; AUC, area under the curve; i.p.t., intraportal; V_{dss}, steady-state volume of distribution.

submicromolar concentrations of calcium (Clark et al., 1995). The catalytic mechanism of $cPLA_2$ is thought to be much like that of serine esterases and proteases. The enzyme forms an acyl enzyme intermediate between arachidonate of the phospholipid substrate and an active site serine residue. Site-directed mutagenesis has been used to provide evidence that serine-228 is this putative active site nucleophile (Sharp et al., 1994). Since the phospholipid substrate on which the enzyme acts is in the form of an aggregate rather than water-soluble monomers, it has been difficult to identify inhibitors of the enzyme that have suitable pharmaceutical properties for in vivo measurements of anti-inflammatory activity.

The identification of inhibitors of $cPLA_2$ is further complicated since the enzyme must first bind to the surface of the lipid/water interface before abstracting a single phospholipid into its active site. Therefore, four classes of inhibitors can be envisioned: 1) compounds that act to promote the desorption of the interface-bound enzyme by altering the physical nature of the interface (i.e., a "detergent" effect); 2) compounds that bind to the interfacial recognition site of the enzyme in the aqueous phase and inhibit the adsorption to the interface; 3) compounds that bind to the active site of the enzyme in the aqueous phase; and 4) compounds that bind to the active site when the enzyme is bound to the lipid/water interface and, therefore, are competitive with respect to individual phospholipid molecules (i.e., competitive inhibition at the interface).

In this report, we investigate the mechanism of action of BMS-229724, a novel inhibitor of $cPLA_2$, and show that it is a competitive inhibitor at the interface. We also demonstrate that it has potent anti-inflammatory activity when administered both topically and orally in skin inflammation models in mice and guinea pigs.

Experimental Procedures

Materials. All nonradiolabeled phospholipids were obtained from Avanti Polar Lipids (Birmingham, AL) except for DMPM, which was from Calbiochem (San Diego, CA). The radiolabeled [^{14}C]PAPC was from PerkinElmer Life Science Products (Boston, MA) (55 mCi/mmol). The human, premonocytic U937 cell line, was obtained from the American Type Culture Collection (Manassas, VA). [^{3}H]Arachidonate-labeled U937 membranes were prepared from U937 cells that had been prelabeled for 16 h with [^{3}H]arachidonate (100 Ci/mmol) as previously described (Burke et al., 1997c). BMS-229724 was synthesized as previously described (Banville et al., 1999).

The human, recombinant $cPLA_2$ was expressed in sf9 insect cells as previously described (Burke et al., 1995b). The human nonpancreatic $sPLA_2$ (group IIA), which had been purified from human platelets, was obtained from Dr. R. C. Franson (Virginia Commonwealth University), along with the [1-^{14}C]oleic acid-labeled *Escherichia coli* membrane substrate (3000 dpm/nmol phospholipid).

Enzyme Assays. Sonicated phospholipid covesicles, comprised of DMPM and containing [^{14}C]PAPC or other phospholipids, were prepared using the general methods described previously (Jain et al., 1989; Jain and Gelb, 1991; Diez et al., 1992). With this synthetic substrate, enzymatic assays were performed using the general procedure of Burke et al. (1995b), in which $cPLA_2$ (210–390 ng/ml) was added to solutions of the radiolabeled covesicles (150–270 μM bulk phospholipid) in 25 mM BisTris propane containing 7 mM $CaCl_2$, 0.4 mg/ml albumin (bovine serum albumin, essentially fatty acid free), and 4 M glycerol at pH 8. All components except enzyme were incubated at 37°C for 5 min before the addition of enzyme. At various times, 100-μl aliquots were removed and quenched by addition into 1.9 ml of tetrahydrofuran. The hydrolyzed, radiolabeled fatty acid was then isolated using aminopropyl solid-phase extraction columns as described previously (Tramposch et al., 1992). The rate of hydrolysis was calculated after an initial burst of product formation (Burke et al., 1999a). Under these assay conditions, the high calcium concentration catalyzes vesicle fusion with continuous exchange of phospholipids (Jain et al., 1986; Burke et al., 1995b).

Assays of $cPLA_2$ activity using the [^{3}H]arachidonate-labeled U937 membranes as substrate typically used 0.6 μg/ml enzyme and membrane substrate (22 μM) in 20 mM HEPES buffer, pH 8, containing 6 mM $CaCl_2$, 0.9 mg/ml human serum albumin, and 0 to 4 M glycerol (Burke et al., 1997c). Enzyme assays were allowed to proceed for 20 min at 37°C before quenching with tetrahydrofuran. The radiolabeled fatty acid product was isolated as described above. Assays of $sPLA_2$ using radiolabeled *E. coli* membrane as substrate were performed as described previously (Tramposch et al., 1992).

Differential Scanning Calorimetry. Differential scanning calorimetry was performed with a MCS DSC instrument as previously described (Burke et al., 1999b).

Cell Assays. For measurements of the effects of $cPLA_2$ inhibitors on cellular arachidonate production, dibutyryl-cAMP-differentiated U937 cells were used according to the previously published procedure (Burke et al., 1997a). In short, cells were preincubated with the inhibitors for 5 min at 37°C prior to stimulating with the chemotactic peptide, fMLP. After 5 min of stimulation, the reactions were terminated in an ice bath and the mass of arachidonate determined by gas chromatography/electron capture of the derivatized product (Burke et al., 1997a).

For the effect on the products of phospholipase C (PLC), the fMLP-induced production of inositol trisphosphates in these same cells was carried out as previously described. (Burke et al., 1997a).

The activation of PLD in differentiated U937 cells was followed by measuring the amount of phosphatidylethanol produced in the presence of ethanol as previously described (Anthes et al., 1991). The radiolabeled production of phosphatidylethanol was monitored by thin-layer chromatography.

The LPS-induced production of eicosanoids and cytokines with peripheral blood monocytes was carried out using the procedure of Liebler et al. (1994). All stimulations were for 4 h except when measuring IL-1β, which required 18 h of stimulation. IL-6, IL-8, TNFα, and IL-1β in the media were measured using specific enzyme immunoassays from Endogen Corporation (Woburn, MA). LTB_4 and PGE_2 were measured using radioimmunoassays from Perceptive Biosystems (Framingham, MA).

For measurements of arachidonate and eicosanoids in platelets, neutrophils, mast cells, and keratinocytes, the procedures of Bartoli et al. (1994), Burke et al. (1997b), Nakamura et al. (1991), and McCord et al. (1994), respectively, were used. The production of PAF in human neutrophils was measured by the procedure of Tramposch et al. (1994).

Chronic Skin Inflammation Model in Mice. A chronic persistent skin inflammation in the ears of CD-1 mice was induced by the repeated treatment of phorbol ester using the procedure of Stanley et al. (1991). Briefly, TPA (10 μl, 0.01% in acetone/water at 99:1) was applied to ears on days 0, 2, 4, 7, and 9. BMS-229724 in the same vehicle was applied twice daily on days 7 to 9 and once (morning) on day 10. Six hours after the last application, ear punch biopsies were removed, weighed for assessment of edema, then snap-frozen for later analysis of myeloperoxidase content, an enzyme marker of neutrophil infiltration, and for eicosanoid levels measured by high-performance liquid chromatography/radioimmunoassay. For PGE_2 levels, biopsies were taken on day 9.

UVB-Induced Erythema in Hairless Guinea Pigs. Hairless guinea pigs (female, 350–400 g, Charles River Laboratories, Wilmington, MA) were used so depilation methods would not interfere with the quality of the skin. Animals were exposed on the masked flanks to UVB radiation (350 mJ) emitted from a bank of six fluo-

rescent tubes. BMS-229724 was administered orally in peanut oil by gavage tube 90 min prior to UVB exposure, and animals were graded for intensity of erythema 6 h after irradiation as follows: grade 0, no erythema; grade 1, very slight erythema; grade 2, mild erythema with uniform redness and well-defined edges; grade 3, moderate erythema (bright even redness); and grade 4, severe erythema (intense red with slight edema). Grading was performed in a blinded fashion. After the guinea pigs were evaluated for erythema, tissue biopsies (5/8 inch) of the inflamed sites were taken for PGE_2 and LTB_4 measurements using the procedure of Tramposch et al. (1994).

Pharmacokinetics in Rats. A solution of BMS-229724 in water/cremophor/ethanol (75:12.5:12.5) at 2.0 mg/ml was administered to one group ($n = 3$) of male Sprague-Dawley-1 rats (cannulated; Hilltop, Scottdale, PA) as a zero order intravenous infusion of 10 min duration or at 1.0 mg/ml to a second group as a zero order intraportal infusion of 20 min duration to provide a dose of 4 mg/kg to each group. A third group of rats ($n = 3$) was administered a solution of BMS-229724 (4.0 mg/ml in water/cremophor/ethanol, 75:12.5:12.5) by oral gavage to provide a dose of 20 mg/kg. Serial blood samples were collected up to 24 h after beginning either infusion or administration of the oral dose. Protein in separated plasma was precipitated with acetonitrile, and the resulting supernatants were analyzed for BMS-229724 by a reverse-phase chromatography (high-performance liquid chromatography system) interfaced to a tandem mass spectrometer operated in the negative ion electrospray, selected reaction monitoring mode.

Results

U937 cells treated with [^{3}H]arachidonate incorporate this radiolabeled fatty acid into phospholipid pools. An assay using membranes isolated from these radiolabeled U937 cells as substrate was used to measure the activity of recombinant, human $cPLA_2$. When assayed in this manner, BMS-229724 was identified as an inhibitor of $cPLA_2$. As shown in Fig. 1, the dose-dependent inhibition gave an IC_{50} value of 2.8 μM. Against the group IIA $sPLA_2$ from human platelets using membranes from *E. coli* as substrate ($sPLA_2$ does not effectively hydrolyze mammalian membranes), BMS-229724 showed less than 30% inhibition at concentrations as high as 600 μM, which is the solubility limit of the compound in this assay. Additional selectivity measurements were taken in cells (vide infra).

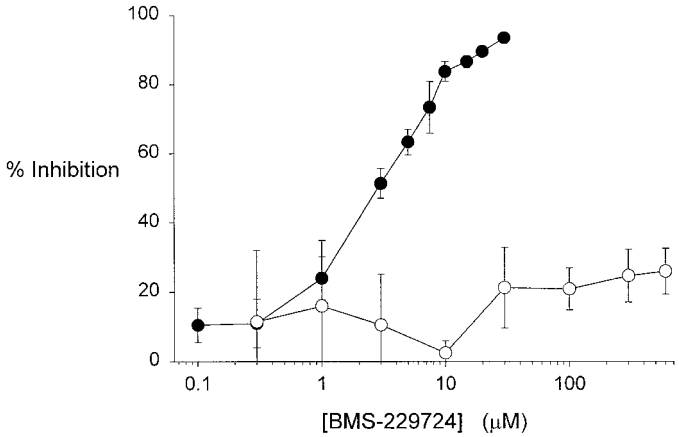
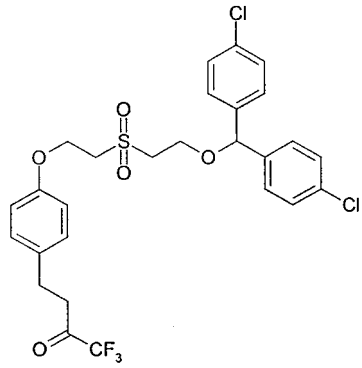
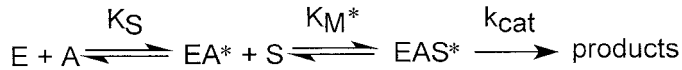


Fig. 1. Dose-dependent inhibition of $cPLA_2$ and $sPLA_2$ by BMS-229724. ●, activity of $cPLA_2$ assayed using membranes from [^{3}H]arachidonate-labeled U937 cells as substrate; ○, activity of $sPLA_2$. Data are represented as the percentage of control without inhibitor and are the average of triplicate measurements. See *Experimental Procedures* for details.



Scheme 1. BMS-229724.



Scheme 2. Kinetic scheme for binding of $cPLA_2$ for phospholipid.

Mechanism of Inhibition. Because the enzyme acts at the lipid/water interface, kinetic analysis of the enzyme (and the inhibition mechanism) are quite different from solution-phase enzymology. Indeed, the equilibrium binding of $cPLA_2$ to the substrate involves a dependence on two processes as defined in Scheme 2 (Burke et al., 1995b) where E is defined as the free enzyme, A is the phospholipid vesicle, EA* is the enzyme bound to the lipid/water interface, S is the phospholipid substrate within the vesicle, and EAS* is the interface-bound enzyme containing an active site-bound phospholipid substrate. These include the intrinsic equilibrium binding to the interface (defined by a dissociation constant, K_S) and the equilibrium binding of phospholipid substrate to the active site at the interface (defined by a dissociation constant, K_M*).

To characterize the mechanism of inhibition by BMS-229724, a covesicle substrate system comprised of PAPC dispersed within DMPM (which is not hydrolyzed by the enzyme) was used. This substrate system greatly simplifies the kinetic analysis of the enzyme at the interface, since there is only one substrate (PAPC) within the bilayer, and its concentration (in units of mole fraction of the bilayer) at the interface can be easily controlled. Indeed, this covesicle substrate allows for the determination of the equilibrium dissociation constants of phospholipids and inhibitors from the active site at the interface (Burke et al., 1995b), and the benefits of using this covesicle substrate system when performing kinetic analyses of inhibitors has recently been demonstrated (Burke et al., 1999a). Using these covesicles, the concentration of BMS-229724 was varied while measuring the $cPLA_2$-catalyzed rate of hydrolysis of the covesicles containing different mole percentages of [^{14}C]PAPC. As long as the bulk phospholipid concentration is large enough to ensure that essentially all of the enzyme is at the interface (e.g., at 270 μM phospholipid over 98% of the enzyme is interface-bound; Burke et al., 1995b), the following equation describing competitive inhibition of $cPLA_2$ at the interface is valid (Burke et al., 1995b, 1997d).[1]

[1] This equation is valid, since the active site dissociation constant for DMPM has been shown to be more than 330 times greater than the value for PAPC (Burke et al., 1997d).

$$\frac{(v_0)^o}{(v_0)^I} = 1 + \left(\frac{1/K_I^{*app}}{1 + \frac{X_S^o}{K_M^{*app}}}\right) X_I/(1 - X_I) \quad (1)$$

where $(v_0)^o/(v_0)^I$ is the ratio of initial rates in the absence to that in the presence of a competitive inhibitor; K_M^{*app} and K_I^{*app} are defined as the apparent dissociation constants for the substrate and inhibitor, respectively,[2] X_I is the concentration of inhibitor (in units of mole fraction), and X_S^o is the mole fraction of radiolabeled substrate ([^{14}C]PAPC) in the absence of inhibitor. The unit of concentration in the interface is a mole fraction that is related to the surface concentration of substrate, rather than bulk concentration, which has units of molarity. With BMS-229724, the X_I value was determined by assuming that all of the inhibitor was partitioned within the phospholipid bilayer (later shown not to be true).

Using these covesicles as substrate for $cPLA_2$, the dose-dependent inhibition by BMS-229724 was plotted in Fig. 2 as $(v_0)^o/(v_0)^I$ versus $X_I/(1 - X_I)$ at each X_S^o value.[3] As predicted by eq. 1, linear correlations were obtained. Nonlinear regression analysis of the data as fit to eq. 1 yielded K_M^{*app} and K_I^{*app} values of 0.35 and 0.005 mol%, respectively. The value of K_M^{*app} determined here is in agreement with the value of 0.3 ± 0.1 mol% determined previously (Burke et al., 1995b, 1997d). Only an inhibitor that acts competitively at the interface would show this relationship.

Verification that BMS-229724 partitions into the bilayer was provided by the effect of the compound on the thermal transition of DMPM vesicles. Differential scanning calorimetry showed that the addition of BMS-229724 at 10 and 100 μM resulted in a dose-dependent lowering and broadening of the phase transition temperature of these vesicles, consistent with a partitioning of the inhibitor into the bilayer (results not shown).

Additional evidence that BMS-229724 acts as a competitive, reversible inhibitor at the lipid/water interface comes from the observation that BMS-229724 was 10 times less potent against $cPLA_2$ with covesicle substrate containing high amounts of cholesterol (cholesterol/phospholipid ratio of 0.6:1, results not shown). The presence of cholesterol in a phospholipid bilayer at this concentration has been shown to greatly reduce the partition coefficient of drugs into phospholipid bilayer vesicles (Herbette et al., 1991). This results, presumably, from an increase in the packing density of the bilayer. This effect of cholesterol on the inhibition by BMS-229724 provides further support to the conclusion that BMS-229724 acts by partitioning into the phospholipid bilayer and competing with phospholipid monomers for the active site of $cPLA_2$.

The assumption that the inhibitor was completely partitioned into the phospholipid bilayer of covesicle substrate (without cholesterol) was tested by determining the effect of reaction volume on the inhibition. If the inhibitor is completely partitioned into the vesicle, the degree of inhibition from a constant mole amount of inhibitor will be independent of the reaction volume, as long as all of the enzyme is bound to the interface since the mole fraction of inhibitor within the bilayer will remain constant (Lin and Gelb, 1993). However, if the inhibitor is only partially partitioned into the vesicle, the inhibition will decrease with increasing volume as more of the inhibitor partitions into the aqueous phase. Indeed, Fig. 3 shows that the inhibition of the linear rate was dependent on the reaction volume. The relationship between the degree of inhibition and the reaction volume can be defined by the following (rearranged equation from Burke et al., 1995a):

$$L = \frac{(((v_0)^o/(v_0)^1 - 1)PUC) - (MCUI_T)}{1-((v_0)^o/(v_0)^I)} \quad (2)$$

where L is the volume of the reaction mixture, P and I_T are the mole amounts of phospholipid and inhibitor, respectively, C is the partition coefficient,[4] M is a constant,[5] U is the volume that the bilayer occupies (equal to $1.21 \cdot 10^{-6}$ ml/nmol; see Bassolino-Klimas et al., 1993); and $(v_0)^o/(v_0)^I$ is as defined earlier.

A partition coefficient of 22 was determined using a non-

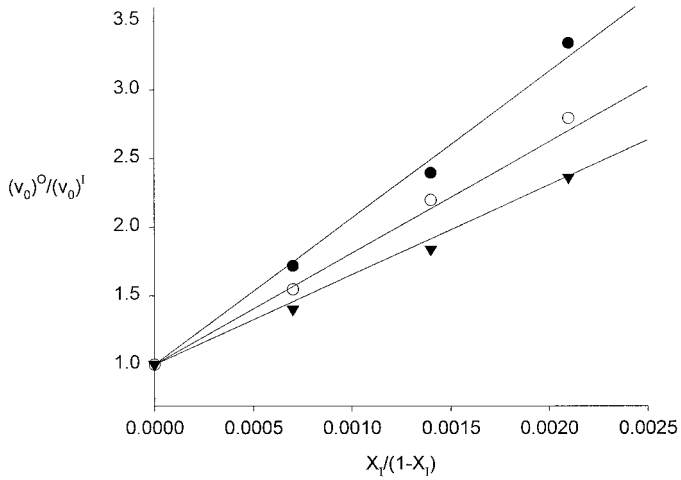


Fig. 2. Correlation of the inhibition of hydrolysis of [^{14}C]PAPC/DMPM covesicles with the concentration of BMS-229724 (X_I). Enzyme was assayed in a solution containing radiolabeled covesicles as substrate. These [^{14}C]PAPC/DMPM covesicles contained 0 to 6.9 mol% BMS-229724 and had X_S^o values (in terms of [^{14}C]PAPC concentrations) of 6 mol% (●), 8 mol% (○), 10 mol% (▼). Data were fit to eq. 1, which describes competitive inhibition at the interface. [Enzyme] = 390 ng/ml, [phospholipid] = 268 μM. The rate was measured at equilibrium, after burst and time-dependent inhibition (see Fig. 4). Data represent the average of duplicate measurements. To determine the X_I values, the inhibitor was assumed to be completely partitioned into the bilayer.

[2] K_M^{*app} and K_I^{*app} are related to the intrinsic dissociation constants (K_M^* and K_I^*) by the equations: $K_M^{*app} = K_M^* (1 + 1/K_L^*)$ and $K_I^{*app} = K_I^* (1 + 1/K_L^*)$, where K_L^* is the active site dissociation constant for DMPM at the interface (Burke et al., 1997d).

[3] When determining the equilibrium dissociation constants from the active site, the mole amount of substrate ([^{14}C]PAPC in this case) and the mole amount of DMPM were held constant, while varying the mole amount of inhibitor (Burke et al., 1995b, 1997d). This has the effect of actually decreasing the mole fraction of both substrate and DMPM as the inhibitor concentration is increased. Thus, X_S^O in eq. 1 equals the mole fraction of substrate phospholipid without inhibitor. The mole fraction of substrate in the presence of the inhibitor is correspondingly less.

[4] The partition coefficient, C, is defined as the concentration (molarity) of inhibitor in the phospholipid bilayer divided by the concentration in the aqueous phase.

[5] In eq. 2, M is defined as:

$$M = I_T\left(\frac{1 + 1/K_I^{*app}}{1 + X_S^o/K_S^{*app}}\right)$$

where I_T equals the total mole amount of inhibitor present. The definitions of all other variables are defined in the text.

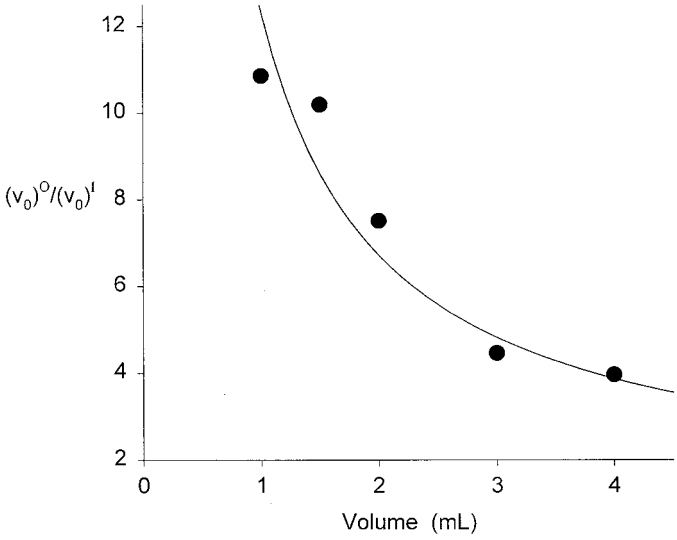


Fig. 3. Effect of the reaction volume on the inhibition of $cPLA_2$-catalyzed hydrolysis of [^{14}C]PAPC/DMPM covesicles by BMS-22974. $cPLA_2$ was assayed in the presence of 536 nmol of phospholipid (covesicles of 92:8 DMPM/[^{14}C]PAPC) with and without 1.43 nmol of BMS-229724 while varying the reaction volume. The solid line represents a fit of the data to eq. 2 to yield a partition coefficient of 22. See text for details.

linear regression analysis to fit the data. This corresponds to partial partitioning of less than 1% BMS-229724 into the bilayer under the conditions of Fig. 2. After correcting for this partial partitioning, the true K_i* value was determined to be $1 \cdot 10^{-5}$ mol%, which is 4 orders of magnitude smaller than the dissociation constant of the phospholipid substrate (K_M* = 0.35 mol%).

Part of the reason that BMS-229724 partitions to such a small extent into phospholipid bilayers in vitro is that it forms aggregates with itself in solution. This was evidenced by laser light-scattering experiments, which showed that aggregate formation of BMS-229724 occurred when the compound was added in dimethyl sulfoxide to aqueous solutions (results not shown). Further evidence of this aggregation was provided by the observation that less than 5% of a 100 μM solution of BMS-229724 was recovered in the filtrate after passing the solution through a 0.10-μm filter (results not shown). In contrast, no light-scattering was observed with a 100 μM solution BMS-229724 in methanol, and complete recovery of compound was obtained with this methanol solution after filtration. The propensity to form aggregates at lower concentrations of BMS-229724 was not measured. The aggregation of BMS-229724 in aqueous solutions shifts the phospholipid-to-water distribution equilibrium toward the aqueous phase. Therefore, most of the enzyme and cell experiments detailed in this section will vastly underestimate the potency of BMS-229724, since the compound is not effectively partitioned into the phospholipid bilayer. Indeed, as will be shown later, BMS-229724 is considerably more potent in vivo, which may reflect a higher degree of partitioning.

Hemiketal Formation with an Active Site Serine. There is NMR evidence that the arachidonyl trifluoromethyl ketone ($AACOCF_3$) forms a hemiketal with a serine residue in the active site of $cPLA_2$ (Trimble et al., 1993). Characteristic of this hemiketal formation is the slow, tight-binding inhibition observed with $AACOCF_3$. The slow-binding inhibition with trifluoromethyl ketone inhibitors of esterases and proteases is thought to result from one of two possible mechanisms: 1) the active site serine can only interact with the nonhydrated trifluoromethylketone, the concentration of which may be so small (BMS-229724 in aqueous solvents is >99% hydrated at the ketone as determined by NMR) that the effect on rate is correspondingly diminished; or 2) a conformational change in the enzyme must occur (e.g., release of a water molecule from the active site) to accommodate the hydrated ketone in the active site (Stein et al., 1987). To determine whether BMS-229724 may also be forming a hemiketal with the active site serine residue, an investigation of the time-dependent (i.e., slow-binding) inhibition with this inhibitor was performed.

As shown in Fig. 4, the $cPLA_2$-catalyzed hydrolysis of synthetic substrate (DMPM/PAPC) was inhibited by the presence of BL-763, a $cPLA_2$ inhibitor that lacks a trifluoromethyl ketone (Burke et al., 1999b). Consistent with the fact that BL-763 is a competitive, readily reversible inhibitor is the observation that there is no time dependence to the inhibition. That is, the inhibition was immediate and constant (an initial burst of product formation was observed under these conditions).

In contrast, the inhibition of the enzymatic hydrolysis by BMS-229724 was time-dependent. In this case, the inhibition was not apparent at the early time points, but was pronounced at later time points. This demonstrates that BMS-229724, like $AACOCF_3$, is a slow-binding inhibitor presumably due to formation of a hemiketal with an active site serine (Scheme 3).

Evidence for tight-binding inhibition of $cPLA_2$ by BMS-229724 was obtained by preincubating the enzyme with BMS-229724 in the presence of phosphatidylmethanol vesicles for 10 min. Dilution of this preincubated enzyme into the synthetic substrate assay (to dilute out the inhibitor) yielded a rate of hydrolysis that was inhibited for several minutes before enzyme activity was eventually regenerated (Fig. 5).

Thus, BMS-229724 is a slow-binding, tight-binding inhib-

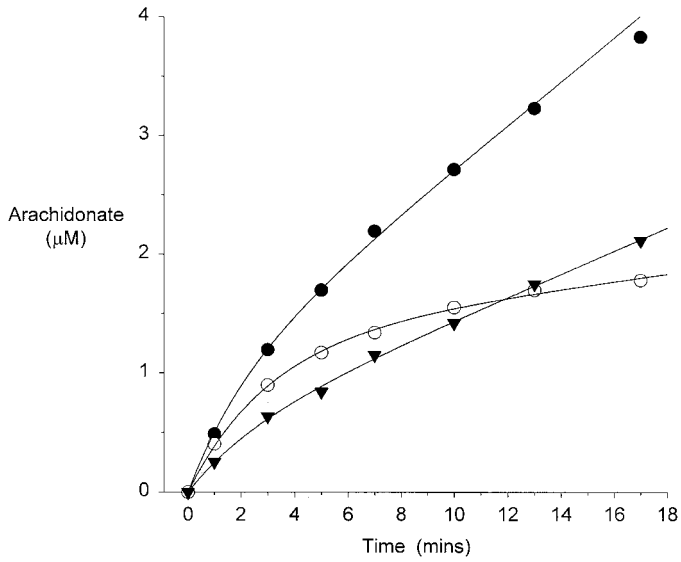
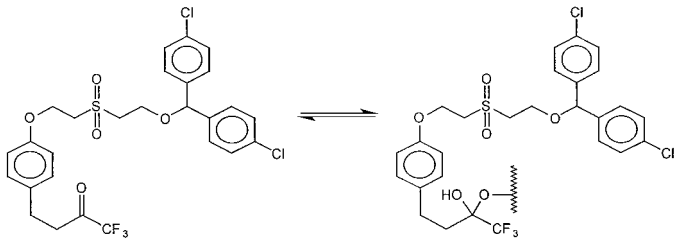


Fig. 4. Time-dependent inhibition of $cPLA_2$. $cPLA_2$-catalyzed hydrolysis of [^{14}C]PAPC/DMPM covesicles by $cPLA_2$ inhibitors. Enzyme was assayed in a solution containing 270 μM radiolabeled covesicles as substrate (8:92 ratio of [^{14}C]PAPC to DMPM). ●, no inhibitor; ▼, 10 μM BL-763; ○, 0.5 μM BMS-229724.



Scheme 3. Putative hemiketal formed between BMS-229724 and an active site serine.

itor while acting competitively at the lipid/water interface. Greatly reduced inhibition measured with methyl ketones and trifluoromethyl alcohols provides additional evidence that BMS-229724 forms a hemiketal with an active site serine (results not shown).

Activity in Cells. It has been shown that stimulation of differentiated U937 cells with the chemotactic peptide fMLP results in a robust release of arachidonate. Moreover, this release is the result of the action of $cPLA_2$ (Burke et al., 1997a). fMLP stimulation of these cells also leads to the activation of PLC and PLD, which appear to be upstream of $cPLA_2$ activation (Burke et al., 1997a). Consistent with the role of $cPLA_2$ in agonist-induced production of arachidonate (and eicosanoids derived from arachidonate), BMS-229724 dose dependently inhibited fMLP-stimulated arachidonate and PGE_2 production in differentiated U937 cells with identical IC_{50} values of 2 μM (Fig. 6). The selectivity for $cPLA_2$ was further evidenced in these cells by the lack of activity against PLC and PLD, as measured by the production of inositol 1,4,5-trisphosphate and phosphatidylethanol, respectively, in U937 cells.

Interestingly, the activity of BMS-229724 against agonist-induced arachidonate (and eicosanoid) production was observed in a wide range of inflammatory cells, including platelets, mast cells, keratinocytes, and monocytes (Table 1). The activity against eicosanoids cannot be explained by inhibition of cyclooxygenase or 5-lipoxygenase, since BMS-229724 did not inhibit either of these enzymes in this concentration range (results not shown). The compound also inhibited the

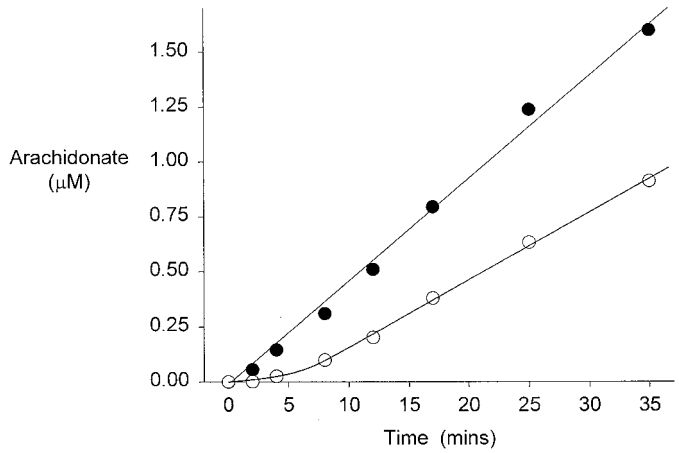


Fig. 5. Slow regeneration of enzymatic activity after preincubation with BMS-229724. Nonradiolabeled (270 μM) PAPC/DMPM (8 mol% PAPC) vesicles were preincubated with BMS-229724 for 10 min, then diluted 14-fold into an assay with 270 μM [^{14}C]PAPC/DMPM covesicles (8 mol% [^{14}C]PAPC) as substrate to dilute out the inhibitor. ●, no inhibitor in preincubation; ○, 1 μM BMS-229724 in preincubation.

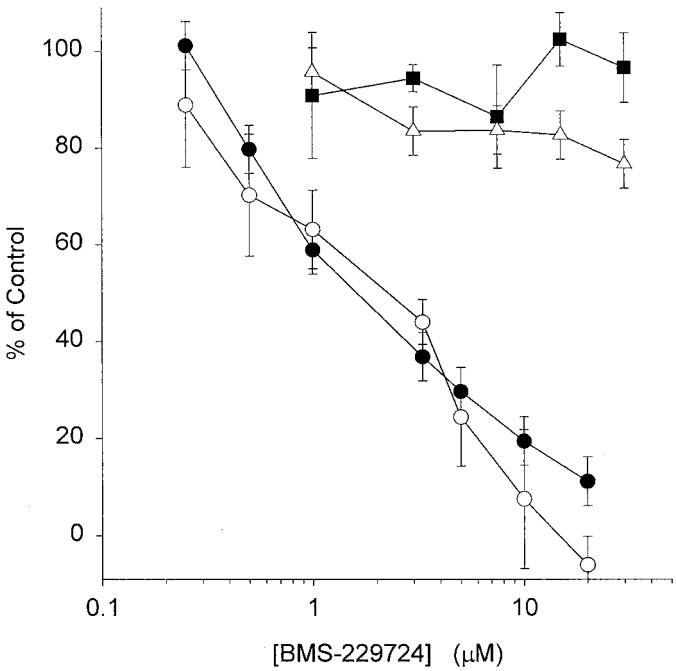


Fig. 6. Inhibition of fMLP-induced production of arachidonate, PGE_2, inositol 1,4,5-trisphosphate, or phosphatidylethanol in differentiated U937 cells. ●, mass of arachidonate produced; ○, amount of PGE_2 produced; ■, amount of PLC-dependent [^{3}H]inositol 1,4,5-trisphosphate produced; △, amount of PLD-dependent phosphatidylethanol produced (stimulated in the presence of ethanol). Data are represented as the percentage of control without inhibitor after subtracting unstimulated values and are the average of triplicate measurements. See *Experimental Procedures* for details.

TABLE 1

Inhibition of arachidonate and eicosanoids by BMS-229724 in inflammatory cells

See *Experimental Procedures* for details.

Cell	Agonist	Lipid Mediator Measured	IC_{50} with BMS-229724
			μM
Platelets, human	Thrombin	Arachidonate	4
Neutrophils, human	LTB_4	Arachidonate	12
Neutrophils, human	A23187	LTB_4	3
Mast cells, mouse	Antigen	Arachidonate	3
Monocytes, human	LPS	PGE_2	6
Keratinocytes, human	A23187	PGE_2	8
Neutrophils, human	A23187	Platelet-activating factor	15

production of platelet-activating factor, a pro-inflammatory lipid mediator resulting from the lysophospholipid product of $cPLA_2$-catalyzed hydrolysis. These results point to the important role of $cPLA_2$ in generating inflammatory mediators.

Recent reports have implicated a role for arachidonate in the activation of NF-κB (Camandola et al., 1996; Thommesen et al., 1998). Accordingly, we tested BMS-229724 as an inhibitor of LPS-stimulated production of TNFα, IL-6, IL-1β, and IL-8 in human monocytes. As shown in Fig. 7, BMS-229724 inhibited production of these cytokines with IC_{50} values in the range of 4 to 7 μM. This tracked with the IC_{50} value against PGE_2 of 6 μM in these cells (arachidonate release could not be directly measured in this experiment due to the small number of cells). Consistent with this effect being regulated on the level of arachidonate rather than the eicosanoid metabolites, neither indomethacin nor MK-886

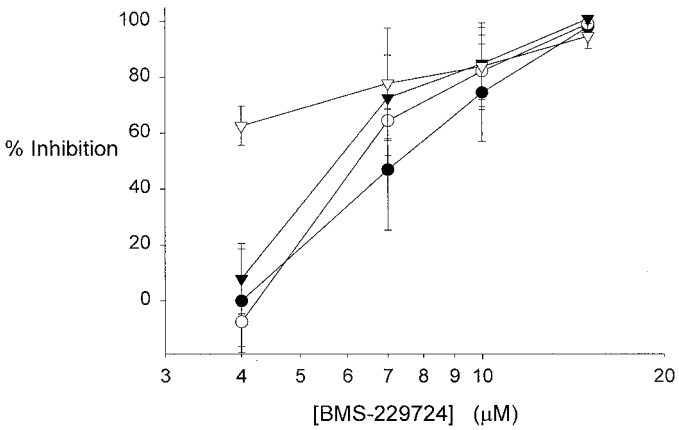


Fig. 7. Inhibition by BMS-229724 of LPS-stimulated cytokine production in human monocytes. ○, IL-6; ●, TNFα; ▼, IL-8; ▽, IL-1β. Data are represented as the percentage of control without inhibitor and are the average of triplicate measurements. All LPS stimulations were for 4 h, except measurements of IL-1β where the stimulation was allowed to proceed for 18 h to achieve measurable levels.

(inhibitors of the cyclooxygenase and 5-lipoxygenase pathways, respectively) inhibited the production of these cytokines at concentrations where complete inhibition of PGE_2 and LTB_4 production, respectively, was observed (results not shown). BMS-229724 was also shown to inhibit LPS-induced cytokine and nitric oxide production in primary cultured microglia (R. Pasmantur, unpublished observation).

Pharmacology and Pharmacokinetics. As a first measure of the anti-inflammatory activity of BMS-229724, the compound was administered topically in a mouse model of chronic skin inflammation induced by repeated exposure to phorbol ester. The skin inflammation in this model is persistent and has been useful in assessing whether topically applied compounds are able to resolve an existing inflammatory lesion (Stanley et al., 1991). A 5% (w/v) solution of BMS-229724 significantly reduced the edema (Fig. 8A) and cell infiltration (Fig. 8B) in this model. Consistent with its mechanism of action, BMS-229724 reduced the levels of prostaglandin and leukotriene biosynthesis in the inflamed skin with reductions of 96% and 74%, respectively, measured at the 5% dose (Fig. 9). Since systemic anti-inflammatory agents, including glucocorticoids, are not active in this model, another model was used to assess the systemic anti-inflammatory activity of BMS-229724 (see below).

To assess the pharmakinetic profile of BMS-229724, intra-arterial- or intraportal-cannulated rats were used. After i.a. administration, the plasma concentrations of BMS-229724 were observed to decline with a mean elimination half-life estimated to be 2.5 ± 0.5 h. The total clearance and steady-state volume of distribution (V_{dss}) were estimated to be 46.1 ± 15.1 ml/min · kg and 1.2 ± 0.5 l/kg, respectively. Based on a comparison of the dose-normalized mean AUC after intraportal (i.p.t.) and oral administration with the mean AUC after i.a. administration, the estimated i.p.t. and oral bioavailabilities of BMS-229724 in the rat were 91% and 12%, respectively. The high i.p.t. bioavailability indicates that the relatively low oral bioavailability is not due to first-pass hepatic metabolism. Nearly identical oral exposure was obtained when BMS-229724 was administered in peanut oil. A summary of the mean pharmacokinetic parameters for BMS-229724 in the rat is shown in Table 2.

To evaluate its systemic activity against skin inflammation, BMS-229724 was tested in hairless guinea pigs in which skin erythema was induced by UVB irradiation. In this model, increased synthesis of PGE_2 is characteristic of the inflammatory response after acute exposure to UVB light (Hruza and Pentland, 1993), and these increases parallel the development of the erythema (Snyder, 1976). The role of PGE_2 in mediating UV-induced erythema was demonstrated by the ability of cyclooxygenase inhibitors such as indomethacin to suppress erythema up to 24 h after irradiation (Snyder, 1976). Recent results have shown that $cPLA_2$ synthesis occurs in skin when exposed to doses of UV sufficient to cause erythema, suggesting that $cPLA_2$ participates in UVB-induced inflammation (Gresham et al., 1996).

When dosed orally in this model, BMS-229724 exhibited a dose-response effect on erythema (Fig. 10A). At 10 mg/kg, the erythema was significantly reduced and the response was similar to the positive control, ibuprofen. BMS-229724 also produced a corresponding dose-responsive reduction of PGE_2 (Fig. 10B). These data are consistent with recent work suggesting that $cPLA_2$ plays an important role in the prostaglandin-dependent erythema in skin induced by UVB irradiation.

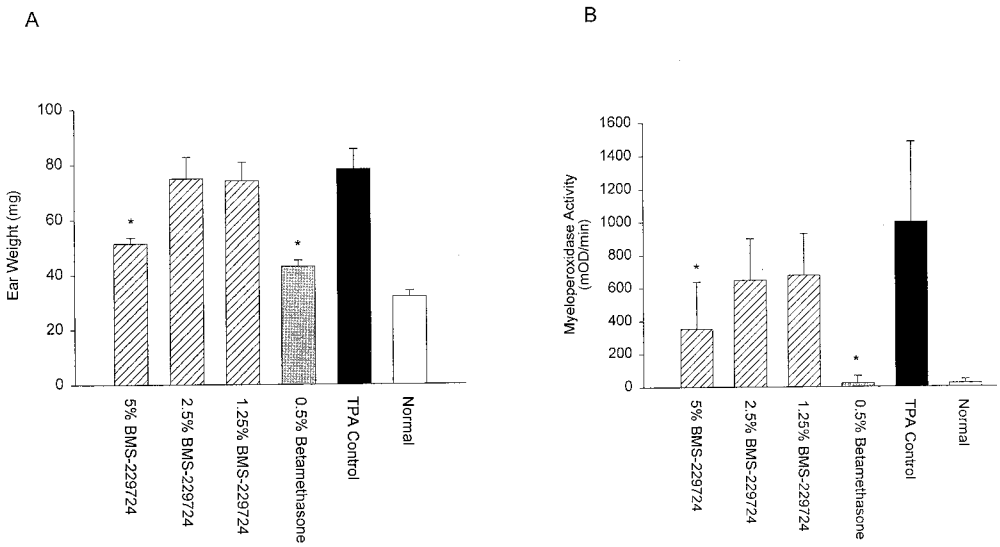


Fig. 8. Effect of topical application of BMS-229724 on ear weight (A) and myeloperoxidase activity (B) in the phorbol ester-induced chronic model of skin inflammation in mice. Mice were treated with topical phorbol ester over 11 days (see *Experimental Procedures* for details). Beginning on day 7, BMS-229724 was applied topically in acetone/water (99:1) to inflamed skin twice daily. Ears were excised on day 10 and weighed to measure edema. Tissue myeloperoxidase content was determined as a measure of neutrophil influx. $N = 10$ animals per group, with the error bars representing standard deviations. Drug concentrations are in w/v percentage. $^*p < 0.05$, compared with TPA control.

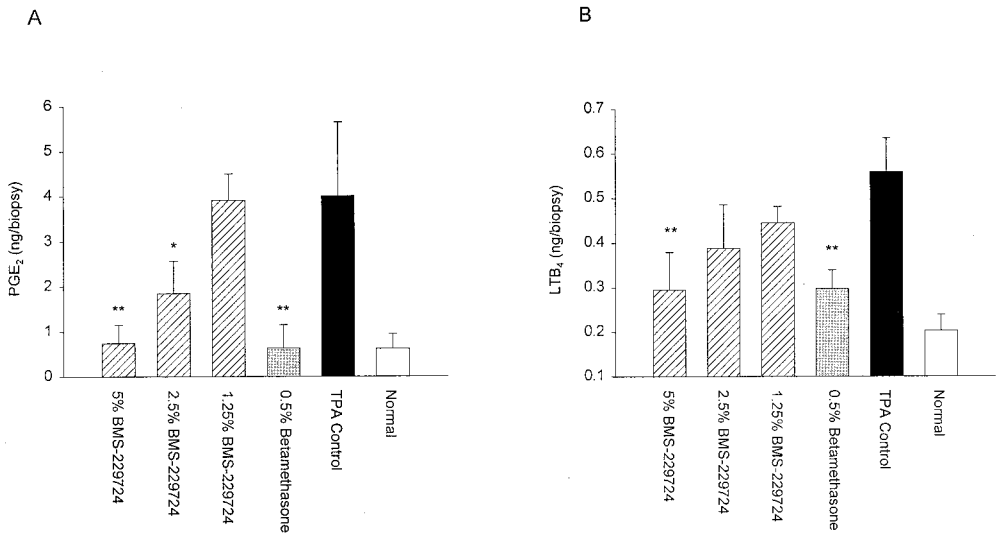


Fig. 9. Effect of topical application of BMS-229724 on PGE_2 (A) and LTB_4 (B) in inflamed skin from the phorbol ester-induced chronic model of skin inflammation in mice. Mice were treated with topical phorbol ester over 11 days (see *Experimental Procedures* for details). Beginning on day 7, BMS-229724 was applied topically in acetone/water (99:1) to inflamed skin twice daily. Ears were excised on either day 9 for PGE_2 or day 10 for LTB_4 determinations. $N = 10$ animals per group, with the error bars representing standard deviations. Drug concentrations are in w/v percentage. $^*p < 0.05$, $^{**}p < 0.01$, compared with TPA control.

TABLE 2

Summary of mean pharmacokinetic parameters for BMS-229724

BMS-229724 was administered by oral (20 mg/kg), intraportal (4 mg/kg), or intra-arterial (4 mg/kg) routes to the rat in cremophor/ethanol/water vehicle except as noted.[a]

Route	C_{max}	T_{max}	$t_{1/2}$	Cl	V_{dss}	F
	ng/ml	*h*	*h*	*ml/min×kg*	*l/kg*	*%*
i.a.	5190 ± 2105	0.17	2.5 ± 0.5	46.1 ± 15.1	1.2 ± 0.5	
i.p.t.	3007	0.33				91
p.o.	361 ± 81	1.7 ± 0.3				12
p.o., peanut oil	270 ± 61	0.9 ± 0.5				10

[a] Note: after i.p.t. administration, F was estimated by comparison of the mean dose normalized $AUC_{0\to\infty}$ from two animals with that after i.a. administration.

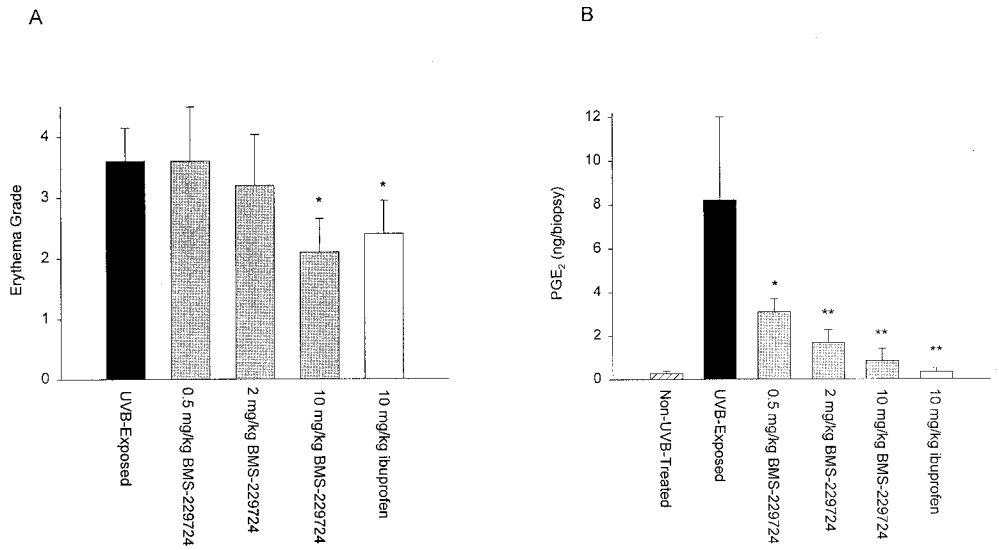


Fig. 10. Effects of BMS 229724 on erythema (A) and PGE_2 (B) levels in the guinea pig UVB model of inflammation. BMS-229724 was administered by oral gavage in peanut oil to hairless guinea pigs. Erythema was graded 6 h after UVB irradiation and skin biopsies taken for PGE_2 level determinations. $N = 7$ animals per group with the error bars representing standard deviations. $^*p < 0.05$, $^{**}p < 0.01$, compared with UVB-exposed, vehicle control.

Discussion

We have shown that BMS-229724 inhibits $cPLA_2$ at the interface in a manner that is competitive with respect to phospholipid molecules for the active site. Inhibitors of this type may be pharmacologically advantageous in vivo, compared with inhibitors that act on the enzyme in the aqueous phase since the degree of inhibition for the latter will depend on the fraction of enzyme bound to the interface.

After taking into account the partial partitioning of BMS-229724 into the bilayer in these in vitro kinetic analyses, the inhibitor was shown to have a dissociation constant 30,000 times lower than that of the arachidonoyl-containing phospholipid substrate. This corresponds to needing only one molecule of BMS-229724 in a sea of over 10,000 arachidonoyl-containing phospholipid molecules to show inhibition. This potency appears to result primarily from the reversible formation of a covalent bond between a serine residue and the carbonyl carbon of the inhibitor (i.e., formation of a hemiketal) as evidenced by the slow-binding, tight-binding inhibition observed with BMS-229724.

Consistent with the role of $cPLA_2$ in the production of arachidonate used for eicosanoid production by cells, BMS-229724 was active in a wide range of cell types in reducing a number of inflammatory mediators, including PGE_2, LTB_4,

and PAF. An inhibitor of $cPLA_2$, therefore, would be expected to have anti-inflammatory efficacy greater than that of cyclooxygenase inhibitors and leukotriene D_4/E_4 antagonists that individually affect only a subset of these lipid mediators.

Of additional interest is the observation that BMS-229724 inhibited the NF-κB-dependent expression of pro-inflammatory cytokines, such as TNFα, IL-1β, IL-8, and IL-6 in human monocytes. Although the exact mechanism is unclear, it appears that $cPLA_2$ regulates NF-κB or AP-1 activation (Camandola et al., 1996; Woo et al., 2000). The results shown here indicate that the role of $cPLA_2$ may be mediated through arachidonate directly rather than the cyclooxygenase- or 5-lipoxygenase-derived metabolites (although arachidonate metabolites derived from routes other these oxygenases cannot be ruled out). Indeed, it has been shown that dual inhibition of the cyclooxygenase and 5-lipoxygenase pathways by tebufelone actually enhanced LPS-stimulated TNFα and IL-1β production in human monocytes (Sirko et al., 1991), presumably due to the build up of arachidonate in these cells in the presence of tebufelone. Inhibition of cytokine production by BMS-229724 suggests that a $cPLA_2$ inhibitor may have a more efficacious and broader anti-inflammatory activity in vivo than the classical nonsteroidal anti-inflammatory drugs, which do not directly affect cytokine production.

The chronic skin inflammation model in mouse ears provided a good opportunity to test for in vivo anti-inflammatory activity with BMS-229724. First, there is evidence that this inflammation is mediated by prostaglandins and leukotrienes (Tramposch et al., 1994). Second, the model allows for compounds to be evaluated for activity against an existing inflammatory lesion since the inflammation is established prior to drug administration. The observation that BMS-229724 displayed topical anti-inflammatory activity in this animal model is particularly relevant, since the model can be considered to be clinically relevant and suitable for selecting useful drug candidates for the treatment of chronic skin diseases, such as psoriasis and atopic dermatitis.

The compound was also quite effective when dosed orally to hairless guinea pigs in a UVB-induced erythema model. Although this erythema does not strictly model any chronic inflammatory skin disease, it is prostaglandin-mediated and would, therefore, be predictive of activity in inflammatory skin disorders, such as psoriasis and atopic dermatitis, where eicosanoids derived from the $cPLA_2$-mediated production of arachidonate may play a role in pathogenesis (Duell et al., 1988; Fogh et al., 1989).

These results together demonstrate that the potent $cPLA_2$ inhibitor BMS-229724 is orally bioavailable and possesses anti-inflammatory activity both topically and orally in skin inflammation models. BMS-229724 is the first demonstration of a $cPLA_2$ inhibitor with anti-inflammatory activity in vivo. We will be performing additional studies to determine whether BMS-229724 is active in other models of inflammatory disorders, such as asthma and arthritis.

Acknowledgments

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Address correspondence to: Dr. James R. Burke, Bristol-Myers Squibb, P.O. Box 4000, Princeton, NJ 08543. E-mail: james.burke@bms.com