Phospholipase C Inhibitors: A New Class of Cytotoxic Agents†

Frank W. Perrella,‡ Shih-Fong Chen,*,‡ Davette L. Behrens,‡ Robert F. Kaltenbach III,§ and Steven P. Seitz§

Cancer Research Group, The DuPont Merck Pharmaceutical Company, Glenolden Laboratory, Glenolden, Pennsylvania 19036, and Experimental Station, Wilmington, Delaware 19898

Received December 15, 1993•

A series of nitrocoumarin and nitrochromene derivatives have been prepared and shown to inhibit the phosphatidylinositol-specific phospholipase C(PLC)(IC_{50} < 10 μg/mL) isolated from human melanoma. The inhibition of PLC by nitrocoumarin **4a** was time-dependent and irreversible. The inhibition of PLC was shown to interfere with inositide metabolism in whole cells (IC_{50} = 4 μg/mL) in a manner consistent with their proposed mode of activity. Finally, the compounds were shown to be growth inhibitory to cultured melanoma cells (ID_{50} = 2 μg/mL), suggesting that PLC may be an attractive new target for chemotherapeutic intervention.

Introduction

Increased metabolism of inositol phospholipids has been observed in response to peptide growth factors.[1] Inositol phospholipid-specific phospholipase C (PLC) catalyzes the hydrolysis of phosphatidylinositols to yield two second-messenger molecules (Figure 1), diacylglycerol (DAG) and inositol phosphates (InPs).[2] It is believed that these second messengers are involved in modulating cell growth.[3] Elevated levels of InPs and DAG have been reported to occur in transformed cell lines.[1,4]

Several forms of phospholipase C are now known.[2,3] These include the isoform types α, β, γ, and δ. These enzymes differ in a number of aspects including size, substrate preference, and tissue distribution. The heterogeneity in the primary sequence of the different PLC isoenzymes suggests that they may be involved in distinct functions within the cell and among different tissues. The elucidation of the role of these enzymes will await the availability of specific inhibitors or genetic engineering approaches. We have been interested in a form of PLC that is present in the human melanoma cell line RPMI 7272.[5] This enzyme has a preference for the substrate phosphatidylinositol in the presence of calcium, has a molecular weight determined by SDS polyacrylamide gel electrophoresis of 150 kDa, and is of the PLC-γ type.

Phospholipase C plays a pivotal role in the transduction of signals of a variety of growth factors, implicating this enzyme in growth-related pathologies such as cancer. Inhibitors of PLC could provide a rational, mechanistic based approach to cancer chemotherapy. A variety of inhibitors have been described[6] with either direct or indirect inhibitory effects on the enzyme PLC or phosphoinositide metabolism. Inhibitors specific for phospholipase C would have potential therapeutic value by shutting down the phosphoinositide signal transduction pathway in cancer cells, which might contribute to tumor cell growth inhibition.

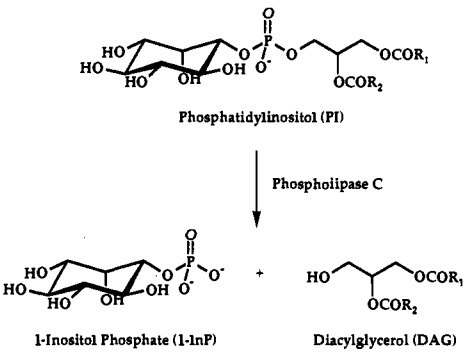
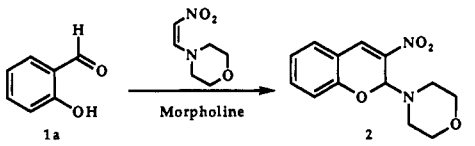


Figure 1. Phospholipase C reaction.

Scheme 1



We describe here the synthesis and biochemical evaluation of novel chromene-based inhibitors of human melanoma phosphatidylinositol-specific PLC. These compounds were evaluated for their inhibitory activity on the enzyme phospholipase C, phosphatidylinositol metabolism, and cell growth in melanoma cells.

Chemistry

Nitrochromene Derivatives. Compound **2** was prepared as described by Rene and Royer[7] (Scheme 1). This material was the common precursor for the derivatives **3a–j** shown in Table 1. Exchange of the morpholine moiety was accomplished by exposure of **2** to an excess of amine in the presence of an acid catalyst (Table 1).

Nitrocoumarin Derivatives. The substituted nitrocoumarins were prepared as shown in Scheme 2. Lewis acid-catalyzed condensation of the appropriately substituted salicylaldehydes **1a–e** with ethyl nitroacetate followed by treatment with *N*-methylmorpholine gave the 3-nitrocoumarin derivatives **4a–e**. The ester-substituted coumarins **5a–j** were prepared by acylation of the hydroxy derivatives **4b–d** using the appropriate acyl chloride and 2,6-lutidine in the presence of a catalytic amount of (dimethylamino)pyridine. The ether derivatives **6a–h**

† Abbreviations: PLC, phosphoinositide phospholipase C; PI, phosphatidylinositol; PIP2, phosphatidylinositol 4,5-bisphosphate; InPs, inositol phosphate(s); SDS, sodium dodecylsulfate; PMSF, phenylmethanesulfonyl fluoride; TCA, trichloroacetic acid; DAG, 1,2-diacylglycerol; IC_{50}, concentration that inhibits by 50%; ID_{50}, dose that inhibits by 50%; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO, dimethyl sulfoxide.

* To whom requests for reprints should be addressed at the Institute for Drug Development, Cancer Therapy and Research Center, 14960 Omicron Dr., San Antonio, TX 78245.

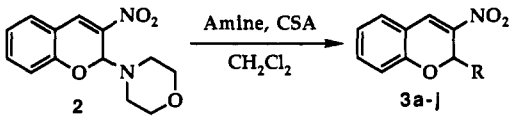
‡ Glenolden Laboratory.

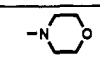
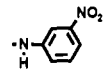
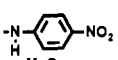
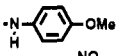
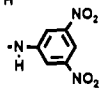
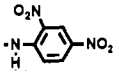
§ Experimental Station.

• Abstract published in *Advance ACS Abstracts*, June 1, 1994.

0022-2623/94/1837-2232$04.50/0 © 1994 American Chemical Society

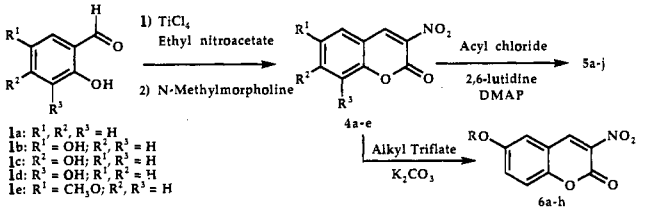
Table 1. Substituted Aminochromenes



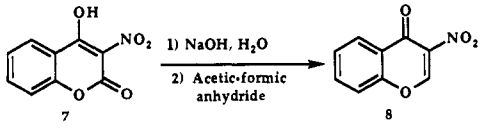
compd[a]	R	yield (%)	Anal.	pK_a[b]	IC_{50} (μg/mL)[c]
2			C,H,N	8.33	5.0 ± 1.7
3a		43	C,H,N	4.4	11.7 ± 0.4
3b		12	C,H,N	−0.26	6.0 ± 0.1
3c		33	C,H,N	2.47	7.5 ± 2.3
3d		39	C,H,N	1.00	14.4 ± 3.1
3e		55	C,H,N	2.52	4.5 ± 0.4
3f		42	C,H,N	4.20	6.6 ± 0.2
3g		24	C,H,N	5.31	12.9 ± 4.0
3h		21	C,H,N	−4.53	inactive[d]
3i		53	C,H,N	0 (est)	inactive
3j		28	C,H,N	2.48	21.0

[a] All compounds have satisfactory IR, NMR, MS, and analytical data. [b] pK_a values are those for the parent amines used in the reaction. [c] The IC_{50} values for PLC inhibition were determined from computer-fitted dose–response curves using the enzyme analysis software EZ-FIT.[16] [d] Inactive represents less than 50% inhibition of PLC at an inhibitor concentration of 80 μg/mL.

Scheme 2



Scheme 3



were obtained by treating **4b** with the appropriate alkyl triflate with K_2CO_3 as a heterogenous acid scavenger. The use of the highly reactive triflate was necessary to obtain adequate yields of the ethers.

The isomeric 3-nitrochromone (**8**) was prepared according to the procedure of Ellis[8] (Scheme 3). Decarbonylation of 4-hydroxy-3-nitrocoumarin (**7**) with aqueous sodium hydroxide followed by condensation with acetic formic anhydride gave the nitrochromone **8** in good yield.

Results and Discussion

Phosphoinositide phospholipase C was partially purified from human melanoma cells grown as solid tumor xenografts in nude mice.[5] A high throughput microtiter plate colorimetric assay with phosphatidylinositol as the substrate was used to evaluate inhibitors of the melanoma PLC. The screening effort uncovered the nitrochromene **2** as an interesting lead compound.

Initial studies on **2** examined the effect of the basicity of the pendant nitrogen on the inhibitory activity of PLC. Protonation of this nitrogen was expected to generate a potent electrophile capable of inactivating this enzyme. As summarized in Table 1, a broad range of amines were surveyed, spanning nearly 13 logs of thermodynamic acidity (pK_a's). The PLC inhibitory activity of this series of compounds was unchanged with the exception of **3h,i**. Compounds **3h,i** were extremely nonbasic compounds and the most substituted examples prepared. It is possible that the highly substituted pendant nitrogen may have sterically hindered these compounds from interacting with the enzyme. These data suggest that protonation on the pendant nitrogen of aminochromene may not be necessary for enzyme inhibition. Since **2** and **3a–j** contain a good electrophile in the nitroalkene functionality, the simplest explanation of these results is that the enzyme is inactivated by Michael addition of an enzyme nucleophile.

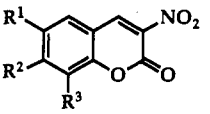
A series of related nitrocoumarins were prepared where the animal functionality of **2** was replaced by a carbonyl group (Table 2). This modification resulted in compounds which were less chemically reactive and more stable in aqueous solution.

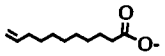
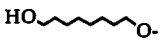
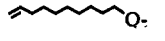
The compound 3-nitrocoumarin (**4a**) produced a dose-dependent inhibition of enzyme activity with a mean IC_{50} of 16 μg/mL. In contrast, the control compound 3-nitrochromenone (**8**) (Scheme 3) with a comparable intrinsically reactive double bond failed to show significant PLC inhibitory activity even up to 100 μg/mL (Figure 2 top). These results suggest that both the position of the carbonyl functionality and the presence of a reactive double bond are important for enzyme inactivation.

The nature of the enzyme inhibition was examined in more detail by preincubation studies with 3-nitrocoumarin (**4a**) and PLC. Irreversible enzyme inhibitors are known to elicit a pattern of inhibition which is time-dependent.[9] As shown in Figure 3, the preincubation of the enzyme at 37 °C with **4a** in the absence of substrate resulted in a progressive loss of PLC activity. In comparison, 3-nitrochromenone (**8**) had no effect on PLC activity over the same time course under conditions known to maintain the stability of the enzyme. The time-dependent loss of enzyme activity by **4a** was believed to be irreversible because extensive dialysis of the inhibited enzyme derived from the preincubation studies failed to restore the catalytic activity (data not shown). These results suggest that **4a** might form a covalent linkage to PLC and thereby inhibit the enzyme irreversibly.

To test whether 3-nitrocoumarin could also interrupt signaling pathways in whole cells, the effect of **4a** was examined on inositide metabolism in cell culture. The incorporation of tritiated inositol into lipids of B16 melanoma cells in culture was studied. The conversion of inositol phosphates back to inositol was blocked in these

Table 2. Substituted 3-Nitrocoumarins



no.[a]	R^1	R^2	R^3	yield (%)	Anal.	IC_{50} (μg/mL)[b]
4a	H	H	H	80	C,H,N	16.1 ± 1.4
4b	OH	H	H	25	C,H,N	10.9 ± 2.1
4c	H	OH	H	17	C,H,N	inactive[d]
4d	H	H	OH	42	C,H,N	24.8 ± 11.2
5a	CH_3CO_2-	H	H	66	C,H,N	16.0 ± 2.6
5b	$C_7H_{15}CO_2$-	H	H	83	C,H,N	4.3 ± 2.1
5c	$C_{15}H_{31}CO_2$-	H	H	61	C,H,N	28.5 ± 5.5
5d		H	H	72	C,H,N	3.2[c]
5e	H	CH_3CO_2-	H	76	C,H,N	inactive
5f	H	$C_7H_{15}CO_2$-	H	81	C,H,N	inactive
5g	H	$C_{15}H_{31}CO_2$-	H	83	C,H,N	inactive
5h	H	H	CH_3CO_2-	88	C,H,N	36.0 ± 5.0
5i	H	H	$C_7H_{15}CO_2$-	96	C,H,N	17.0 ± 6.1
5j	H	H	$C_{15}H_{31}CO_2$-	7	C,H,N	47.0 ± 2.0
4e	CH_3O-	H	H	45	C,H,N	31.0
6a	C_4H_9O-	H	H	49	C,H,N	33.9
6b	$C_6H_{13}O$-	H	H	77	C,H,N	inactive
6c	$C_7H_{17}O$-	H	H	48	C,H,N	inactive
6d	$C_{10}H_{21}O$-	H	H	32	C,H,N	inactive
6e	$C_{12}H_{25}O$-	H	H	43	C,H,N	68.6
6f	*i*-PrO-	H	H	84	C,H,N	23.5
6g		H	H	11	C,H,N	9.6
6h		H	H	60	C,H,N	45.1

[a] All compounds have satisfactory IR, NMR, MS, and analytical data. [b] The IC_{50} values for PLC inhibition were determined from computer-fitted dose–response curves using the enzyme analysis software EZ-FIT.[16] [c] Each datum point is the mean of triplicate determinations from a single representative experiment. [d] Inactive represents less than 50% inhibition of PLC at an inhibitor concentration of 80 μg/mL.

studies by preventing the reincorporation of label into phospholipid membranes using lithium chloride.[10] Under these conditions, inositol phosphates were produced enzymatically by PLC and accumulated in the cells. Figure 2, middle, shows that **4a** completely inhibited cellular phosphoinositide metabolism in a dose-dependent manner; the accumulation of inositol phosphates was inhibited by 100% at 10 μg/mL concentration. An IC_{50} value of 4 μg/mL for the inhibition of inositol phosphate accumulation was comparable to the enzymatic inhibition constant for 3-nitrocoumarin (**4a**). These data provide support that the nitrocoumarin analogs enter cells and interrupt inositol metabolism, possibly through their action on the biological target PLC.

PLC-linked inositol signaling pathways are believed to be involved in mitogenic signaling in cells. The specific inhibition of this enzyme could therefore be growth inhibitory to tumor cells. In this regard, cultured human melanoma cells RPMI 7272 were grown in the presence of increasing concentrations of **4a** and 8. Figure 2, bottom, illustrates that the PLC inhibitor 3-nitrocoumarin (**4a**) was a potent antagonist of cell growth, with an IC_{50} value of approximately 2 μg/mL. Consistent with the previous results, 3-nitrochromenone (8) had little effect on cell growth in this assay system.

It is known that other lipid-utilizing enzymes such as phospholipase A_2 have hydrophobic clefts for interacting with fatty acyl side chains.[11] We therefore considered that the initial binding of **4a** to PLC might be improved by the inclusion of a hydrocarbon pharmacophore to 3-nitrocoumarin so that it more closely resembles the PLC substrate phosphatidylinositol. Alkoxy and carboalkoxy chains were appended to the 3-nitrocoumarin nucleus at the 6-, 7-, and 8-positions. The 6-position was the most tolerant of substitution. Attachment of octanoyl (**5b**) and 10-undecenoyl (**5d**) groups resulted in improved inhibitors. Substitution was not tolerated at the 7-position (**5e–g**), while the 8-isomers were of slightly diminished activity. The poor solubility of analogs **5c,g,j** which were substituted with long-chain fatty acids may contribute to their reduced potency.

Ether substitutions to the 6-position of the 3-nitrocoumarin nucleus were prepared to prevent hydrolysis of the linkages by esterases *in vivo*. Of the ether analogs, the hydroxyl-substituted (8-hydroxyoctanoxy)-3-nitrocoumarin **6g** was the most active inhibitor of the enzyme with an IC_{50} value of 9.6 μg/mL. The attachment of an 9-decenoxy group (**6h**) was less effective in inhibiting the activity of PLC than the alcohol-substituted 3-nitrocoumarin **6g**. Ether linkages of 6, 8, and 10 carbons substituted to 3-nitrocoumarin (**6b–d**) were inactive. The SAR studies of 3-nitrocoumarin analogs suggest that a 6-position substitution with an ester of 8 (**5b**)–10 carbons (**5d**) was optimal for PLC inhibition.

We have described a series of nitrocoumarin and aminochromene inhibitors of the enzyme PLC. The compound 3-nitrocoumarin (**4a**) inhibits PLC activity in a time-dependent and irreversible fashion. Additionally, the cellular metabolism of phosphoinositides is inhibited by **4a** in a manner predicted for the subcellular inhibition of PLC activity. An inhibitor that interrupts the cellular mitogenic signals mediated through PLC and the phosphatidylinositol pathway might be expected to inhibit the growth of tumor cells. Therefore, the inhibition of melanoma cell growth by **4a** may be due to the inhibition of PLC and phosphoinositide metabolism in these cells.

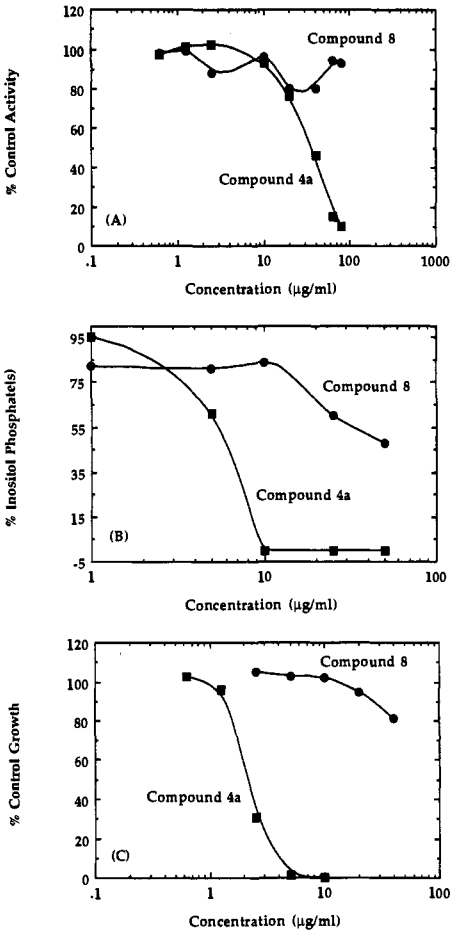


Figure 2. Specificity of 3-nitrocoumarin (**4a**) for phospholipase C (top), inositol metabolism (middle), and cell growth inhibition (bottom). The indicated compounds were incubated with melanoma (top) PLC and assayed for 15 min in the presence of 1 mM phosphatidylinositol to determine the enzyme inhibitory activity and cells in culture for (middle) 30 min to determine their effects on inositol metabolism and (bottom) 72 h to determine their growth inhibitory activity using the MTT assay. All methods were as described under the Experimental Section. The data are presented as the mean of quadruplicate determinations; the standard error is no greater than ±10%.

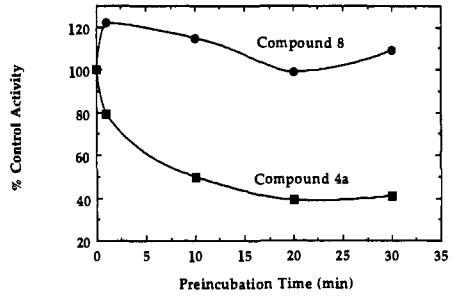


Figure 3. Time-dependent inhibition of PLC by 3-nitrocoumarin (**4a**) and 3-nitrochromone (**8**). The compounds were preincubated with PLC at 30 μg/mL for the indicated periods of time, the substrate phosphatidylinositol was added (1 mM), and the reaction was terminated 15 min later. The enzyme assay conditions were as described under the Experimental Section.

The further development of inhibitors of PLC may have potential therapeutic value as anticancer agents by down-modulating a proliferative pathway in tumor cells and thus inhibiting tumor growth and metastasis.

Experimental Section

Chemistry. All reactions were run under a dry nitrogen atmosphere with appropriate stirring unless otherwise specified. Column chromatography was performed with E. Merck silica gel 60 (230–400 mesh) under low-pressure nitrogen. Melting points were determined with a Thomas-Hoover melting point apparatus and are uncorrected. NMR spectra were obtained in $CDCl_3$ (unless stated otherwise) by using a Varian VXR 300S, 300-MHz spectrometer with chemical shifts recorded as parts per million (ppm) downfield from tetramethylsilane. Infrared spectra were recorded on a Perkin-Elmer 1600 series FTIR. Chemical ionization MS spectra were recorded with a Hewlett-Packard 5988A mass spectrometer. Elemental analyses were performed by Quantitative Technologies, Inc., Bound Brook, NJ, and agree to within 0.4% of the calculated values. Yields were not optimized.

[*N*,*N*-(3-Oxapentanediyl)amino]-3-nitro-2*H*-chromene (2). This compound was synthesized according to the procedure of Rene and Royer:[7] mp 133–134 °C (lit. mp 134 °C); ^{1}H NMR δ 8.09 (1H, s, H4), 7.45–7.26 (2H, m, aromatic), 7.05 (2H, m, aromatic), 6.21 (1H, s, H2), 3.58 (4H, m, CH_2), 2.86 (2H, m, CH_2), 2.60 (2H, m, CH_2); IR (KBr) ν 2955, 2855, 2834, 1654, 1603, 1514, 1454, 1328 cm^{-1}; MS (CI, CH_4) *m/e* 263 (M^+). Anal. ($C_{13}H_{14}N_2O_4$) C,H,N.

General Procedure for the Synthesis of Aminochromenes 3a–j: 2-(Phenylamino)-3-nitro-2*H*-chromene (3a). To a solution of 1.0 g (3.8 mmol) of aminochromene **2** in 20 mL of methylene chloride were added 0.69 mL (7.6 mmol) of aniline and 180 mg (0.76 mmol) of (+)-10-camphorsulfonic acid. After the mixture was stirred overnight, the solvent was removed under reduced pressure and the residue was chromatographed on silica gel (elution with methylene chloride). Repurification (silica gel, elution with 20% ether:hexanes) gave 440 mg (43%) of **3a** as a yellow solid: mp 153–154 °C; ^{1}H NMR δ 8.06 (1H, s, H4), 7.45–6.83 (9H, m, aromatic), 4.55 (1H, d, J = 9.6 Hz, NH); IR (KBr) ν 3401, 1642, 1605, 1531, 1499, 1336, 1321 cm^{-1}; HRMS *m/e* 268.0848 (M^+). Anal. ($C_{15}H_{12}N_2O_3$) C,H,N.

General Procedure for the Synthesis of Nitrocoumarins 4a–e: 3-Nitrocoumarin (4a). To a solution of 13.3 mL of titanium tetrachloride (121 mmol) in 240 mL of methylene chloride at 0 °C was added 6.5 mL (61 mmol) of salicylaldehyde (**1a**) in 40 mL of tetrahydrofuran over 15 min. The resulting red solution was stirred at 0 °C for 15 min followed by the addition of 7.4 mL (64 mmol) of ethyl nitroacetate in 40 mL of tetrahydrofuran dropwise. After 2 h, 27 mL (243 mmol) of *N*-methylmorpholine was added dropwise. The resulting slurry was stirred overnight at room temperature followed by the addition of an equal amount of water. The mixture was extracted with methylene chloride, and the combined organic layers were washed with water and dried over anhydrous $MgSO_4$. The solvent was removed under reduced pressure, and the residue was purified by recrystallization from methylene chloride followed by recrystallization in toluene to give 9.3 g (80%) of the coumarin as a yellow solid: mp 141–142 °C (lit.[12] mp 143–144 °C); ^{1}H NMR δ 8.75 (1H, s, H4), 7.83–7.71 (2H, m, aromatic), 7.47 (2H, m, aromatic); IR (KBr) ν 1749, 1608, 1562, 1523, 1350 cm^{-1}; MS (CI, CH_4) *m/e* 192 (M^+). Anal. ($C_9H_5NO_4$) C,H,N.

6-Hydroxy-3-nitrocoumarin (4b). To a solution of 100 mL of titanium tetrachloride (600 mmol) in methylene chloride was added 2.9 g (21 mmol) of 5-hydroxysalicylaldehyde (**1b**) in 20 mL of tetrahydrofuran over 20 min. The resulting red solution was stirred 10 min followed by the addition of 2.56 mL (22 mmol) of ethyl nitroacetate at 0 °C. After 2.5 h, 9.2 mL (84 mmol) of *N*-methylmorpholine was added dropwise. The resulting slurry was stirred overnight at room temperature followed by the addition of an equal amount of water. The mixture was extracted with a 5% solution of methanol in methylene chloride, and the combined organic layers were washed with water and dried over anhydrous $MgSO_4$. The solvent was removed under reduced pressure, and the residue was triturated with methylene chloride to give 1.1 g (25%) of the coumarin as a yellow solid: mp 215–218 °C dec; ^{1}H NMR (CD_3OD) δ 8.89 (1H, s, H4), 7.33–7.18 (3H, m, aromatic); IR (KBr) ν 3249, 1727, 1706, 1574, 1447 cm^{-1}; MS (CI, CH_4) *m/e* 208 (M^+). Anal. ($C_9H_5NO_5$) C,H,N.

General Procedure for the Synthesis of Esters 5a–j: 6-Acetoxy-3-nitrocoumarin (5a). To a stirred solution of 150 mg (0.72 mmol) of nitrocoumarin **4b** in 5 mL of tetrahydrofuran were added 0.25 mL (2.17 mmol) of 2,6-lutidine, 77 mL (1.09 mmol) of acetyl chloride, and 25 mg of 4-(dimethyl-

amino)pyridine. After stirring 3 h, the mixture was diluted with water and extracted with methylene chloride, and the combined organic layers were dried over anhydrous $MgSO_4$. The solvent was removed under reduced pressure, and the residue was chromatographed on silica gel. Elution with methylene chloride gave 119 mg (66%) of the ester as a yellow powder: mp 137–139 °C; ^{1}H NMR δ 8.66 (1H, s, H4), 7.50 (3H, m, aromatic), 2.37 (3H, s, CH_3CO_2); IR (KBr) ν 1758, 1575, 1543, 1367, 1203 cm^{-1}; MS (CI, CH_4) *m/e* 250 (M^+). Anal. ($C_{11}H_7NO_6$) C,H,N.

General Procedure for the Synthesis of Ethers 6a–h: 6-(Hexyloxy)-3-nitrocoumarin (6b). To a solution of 0.56 mL (4.46 mmol) of hexyl alcohol and 0.36 mL (4.46 mmol) of pyridine at 0 °C was added 0.75 mL (4.46 mmol) of triflic anhydride dropwise. After stirring 1 h, the suspension was rapidly filtered through silica gel with methylene chloride, and the solvent was removed under reduced pressure. The resulting triflate was diluted with 3 mL of methylene chloride, and 100 mg (0.48 mol) of nitrocoumarin **4b** and 500 mg of K_2CO_3 was added. After stirring 18 h, the suspension was chromatographed on silica gel. Elution with methylene chloride gave 107 mg (77%) of the ether as a yellow solid; mp 90–93 °C; ^{1}H NMR δ 8.68 (1H, s, H4), 7.35 (2H, m, aromatic), 7.07 (1H, d, J = 1.1 Hz, aromatic), 4.01 (2H, t, J = 6.2 Hz, CH_2O), 1.83 (2H, p, J = 6.6 Hz, CH_2), 1.47 (2H, m, CH_2), 1.36 (4H, m, CH_2), 0.92 (3H, t, J = 7.0 Hz, CH_3); IR (KBr) ν 2952, 1758, 1742, 1575, 1530, 1492, 1265 cm^{-1}; MS (CI, CH_4) *m/e* 292 (M^+). Anal. ($C_{15}H_{17}NO_5$) C,H,N.

3-Nitrochromen-4-one (8). This compound was synthesized according to the procedure of Ellis:[8] mp 149–151 °C (lit. mp 151–152 °C; ^{1}H NMR δ 9.07 (1H, s, H2), 8.37 (1H, dd, J = 8.1, 1.9 Hz, aromatic), 7.80 (1H, m, aromatic), 7.54 (2H, m, aromatic); IR (KBr) ν 1676, 1620, 1525, 1461, 1357 cm^{-1}; MS (CI, CH_4) *m/e* 192 (M^+). Anal. ($C_9H_5NO_4$) C,H,N.

Partial Purification of Human Melanoma Phospholipase C. Phosphatidylinositol was purchased from Avanti Polar Lipids Inc., Birmingham, AL. Alkaline phosphatase (bovine intestinal mucosa, type VII-T) and other chemicals were from Sigma Chemical Co., St. Louis, MO.

Mice for xenografts were 6-week-old Nu/Nu Swiss mice weighing from 24 to 30 g. Animals were maintained in a regulated lighting cycle of 12 h of light and 12 h of dark. The human melanoma cell line RPMI 7272 was introduced by sc implantation of cultured tumor cells in nude mice. The mice were sacrificed by cervical dislocation, and the tumors (weighing 1–2 g wet weight) were rapidly removed and frozen at −70 °C in hexane on solid CO_2. Frozen tumors were thawed, excess fat was removed, and they were placed in 6.5 volumes of homogenization buffer A (10 mM imidazole-HCl, 10 mM EDTA, 0.25 M sucrose, pH 7.2) containing the protease inhibitors 1 mM phenylmethanesulfonyl fluoride (PMSF) and 1 μM leupeptin. The tissue was homogenized on ice by mechanical disruption and sonication using a Tekmar ultrasonic homogenizer for 30 s at 20 000 rpm. Large tissue debris was removed from the homogenate by low-speed centrifugation at 250*g* for 20 min at 4 °C. The sediment (pellet) was washed once with 6.5 volumes of buffer A and centrifuged again at 250*g* for 20 min. The two supernatant fractions were pooled and centrifuged at 200000*g* for 1 h at 4 °C. The fatty layer was removed using a cotton swab, and the supernatant fraction was dialyzed twice against 2 L of buffer B (10 mM imidazole-HCl, 1 mM EGTA, 1 mM dithiothreitol, 5% glycerol, 5% ethylene glycol, 0.005% Triton X-100, pH 7.2) containing 1 mM PMSF and 1 μM leupeptin overnight using dialysis tubing with a molecular weight cutoff of 25 k Da (Spectra/Por 7; Spectrum Medical Industries, Los Angeles). The dialyzed supernatant fraction is referred to as the cytosolic fraction, and PLC was purified further as described previously[5] and as outlined below. The cytosolic fraction was loaded directly on a fast Q-Sepharose (Pharmacia-LKB) column equilibrated with buffer B, and the enzyme was eluted with a gradient of NaCl. The column fractions representing the first peak of activity were pooled and dialyzed against 2 L of buffer B. The dialyzed activity was loaded onto a heparin-Sepharose (Pharmacia-LKB) column equilibrated with buffer B, and the enzyme was eluted with a gradient of NaCl. The PLC activity eluting at a NaCl concentration of 0.35 M was dialyzed against buffer B (without EGTA) and used as the source of enzyme for the inhibition studies.

Microtiter Plate Phospholipase C Assay. PLC activity was measured by determining the formation of inositol 1-phosphate from the substrate phosphatidylinositol using a modification and automation of the method of Palmer.[13] All concentrations are final assay values unless stated otherwise. The assay was prepared in 96-well microtiter plates using a mixture of 25 mM imidazole, pH 7.2, 100 mM KCl, 1 mM $CaCl_2$, 0.8 mg/mL deoxycholate, test compound, 1 mM phosphatidylinositol, and 20–30 μg of PLC protein fraction in a final volume of 100 μL. Phosphatidylinositol was prepared as a 10 mM stock solution in deionized water by brief sonication for 30 s. The enzyme was preincubated with the test compound for 5 min at 37 °C before initiating the assay with the substrate. The enzyme reaction was started by adding 1 mM PI and incubating for 15 min at 37 °C. The calcium-dependent PLC reaction was stopped by the addition of 9 mM EDTA, pH 7.0. Alkaline phosphatase (10 units) was added and the mixture incubated for an additional 30 min (37 °C) to hydrolyze the product of the reaction (inositol 1-phosphate) to inorganic phosphate and inositol. The phosphatase reaction was stopped by adding 3.7% SDS and 29 mM EDTA (pH 4.0) to the assay mixture. Inorganic phosphate was quantitated by adding 36.5 mM $ZnCl_2$ and 5.5 mM ammonium molybdate. The mixture was incubated for 20 min at 37 °C before the absorbance was read spectrophotometrically at 360 nm in a Titertek Multiskan (Flow Labs) microtiter plate reader connected to an IBM PC.

The rate of PI hydrolysis was linear at protein concentrations up to 30 μg per assay volume. Approximately 25% of the phospholipid substrate was hydrolyzed during the first 30 min of the reaction. PI hydrolysis was approximately constant for the first 30 min of incubation and leveled off by 60 min. PLC activity was determined routinely for 15 min at 30 μg of protein, producing a specific activity of about 0.2 μmol/min/mg of protein.

MTT Cell Growth Assay. The MTT assay[14] for growth inhibition of cells was used to assess the growth inhibitory effects of compounds. Human melanoma cells (RPMI 7272) were grown in 96-well microtiter plates (Costar, flat-bottom) seeded at a cell density of 2000 cells/well in 0.1 mL of RPMI 1640 medium plus 10% fetal bovine serum. After 24 h at 37 °C, the test compounds were added in 0.1 mL of medium plus DMSO solution. The final DMSO concentration in the medium was 0.2%. The cells were cultured for another 72 h, after which half of the medium was removed and 0.05 mL of MTT solution (1 mg/mL in PBS) was added to each well. The plates were shaken and incubated for 4 h at 37 °C. At the end of the incubation period, the medium and MTT solution were removed from the adherent cells by inverting and blotting the plates. Acidic isopropyl alcohol (0.04 N HCl), 0.1 mL, was added to each well and mixed by aspirating up and down with a pipette to dissolve the blue formazan crystals. The optical density of each well was measured using an automated multiwell plate spectrophotometer (Titertek Multiskan; Flow Labs) at a 570-nm test wavelength and a 630-nm reference wavelength.

Cellular Phosphatidylinositol Metabolism. A single cell suspension of B16 melanoma cells was plated at 1 × 10^6 cells/60-mm culture plates in RPMI 1640 medium supplemented with 10% fetal bovine serum and incubated at 37 °C for 24 h to allow for cell attachment. Monolayer cells were rinsed with M199 medium, and 4 mL of M199 medium containing 10% dialyzed fetal bovine serum and 1.25 μCi/mL of myo-[2-^{3}H]inositol (DuPont New England Nuclear Res. Prod.) was added to the plates. The plates were incubated at 37 °C for 24 h to allow the myo-[2-^{3}H]inositol to be incorporated into phospholipids. To determine the effect of inhibitors of phospholipase C on PI turnover, 1 mL of M199 medium containing 62.5 mM LiCl with or without test compounds was added to the cells, and they were incubated at 37 °C for 30 min. The incubation was terminated by removing the medium and rinsing the monolayers with cold saline. A modification of the trichloroacetic acid (TCA) precipitation procedure described by Davis[15] was followed in which the cells were precipitated with 0.5 mL of cold TCA. The precipitated cells were scraped with a rubber policeman and transferrred to a microfuge tube. The plate was rinsed with 0.5 mL of TCA three times, and the TCA washes were combined in the same microcentrifuge tube. The TCA suspensions were centrifuged for 12 min at 10000*g* (4 °C).

The TCA soluble fraction was carefully removed from the pellet

and placed in a glass tube. Two milliliters of H_2O-saturated ether was added to each tube to remove the TCA. The samples were vortexed and centrifuged and the upper ether layer removed by aspiration. The ether extraction was repeated three times. The aqueous layer was neutralized with 0.2 mL of 500 mM potassium phosphate buffer (pH 7.4) and 6 μL of 0.5 N KOH. The total radioactivity in the TCA soluble fraction was determined by adding ACS (Amersham) for liquid scintillation analysis.

References

(1) (a) Margolis, B.; Zilberstein, A.; Franks, C.; Felder, S.; Kremer, S.; Ullrich, A.; Rhee, S. G.; Skorecki, K.; Schlessinger, J. Effect of Phospholipase C-γ Overexpression on PDGF-Induced Second Messengers and Mitogenesis. *Science* **1990**, *248*, 607–610. (b) Williams, L. T. Signal Transduction by the Platelet-Derived Growth Factor Receptor. *Science* **1989**, *243*, 1564–1570. (c) Gilligan, A.; Prentki, M.; Glennon, M. C.; Knowles, B. B. Epidermal Growth Factor-Induced Increases in Inositol Trisphosphates, Inositol Tetrakisphosphates and Cytosolic Calcium in a Human Hepatocellular Carcinoma-Derived Cell Line. *FEBS Lett.* **1988**, *233*, 41–46.

(2) Rhee, S. G.; Suh, P.-G.; Ryu, S.-H.; Lee, S. Y. Studies of Inositol Phospholipid-Specific Phospholipase C. *Science* **1989**, *244*, 546–550.

(3) (a) Dennis, E. A.; Rhee, S. G.; Billah, M. M.; Hannun, Y. A. Role of Phospholipases in Generating Lipid Second Messengers in Signal Transduction. *FASEB J.* **1991**, *5*, 2068–2077. (b) Fain, J. N. Regulation of Phosphoinositide-Specific Phospholipase C. *Biochim. Biophys. Acta* **1990**, *1053*, 81–88.

(4) Smith, M. R.; Liu, Y.-L.; Kim, H.; Rhee, S. G.; Kung, H.-F. Inhibition of Serum- and Ras-Stimulated DNA Synthesis by Antibodies to Phospholipase C. *Science* **1990**, *247*, 1074–1077.

(5) (a) Perrella, F. W. Characterization of Phosphatidylinositol Phospholipase C Activity in Human Melanoma. *Biochem. Biophys. Res. Commun.* **1990**, *166*, 715–722. (b) Perrella, F. W.; Jankewicz, R.; Dandrow, E. A. Phospholipase C from Human Melanoma: Purification and Characterization of a Phosphatidylinositol-Selective Enzyme. *Biochim. Biophys. Acta* **1991**, *1076*, 209–214.

(6) (a) Yamada, K.; Kumada, Y.; Kubo, K. KF4939, a New Anti-platelet Agent, Inhibits Activation of Phospholipase C and A_2 in Rabbit Platelets. *Jpn. J. Pharmacol.* **1985**, *39*, 108–111. (b) Clark, M. T.; Chang, J.; Navran, S. S.; Huzoor-Akbar; Mukhopadhyay, A.; Amin, H.; Fuller, D. R.; Miller, D. D. Synthesis and Investigation of the β-Adreno-receptor Agonist and Platelet Antiaggregatory Properties of 1,7,8-Trisubstituted 2,3,4,5-Tetrahydro-1H-2-benzazepine Analogues of Trimetoquinol. *J. Med. Chem.* **1986**, *29*, 181–185. (c) Bronner, C.; Wiggins, C.; Monte', D.; Marki, F.; Capron, A.; Landry, Y.; Franson, R. C. Compound 48/80 is a Potent Inhibitor of Phospholipase C and a Dual Modulator of Phospholipase A_2 from Human Platelet. *Biochim. Biophys. Acta* **1987**, *920*, 301–305. (d) Imoto, M.; Yamashita, T.; Sawa, T.; Kurasawa, S.; Naganawa, H.; Takeuchi, T.; Bao-quan, Z.; Umezawa, K. Inhibition of Cellular Phosphatidylinositol Turnover by psi-Tectorigenin. *FEBS Lett.* **1988**, *230*, 43–46. (e) Bennett, C. F.; Mong, S.; Wu, H.-L. W.; Clark, M. A.; Wheeler, L.; Crooke, S. T. Inhibition of Phosphoinositide-Specific Phospholipase C by Manoalide. *Mol. Pharmacol.* **1987**, *32*, 587–593. (f) Yang, S. S.; Beattie, T. R.; Durette, P. L.; Gallagher, T. F.; Shen, T.-Y. Phosphatidyl Inositol Analogs Useful as Anti-Inflammatory/Analgesic Agents. US Patent 4,515,722, May 7, 1985. (g) Powis, G.; Seewald, M. J.; Gratas, C.; Melder, D.; Riebow, J.; Modest, E. J. Selective Inhibition of Phosphatidylinositol Phospholipase C by Cytotoxic Ether Lipid Analogues. *Cancer Res.* **1992**, *52*, 2835–2840. (h) Kohn, E. C.; Sandeen, M. A.; Liotta, L. A. In Vivo Efficacy of a Novel Inhibitor of Selected Signal Transduction Pathways Including Calcium, Arachidonate, and Inositol Phosphates. *Cancer Res.* **1992**, *52*, 3208–3212.

(7) Rene, L.; Royer, R. Recherches sur les Derives Nitres d'Interet Biologique. XXV.-Synthese et Essais Preliminaires de Dialcoylamino-2 nitro-3-2H-chromenes. (Research on Nitro-Derivatives of Biological Interest. XXV. Synthesis and Preliminary Study of 2-Dialkylamino-3-nitro-2H-chromenes.) *Eur. J. Med. Chem.* **1982**, *17*, 89–90.

(8) Becket, G. J. P.; Ellis, G. P. Benzopyrones. Part XII. Novel Synthesis of some 3-Substituted Chromones. *Tetrahedron Lett.* **1976**, *9*, 719–720.

(9) Silverman, R. B. *Mechanism-Based Enzyme Inactivation: Chemistry and Enzymology*; CRC Press: Boca Raton, FL, 1988; Vol. I, pp 3–22.

(10) (a) Hallcher, L. M.; Sherman, W. R. The Effects of Lithium Ion and Other Agents on the Activity of Myo-inositol-1-phosphatase from Bovine Brain. *J. Biol. Chem.* **1980**, *255*, 10896-10901. (b) Sherman, W. R.; Leavitt, A. L.; Honchar, M. P.; Hallcher, L. M.; Phillip, B. E. Evidence that Lithium Alters Phosphoinositide Metabolism: Chronic Administration Elevates Primary D-myo-inositol-1-phosphate in Cerebral Cortex of the Rat. *J. Neurochem.* **1981**, *36*, 1947–1951.

(11) (a) White, S. P.; Scott, D. L.; Otwinowski, Z.; Gelb, M. H.; Sigler, P. B. Crystal Structure of Cobra-Venom Phospholipase A_2 in a Complex with a Transition-State Analogue. *Science* **1990**, *250*, 1560–1563. (b) Scott, D. L.; Otwinoswki, Z.; Gelb, M. H.; Sigler, P. B. Crystal Structure of Bee-Venom Phospholipase A_2 in a Complex with a Transition-State Analogue. *Science* **1990**, *250*, 1563–1566. (c) Scott, D. L.; White, S. P.; Browning, J. L.; Rosa, J. J.; Gelb, M. H.; Sigler, P. B. Structures of Free and Inhibited Human Secretory Phospholipase A_2 from Inflammatory Exudate. *Science* **1991**, *254*, 1007–1010.

(12) Dauzonne, D.; Royer, R. A Convenient One-Flask Synthesis of 3-Nitrocoumarins. *Synthesis* **1983**, 836–837.

(13) Palmer, F. B. S. C. An Enzymatically Coupled Assay for Rat Brain Polyphosphoinositide Phosphodiesterase in an Optimized Reaction Mixture. *Anal. Biochem.* **1985**, *150*, 345–352.

(14) (a) Mosmann, T. Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assays. *J. Immunol. Methods* **1983**, *65*, 55–63. (b) Denizot, F.; Lang, R. Rapid Colorimetric Assay for Cell Growth and Survival. Modifications to the Tetrazolium Dye Procedure Giving Improved Sensitivity and Reliability. *J. Immunol. Methods* **1986**, *89*, 271–277.

(15) Davis, J. S.; Weakland, L. L.; West, L. A.; Farese, R. V. Luteinizing Hormone Stimulates the Formation of Inositol Triphosphate and Cyclic AMP in Rat Granulosa Cells. *Biochem. J.* **1986**, *238*, 597–604.

(16) Perrella Scientific, Inc., P.O. Box 80593, Conyers, GA 30208. Perrella, F. W. EZ-Fit: A Practical Microcomputer Program for the Analysis of Enzyme Kinetic Data on IBM-PC Compatible Computers. *Anal. Biochem.* **1988**, *174*, 437–447.