Design of Potent and Selective Inhibitors of *myo*-Inositol 1,4,5-Trisphosphate 5-Phosphatase†

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ABSTRACT: The interactions of synthetic analogues of D-*myo*-inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] with the Ins(1,4,5)P_3 receptor in permeabilized SH-SY5Y cells and with two key metabolic enzymes, Ins(1,4,5)P_3 3-kinase from a supernatant preparation of rat brain homogenates and Ins(1,4,5)P_3 5-phosphatase from human erythrocyte ghosts, have been examined. L-*chiro*-Inositol 2,3,5-trisphosphorothioate [L-*chiro*-Ins(2,3,5)PS_3], which we have previously identified as a partial agonist at the Ins(1,4,5)P_3 receptor [Safrany, S. T., Wilcox, R. A., Liu, C., Dubreuil, D., Potter, B. V. L., & Nahorski S. R. (1993) *Mol. Pharmacol. 43,* 499–503], is identified as the most potent 5-phosphatase inhibitor yet described {inhibiting dephosphorylation of [^{3}H]Ins(1,4,5)P_3 with K_i = 230nM}. L-*chiro*-Ins(2,3,5)PS_3 was also found to be the most potent small-molecule inhibitor of 3-kinase (K_i = 820 nM). The properties of three novel, potent, and *selective* inhibitors of 5-phosphatase are described. L-*myo*-Inositol 1,4,5-trisphosphorothioate inhibited 5-phosphatase with K_i = 430 nM, showing 250-fold selectivity over 3-kinase (K_i = 108 μM); *myo*-inositol 1,3,5-trisphosphorothioate inhibited 5-phosphatase with 475-fold selectivity over 3-kinase (K_i = 520 nM and 247 μM, respectively). The most potent, selective inhibitor of 5-phosphatase was L-*chiro*-inositol 1,4,6-trisphosphorothioate [L-*chiro*-Ins(1,4,6)PS_3]. L-*chiro*-Ins(1,4,6)PS_3 inhibited 5-phosphatase with K_i = 300 nM and did not interact with the Ins(1,4,5)P_3 receptor or 3-kinase at doses tested. These studies, therefore, identify a highly potent and selective inhibitor of 5-phosphatase, which should be considered the tool of choice when inhibiting this enzyme in a broken cell or cell-free system.

D-*myo*-Inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3][1] (**1**, Figure 1) is a second messenger which mediates the release of Ca^{2+} from nonmitochondrial intracellular stores (Berridge, 1993), via a receptor which has been purified, cloned, and sequenced (Ferris & Snyder, 1992a,b). Because of the fundamental importance of the phosphoinositide signaling system in cell biology, it is desirable to have access to pharmacological tools which can facilitate intervention at the Ins(1,4,5)P_3 receptor and inhibit the metabolic enzymes acting upon this second messenger. Ins(1,4,5)P_3 antagonists and compounds which block the polyphosphoinositide pathway may have a therapeutic role as drugs, provided they can gain access to the interior of cells. Heparin (Ghosh *et al.*, 1988) and decavanadate (Strupish *et al.*, 1991) have been found to act as Ins(1,4,5)P_3 receptor antagonists, but few such tools have yet been identified, and there are certain difficulties intrinsic to a drug design strategy based upon Ins(1,4,5)P_3, the most prominent being those of cell selectivity and membrane permeability.

Initial synthetic approaches to inositol polyphosphates addressed the central problems of selective hydroxyl group protection, phosphorylation, optical resolution, and deblocking (Billington, 1989, 1993; Potter, 1990). Now that these problems have been resolved, the rational design and chemical synthesis of many inositol polyphosphate analogues can be envisaged (Potter, 1993). Nevertheless, the chemistry remains complex and relatively few reports on structurally modified compounds possessing useful biological activity have been published.

Inositol ring-modified and phosphate-modified analogues have been synthesized, and some progress has been made to understand the role of the three phosphate and hydroxyl groups

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[1] Abbreviations: 2,3-BPG, D-2,3-bisphosphoglycerate; EC_{50}, concentration of agent producing 50% of maximal response; EDTA, ethylene diaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IC_{50}, concentration of agent inhibiting a response by 50%; K_i, dissociation constant for an inhibitor–enzyme complex; K_m, Michaelis–Menten constant; PtdIns, phosphatidylinositol; PtdIns(4)P, phosphatidylinositol 4-monophosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; Tris, tris(hydroxymethyl)aminomethane; V_{max}, maximum velocity of an enzyme-catalyzed reaction; Ins(1,4,5)P_3, D-*myo*-inositol 1,4,5-trisphosphate; L-Ins(1,4,5)P_3, L-*myo*-inositol 1,4,5-trisphosphate; Ins(1,4,5)PS_3, *myo*-inositol 1,4,5-trisphosphorothioate; Ins-(1,4,5)P_3-5S, *myo*-inositol 1,4-bisphosphate 5-phosphorothioate; Ins-(1,4,5)P_3-4,5S_2, *myo*-inositol 1-phosphate 4,5-bisphosphorothioate; Ins-(1,4,5)P_3(PP), *myo*-inositol 1-phosphate 4,5-pyrophosphate; Ins(1,3,5)-P_3, *myo*-inositol 1,3,5-trisphosphate; Ins(1,3,5)PS_3, *myo*-inositol 1,3,5-trisphosphorothioate; 6-deoxy-Ins(1,4,5)P_3, 6-deoxy-*myo*-inositol 1,4,5-trisphosphate; 6-deoxy-Ins(1,4,5)PS_3, 6-deoxy-*myo*-inositol 1,4,5-trisphosphorothioate; Ins(1,3,4,5)P_4, D-*myo*-inositol 1,3,4,5-tetrakisphosphate; Ins(1,3,4,6)P_4, *myo*-inositol 1,3,4,6-tetrakisphosphate; Ins(1,3,4,6)PS_4, *myo*-inositol 1,3,4,6-tetrakisphosphorothioate; Ins(1,2,4,5)P_4, *myo*-inositol 1,2,4,5-tetrakisphosphate; 2,5-diMe-Ins(1,3,4,6)P_4, 2,5-di-*O*-methyl-*myo*-inositol 1,3,4,6-tetrakisphosphate; 2,5-diMe-Ins(1,3,4,6)PS_4, 2,5-di-*O*-methyl-*myo*-inositol 1,3,4,6-tetrakisphosphorothioate; 3,6-diBz-Ins-(1,2,4,5)P_4, 3,6-di-*O*-benzoyl-*myo*-inositol 1,2,4,5-tetrakisphosphate; L-*chiro*-Ins(1,4,6)P_3, L-*chiro*-inositol 1,4,6-trisphosphate; L-*chiro*-Ins-(1,4,6)PS_3, L-*chiro*-inositol 1,4,6-trisphosphorothioate; L-*chiro*-Ins(2,3,5)-P_3, L-*chiro*-inositol 2,3,5-trisphosphate; L-*chiro*-Ins(2,3,5)PS_3, L-*chiro*-inositol 2,3,5-trisphosphorothioate.

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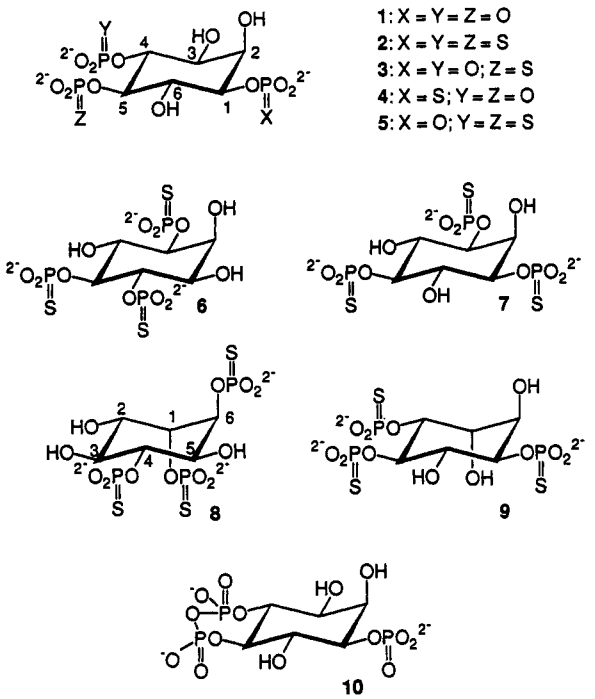

FIGURE 1: Structures of Ins(1,4,5)P_3 (**1**), phosphorothioate analogues (**2–5**), submicromolar inhibitors of 5-phosphatase (**6–9**), and Ins(1,4,5)P_3(PP) (**10**).

of Ins(1,4,5)P_3 in receptor binding and enzyme recognition. Advances in inositol phosphate chemistry (Billington, 1989, 1993; Potter, 1990, 1993) and the molecular recognition of inositol polyphosphates by receptors and metabolic enzymes have been reviewed (Taylor & Richardson, 1991; Potter & Nahorski, 1992). As part of an ongoing program aimed to study structure–activity relationships in inositol tris- and tetrakisphosphates, we have been engaged in the synthesis of inositol polyphosphates and their analogues. Our synthesis of DL-*myo*-inositol 1,4,5-trisphosphorothioate [DL-Ins(1,4,5)-PS_3] (**2**, Figure 1) provided an analogue that releases Ca^{2+} potently and is resistant to metabolism by either Ins(1,4,5)P_3 3-kinase or Ins(1,4,5)P_3 5-phosphatase (Safrany *et al.*, 1991a). DL-Ins(1,4,5)PS_3 is also a potent 5-phosphatase inhibitor (Cooke *et al.*, 1989). It is clear that such analogues (for example, **1–5**, Figure 1) offer considerable potential for investigation and modification of the complex metabolism of Ins(1,4,5)P_3 [see Wojcikiewicz *et al.* (1990a) and Potter (1993)].

Two recent reports have focused upon potential links between certain disease states and malfunctions of the polyphosphoinositide signaling pathway of cellular signaling exemplified by defects in 5-phosphatase activity: Lowe's oculocerebrorenal syndrome affects the development of the eye lens, brain, and kidneys and is characterized by a mutated gene bearing 71% homology to the type II 5-phosphatase (Attree *et al.*, 1992). Furthermore, in HIV-infected lymphocytes the Ins(1,4,5)P_3/Ins(1,3,4,5)P_4 5-phosphatase is either inhibited or defective (Nye *et al.*, 1992). Additionally, a recent report has focused upon the potential alteration of Ins(1,4,5)P_3-mediated Ca^{2+} mobilization in fibroblasts from patients with Alzheimer's disease (Ito *et al.*, 1994). Thus, a highly topical challenge lies in the development of potent and selective synthetic inhibitors of the metabolic enzymes, 5-phosphatase and 3-kinase, which are inactive at the Ins(1,4,5)P_3 receptor. These agents hold the key to understanding further the roles of these enzymes in cellular signaling.

We describe here the biological characteristics of a number of synthetic analogues of Ins(1,4,5)P_3 and identify novel, highly potent inhibitors of 5-phosphatase which interact poorly with both 3-kinase and the Ca^{2+}-mobilizing Ins(1,4,5)P_3 receptor.

MATERIALS AND METHODS

Cell Culture and Ca^{2+} Mobilization. Monolayers of SH-SY5Y human neuroblastoma cells (passage 80–90), initially a kind gift of Dr. J. L. Biedler (Sloane-Kettering Institute, New York), were grown as described (Lambert *et al.*, 1989). Prior to use, cells were harvested in 10 mM HEPES and 0.9% NaCl, pH 7.4, containing 0.02% EDTA. Suspensions of cells were electroporated (Wojcikiewicz *et al.*, 1990b), and $^{45}Ca^{2+}$ loading and mobilization experiments were performed as described (Strupish *et al.*, 1988) in a buffer comprising 120 mM KCl, 20 mM HEPES, 6 mM $MgCl_2$, 5 mM sodium succinate, 5 mM Na_2ATP, 2 mM KH_2PO_4, and 10 μM EGTA (to reduce free Ca^{2+} concentration to 100–300 nM), pH 6.9. EC_{50} values for Ca^{2+} mobilization were derived using ALLFIT computer-assisted curve fitting (DeLean *et al.*, 1978).

Enzyme Interactions. Inhibition of human erythrocyte ghost 5-phosphatase-catalyzed breakdown of [^{3}H]Ins(1,4,5)-P_3 was examined by incubating 10–40 μM [^{3}H]Ins(1,4,5)P_3 (approximately 10 000 dpm) and increasing amounts of the analogues with human erythrocyte ghosts (0.4 mg of protein/mL) at 37 °C in a buffer comprising 30 mM HEPES and 2 mM $MgCl_2$, pH 7.2, under conditions where no more than 30% of the Ins(1,4,5)P_3 was hydrolyzed. Bis- and trisphosphate fractions were separated on Dowex AG1-X8 resin (Batty *et al.*, 1985). No mono- or tetrakisphosphates were identified. Rates of inositol bisphosphate formation were then calculated, from which Lineweaver–Burk plots were constructed and K_i values obtained. Agents which inhibited 5-phosphatase poorly were tested for activity using 10 μM Ins(1,4,5)P_3 only, and K_i values were calculated from IC_{50} values, according to Cheng and Prusoff (1973), assuming competitive inhibition.

To ascertain whether the analogues were substrates for the 5-phosphatase, Ins(1,4,5)P_3 or its analogues (10 μM) or KH_2PO_4 (0–50 μM) as control was incubated with erythrocyte ghosts (1 mg of protein/mL) at 37 °C for 20 min in a volume of 250 μL. Liberation of inorganic phosphate was determined using a colorimetric assay as described (Cooke *et al.*, 1989).

Inhibition of [^{3}H]Ins(1,4,5)P_3 phosphorylation by the analogues was examined by incubating 1–10 μM [^{3}H]Ins-(1,4,5)P_3 (approximately 10 000 dpm) and increasing amounts of the analogues with a crude 3-kinase preparation (Safrany *et al.*, 1990) (0.1% w/v) at 37 °C in a buffer comprising 50 mM Tris maleate, 20 mM $MgCl_2$, 10 mM Na_2ATP, 5 mM 2,3-bisphosphoglycerate (2,3-BPG), and 0.1% bovine serum albumin, pH 7.5, under conditions where no more than 20% of the Ins(1,4,5)P_3 was phosphorylated. The rate of phosphorylation was linear up to 40% conversion (unpublished data). Tris- and tetrakisphosphate fractions were separated on Dowex AG1-X8 resin (Batty *et al.*, 1985). No mono- or bisphosphates were identified. Rates of Ins(1,3,4,5)P_4 formation were then calculated, from which Lineweaver–Burk plots were constructed and K_i values obtained. Agents which inhibited 3-kinase poorly were tested for activity using 1 μM Ins(1,4,5)P_3 only, and K_i values were calculated from IC_{50} values, according to Cheng and Prusoff (1973), assuming competitive inhibition.

Chemical Synthesis. A series of Ins(1,4,5)P_3 analogues has been synthesized. The structures of the most important of these analogues are given in Figure 1. References for the

Table 1

analogue	Ca^{2+} release, EC_{50} (μM)	enzyme interactions: 3-kinase, K_i (μM)	enzyme interactions: 5-phosphatase, K_i (μM)	synthesis ref
Ins(1,4,5)P_3	0.08	0.85 (K_m)	13.8 (K_m)	
D-Ins(1,4,5)PS_3	ND[f]	>100	1.4	Cooke *et al.*, 1987
DL-Ins(1,4,5)P_3-4,5S_2	1.2	46	1.4	Noble *et al.*, 1992b
DL-Ins(1,4,5)P_3-5S	0.8[a]	5[a]	6.8[a]	Noble *et al.*, 1992a
Ins(1,3,4,6)P_4	19.6	150[g]	7.7	
Ins(1,3,4,6)PS_4	≫30	46[g]	1.9	
2,5-diMe-Ins(1,3,4,6)P_4	≫30	>100	15.9	
2,5-diMe-Ins(1,3,4,6)PS_4	≫30	105[g]	1.4	
L-*chiro*-Ins(2,3,5)P_3	1.4[b]	0.97	7.7[b]	Liu *et al.*, 1992
L-*chiro*-Ins(2,3,5)PS_3	4.9[c]	0.82	0.23	Liu *et al.*, 1994
D-6-deoxy-Ins(1,4,5)P_3	6.4[d]	5.7[d]	88.6	
D-6-deoxy-Ins(1,4,5)PS_3	16.6[c]	7.9	1.4	
L-Ins(1,4,5)P_3	≫30	40.3	15.3	Cooke *et al.*, 1987
L-Ins(1,4,5)PS_3	≫30	108[g]	0.43	Lampe *et al.*, 1994
Ins(1,3,5)PS_3	≫30	247[g]	0.52	Lampe *et al.*, 1994
DL-Ins(1,4,5)P_3(PP)	≫30	>100	200[g]	Noble *et al.*, 1992b
L-*chiro*-Ins(1,4,6)P_3	≫30	>100	44	Lampe *et al.*, 1994
L-*chiro*-Ins(1,4,6)PS_3	≫30	>100	0.30	Lampe *et al.*, 1994
DL-Ins(1,2,4,5)P_4	0.16[e]	>200[e]	2.9[e]	Mills *et al.*, 1993
DL-3,6-diBz-Ins(1,2,4,5)P_4	>100[e]	105[e,g]	15.9[e]	Mills *et al.*, 1993

[a] Taken from Safrany *et al.* (1991a). [b] Taken from Safrany *et al.* (1992b). [c] Taken from Safrany *et al.* (1993). [d] Taken from Safrany *et al.* (1991b). [e] Taken from Mills *et al.* (1993). [f] ND, not determined. [g] Assuming competitive inhibition.

synthesis of these agents, where appropriate, are shown in Table 1. All analogues were purified by ion-exchange chromatography on DEAE-Sephadex or Q-Sepharose, using gradients of triethylammonium bicarbonate as eluant. Analogues were shown to be homogeneous by ^1H and ^{31}P NMR spectroscopy, were quantified by the Briggs phosphate assay (Briggs, 1922), and were used as their triethylammonium salts. D-6-Deoxy-Ins(1,4,5)P_3 was a kind gift of Dr. S. D. Gero (CNRS, Paris, France).

RESULTS

Interaction with the Ins(1,4,5)P_3 Receptor. Ins(1,4,5)P_3 mobilized $^{45}Ca^{2+}$ from electrically permeabilized SH-SY5Y human neuroblastoma cells with an EC_{50} = 0.08 μM (n = 6), releasing maximally 80% of the $^{45}Ca^{2+}$ loaded. In agreement with previous studies, *myo*-inositol 1,3,4,6-tetrakisphosphate [Ins(1,3,4,6)P_4] appeared to be a partial agonist with high intrinsic activity (Gawler *et al.*, 1991), mobilizing only some 85% of the $^{45}Ca^{2+}$ mobilized by Ins(1,4,5)P_3, with EC_{50} = 19.6 μM. Substitution of the two free hydroxyl groups of Ins(1,3,4,6)P_4 with methyl groups, producing 2,5-di-*O*-methyl-*myo*-inositol 1,3,4,6-tetrakisphosphate [2,5-diMe-Ins(1,3,4,6)-P_4], removed all $^{45}Ca^{2+}$-mobilizing activity (≤30 μM), in a fashion reminiscent of that previously observed for the 3- and 6-hydroxyl groups of DL-inositol 1,2,4,5-tetrakisphosphate [DL-Ins(1,2,4,5)P_4] (EC_{50} = 0.16 ± 0.01 μM) using DL-3,6-di-*O*-benzoyl-*myo*-inositol 1,2,4,5-tetrakisphosphate [DL-3,6-diBz-Ins(1,2,4,5)P_4] (EC_{50} ≫ 100 μM; Mills *et al.*, 1993). We have previously reported that L-*chiro*-inositol 2,3,5-trisphosphorothioate [L-*chiro*-Ins(2,3,5)PS_3] (**9**, Figure 1; note the difference in numbering of the *myo*- and *chiro*-inositol rings) and D-6-deoxy-*myo*-inositol 1,4,5-trisphosphorothioate [D-6-deoxy Ins(1,4,5)PS_3] act as partial agonists at the Ins(1,4,5)P_3 receptor, with EC_{50} values of 5.3 ± 0.8 and 16 ± 2 μM, respectively (Safrany *et al.*, 1993). *myo*-Inositol 1,3,4,6-tetrakisphosphorothioate [Ins(1,3,4,6)PS_4], 2,5-di-*O*-methyl-*myo*-inositol 1,3,4,6-tetrakisphosphorothioate [2,5-diMe-Ins(1,3,4,6)PS_4], L-*myo*-inositol 1,4,5-trisphosphate [L-Ins(1,4,5)-P_3], L-*myo*-inositol 1,4,5-trisphosphorothioate [L-Ins(1,4,5)-PS_3] (**6**, Figure 1), L-*chiro*-inositol 1,4,6-trisphosphate [L-*chiro*-Ins(1,4,6)P_3], L-*chiro*-inositol 1,4,6-trisphosphorothioate [L-*chiro*-Ins(1,4,6)PS_3] (**8**, Figure 1), DL-*myo*-inositol 1-phosphate 4,5-pyrophosphate [DL-Ins(1,4,5)P_3(PP)] (**10**, Figure 1), and *myo*-inositol 1,3,5-trisphosphorothioate [Ins(1,3,5)PS_3] (**7**, Figure 1) were poor agonists at the Ins(1,4,5)P_3 receptor, mobilizing <15% of that of a maximal dose of Ins(1,4,5)P_3 at 30 μM (Table 1). $^{45}Ca^{2+}$ mobilization elicited by Ins(1,4,5)P_3 was not antagonized by these "inactive" agents. We have previously reported (Safrany *et al.*, 1991a) that DL-*myo*-inositol 1,4-bisphosphate 5-phosphorothioate [DL-Ins(1,4,5)P_3-5S] (**3**, Figure 1) and DL-Ins(1,4,5)PS_3 mobilize $^{45}Ca^{2+}$ with EC_{50} values of 0.8 and 2.5 μM, respectively. DL-*myo*-Inositol 1-phosphate 4,5-bisphosphorothioate [DL-Ins(1,4,5)P_3-4,5S_2] (**5**, Figure 1) mobilized $^{45}Ca^{2+}$ with EC_{50} = 1.2 μM (Table 1), indicating that the sequential replacement of phosphate groups with phosphorothioate groups decreases the Ca^{2+}-mobilizing ability of these agents by small increments.

Interaction with 3-Kinase. Ins(1,4,5)P_3 was phosphorylated by a crude rat brain homogenate supernatant preparation with K_m = 0.85 μM and V_{max} = 1.1 nmol/(min·mg of protein) [204 nmol/(min·g fresh weight of tissue)] in a heparin-sensitive manner. DL-Ins(1,4,5)P_3-4,5S_2 appeared to be a cosubstrate, inhibiting Ins(1,4,5)P_3 metabolism with an apparent K_i = 46 μM. As with Ca^{2+} mobilization data, this figure is between the K_i values obtained for Ins(1,4,5)P_3-5S (K_i = 5 μM) and DL-Ins(1,4,5)PS_3 (K_i = 230 μM) (Safrany *et al.*, 1991a). L-Ins(1,4,5)P_3 competitively inhibited Ins(1,4,5)P_3 phosphorylation with K_i = 40.3 μM. Replacement of the three phosphate groups of L-Ins(1,4,5)P_3 with phosphorothioate groups decreased the affinity for 3-kinase, L-Ins(1,4,5)PS_3 inhibiting Ins(1,4,5)P_3 phosphorylation with K_i = 108 μM. L-*chiro*-Ins(2,3,5)PS_3 and D-6-deoxy-Ins(1,4,5)PS_3 inhibited Ins(1,4,5)P_3 phosphorylation with K_i values of 0.82 and 7.9 μM, close to those obtained for their corresponding phosphates {K_i[L-*chiro*-Ins(2,3,5)P_3] = 0.97 μM, K_i[D-6-deoxy-Ins(1,4,5)-P_3] = 5.7 μM (Safrany *et al.*, 1991b)}. Ins(1,3,4,6)PS_4 was the only other analogue with significant affinity for 3-kinase, with an apparent K_i = 46 μM. All other analogues tested had affinities of over 100 μM (Table 1). Direct studies on the substrate properties of these analogues, like those reported for other analogues (Safrany *et al.*, 1990, 1991a, 1992a,b),

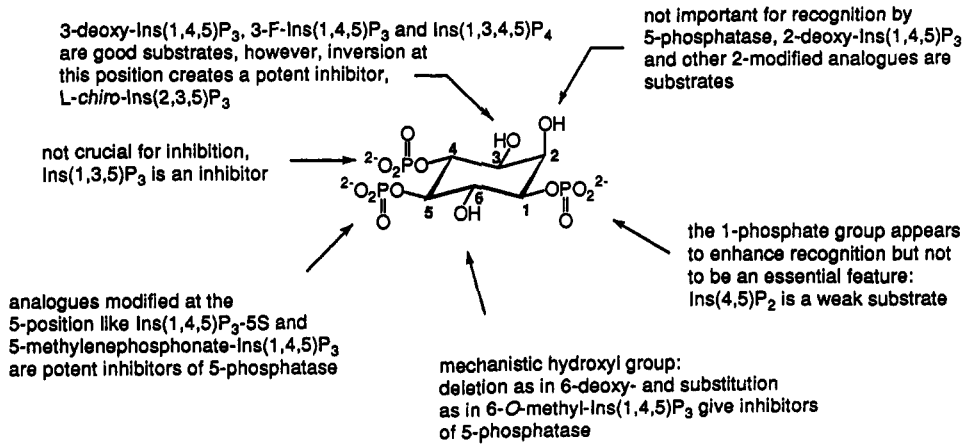

FIGURE 2: Structure-recognition requirements of Ins(1,4,5)P_3 and its analogues by 5-phosphatase.

were not possible due to their low potencies as mobilizers of Ca^{2+}.

Interaction with 5-Phosphatase. Ins(1,4,5)P_3 was dephosphorylated by a human erythrocyte ghost preparation with K_m = 13.8 μM and V_{max} = 1.03 nmol/(mg of protein·min). All the analogues described here were resistant to 5-phosphatase-catalyzed dephosphorylation, with no liberation of inorganic phosphate/phosphorothioate following incubation with human erythrocyte ghosts (data not shown).

The linking together of the 4- and 5-phosphate groups [producing DL-Ins(1,4,5)P_3(PP)] (Noble *et al.*, 1992b) greatly reduced affinity for 5-phosphatase. However, human erythrocyte ghost 5-phosphatase did not appear to exhibit any marked stereoselectivity, as L-Ins(1,4,5)P_3 inhibited dephosphorylation of Ins(1,4,5)P_3 with K_i = 15.3 μM. As observed with Ins(1,4,5)P_3, DL-Ins(1,4,5)P_3-5S, and DL-Ins(1,4,5)PS_3 (Safrany *et al.*, 1991a), phosphorothioate substitution greatly enhanced the affinity of these analogues for 5-phosphatase. However, in this present study DL-Ins(1,4,5)P_3-4,5S_2 and D-Ins(1,4,5)PS_3 were bound by 5-phosphatase with equal affinities. L-Ins(1,4,5)PS_3 was found to be a potent inhibitor of 5-phosphatase, with K_i = 0.43 μM. Ins(1,3,5)PS_3 was also a potent and selective inhibitor of 5-phosphatase, with K_i = 0.52 μM, 85-fold more potent than previous reports for Ins(1,3,5)P_3 inhibiting the bovine aortic smooth muscle enzyme (Polokoff *et al.*, 1988). The affinity of D-6-deoxy-Ins(1,4,5)PS_3 for 5-phosphatase (K_i = 1.4 μM) was also considerably higher than that of D-6-deoxy-Ins(1,4,5)P_3 (K_i = 88.6 μM, compared with 76 μM found previously; Safrany *et al.*, 1991b), whereas L-*chiro*-Ins(2,3,5)PS_3 bound with 36-fold higher affinity than L-*chiro*-Ins(2,3,5)P_3. Thus, L-*chiro*-Ins(2,3,5)PS_3 is the most potent inhibitor of 5-phosphatase yet described, with K_i = 0.23 μM.

We (Mills *et al.*, 1993), and others [e.g., Polokoff *et al.* (1988)], have previously reported that inositol tetrakisphosphates are bound with higher affinity by 5-phosphatase than trisphosphates. Thus Ins(1,3,4,6)P_4 was bound, like DL-Ins(1,2,4,5)P_4 (Mills *et al.*, 1993), with higher affinity than Ins(1,4,5)P_3 (K_i = 7.7 and 2.9 μM, respectively). Phosphorothioate substitution of Ins(1,3,4,6)P_4 again increased affinity for 5-phosphatase {K_i[Ins(1,3,4,6)PS_4] = 1.9 μM}. Methylation of the two free hydroxyl groups of Ins(1,3,4,6)P_4 and Ins(1,3,4,6)PS_4 was without great effect, whereas replacement of the free hydroxyl groups of DL-Ins(1,2,4,5)P_4 with the larger benzoyl groups marginally decreased affinity (Mills *et al.*, 1993).

L-*chiro*-Ins(1,4,6)P_3, which bears very little structural resemblance to Ins(1,4,5)P_3, inhibited 5-phosphatase with K_i = 44 μM. Phosphorothioate substitution greatly enhanced affinity, with L-*chiro*-Ins(1,4,6)PS_3 having a K_i = 0.3 μM.

DISCUSSION

5-Phosphatase is a key enzyme in the polyphosphoinositide signal transduction pathway. Much of the Ins(1,4,5)P_3 generated from agonist-stimulated hydrolysis of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] is rapidly dephosphorylated within seconds of its production (Shears, 1992). This enzyme, however, exhibits poor stereoselectivity, binding L-Ins(1,4,5)P_3 with equal (this study) or only 3-fold lower affinity (Polokoff *et al.*, 1988; Cooke *et al.*, 1989). Indeed, 5-phosphatase appears to interact potently with most Ins(1,4,5)P_3 analogues described to date, with major alterations of the Ins(1,4,5)P_3-like molecule being required before significant loss in affinity is observed (Kozikowski *et al.*, 1993). A summary of current structure-recognition data for 5-phosphatase is shown in Figure 2. Previous studies have identified only a few substrates for 5-phosphatase, with the exception of 2-position-substituted Ins(1,4,5)P_3 analogues. Substitution by large aromatic or aliphatic groups at this position (Hirata *et al.*, 1989) did, however, decrease affinity for 5-phosphatase. A *gem*-2,2-difluoro motif at this position in L-Ins(1,4,5)P_3 resulted in an analogue, L-2-deoxy-2,2-difluoro-*myo*-inositol 1,4,5-trisphosphate, which had higher affinity for 5-phosphatase than its corresponding D-enantiomer, and this compound inhibited the dephosphorylation of this enantiomer in a racemic mixture (Safrany *et al.*, 1992a). Replacement of the 3-hydroxyl group with an equatorial fluorine atom [3-deoxy-3-fluoro-Ins(1,4,5)P_3] (Safrany *et al.*, 1992b) or substitution with a phosphate group [Ins(1,3,4,5)P_4] (Polokoff *et al.*, 1988; Mills *et al.*, 1993), while increasing the affinity, decreased the rate of hydrolysis. L-*chiro*-Ins(2,3,5)P_3, in which the equatorial 3-hydroxyl group of Ins(1,4,5)P_3 is made axial, was found to be resistant (Safrany *et al.*, 1992b). In line with the observations of Polokoff *et al.* (1988) who suggested that the minimum requirements for hydrolysis included phosphate groups at the 4- and 5-position and a free 6-hydroxyl group in a D-isomer, we have previously proposed an explanation for this resistance, suggesting that a free equatorial 6-hydroxyl group is required for 5-phosphatase susceptibility, and L-*chiro*-Ins(2,3,5)P_3 may bind in an inverted manner (Safrany *et al.*, 1992b). Likewise, D-6-deoxy-Ins(1,4,5)P_3 (Safrany *et al.*, 1991b) and DL-6-methoxy-Ins(1,4,5)P_3 (Polokoff *et al.*, 1988) were also found to be resistant to hydrolysis.

The increase in affinity for 5-phosphatase of an analogue upon replacement of its phosphate groups with phosphorothioate groups, as previously observed with DL-Ins(1,4,5)-

P_3-5S and DL-Ins(1,4,5)PS_3 (Safrany *et al.*, 1991a), appears to be universal and was the basis for our design of the highly potent non-Ca^{2+}-mobilizing inhibitors reported here. However, as DL-Ins(1,4,5)P_3-4,5S_2 and D-Ins(1,4,5)PS_3 were bound with equal affinities, we suggest that replacement at the crucial 4- and 5-phosphate groups is the key to this enhanced affinity. The orientation of the group in the 1-position is also important, as described recently by some of us with (1*R*,2*R*,4*R*)- and (1*R*,2*R*,4*S*)-cyclohexane-1,2,4-tris(methylenesulfonate) (Kozikowski *et al.*, 1993). Total phosphorothioate substitution in Ins(1,3,4,6)P_4 enhanced the affinity of the analogue, Ins(1,3,4,6)PS_4, for 5-phosphatase, and similarly for L-*chiro*-Ins(2,3,5)PS_3, D-6-deoxy-Ins(1,4,5)PS_3, L-Ins(1,4,5)PS_3, Ins(1,3,5)PS_3, and L-*chiro*-Ins(1,4,6)PS_3 over their corresponding polyphosphates. As phosphorothioate substitution increases hydrophobicity compared with phosphate groups, this suggests that hydrophobic interactions may be important in the binding of analogues to 5-phosphatase. Alternatively, since the pK_a of a phosphorothioate group is generally lower than that of the corresponding phosphate, the analogue may have a slightly higher net charge than the corresponding polyphosphate at physiological pH. Clearly, the increase in steric bulk on substitution with phosphorothioates is also well tolerated by the enzyme.

It does appear, however, that free phosphate groups are required at the 4- and 5-positions, as an analogue with the 4- and 5-phosphate groups linked together as a pyrophosphate, DL-Ins(1,4,5)P_3(PP), interacts very poorly with 5-phosphatase, and indeed with the receptor and 3-kinase. Ins(1,4,5)P_3(PP) was designed to mimic an Ins(1,4,5)P_3 conformation in which the vicinal 4,5-bisphosphate groups may chelate a metal ion in free solution. While little is known about Ins(1,4,5)P_3 conformation in solution and still less about its conformation on the receptor, the negligible activity of DL-Ins(1,4,5)P_3(PP) in Ca^{2+}-release assays or as an enzyme inhibitor strongly suggests a binding conformation where the 4,5-bisphosphate groups are splayed out, presumably as a result of electrostatic repulsion.

We have previously reported on other Ins(1,4,5)P_3 analogues based on the L-*chiro*-inositol nucleus. L-*chiro*-Ins(2,3,5)P_3, an analogue resembling Ins(1,4,5)P_3 in all respects but one, in that the equatorial 3-hydroxyl of Ins(1,4,5)P_3 is replaced by a 3-axial hydroxyl group, is, surprisingly, resistant to 5-phosphatase, but binds with higher affinity than Ins(1,4,5)-P_3. It is also, as expected, resistant to 3-kinase and has a K_i value equal to the K_m of Ins(1,4,5)P_3 (Safrany *et al.*, 1992b). Its phosphorothioate derivative, L-*chiro*-Ins(2,3,5)PS_3, which we have recently described as a partial agonist at the Ins(1,4,5)P_3 receptor releasing 34% of the Ins(1,4,5)P_3-sensitive pool (Safrany *et al.*, 1993), inhibited 5-phosphatase potently, with K_i = 0.23 μM. This makes L-*chiro*-Ins(2,3,5)PS_3 the most potent 5-phosphatase inhibitor yet described. Its use, however, as a 5-phosphatase inhibitor may be limited as it mobilizes Ca^{2+} from intracellular stores as a partial agonist, thus potentially blocking the actions of Ins(1,4,5)P_3, and also inhibits 3-kinase potently, with a similar affinity to L-*chiro*-Ins(2,3,5)P_3. Likewise, D-6-deoxy-Ins(1,4,5)PS_3, a partial agonist at the Ins(1,4,5)P_3 receptor, mobilized 42% of the Ins(1,4,5)P_3-sensitive pool and inhibited 3-kinase with a similar K_i value to the apparent K_i of D-6-deoxy-Ins(1,4,5)P_3. L-*chiro*-Ins(2,3,5)PS_3 is a key molecule in the development of both enzyme inhibitors and small-molecule Ins(1,4,5)P_3 receptor antagonists. It is the most potent inhibitor of 5-phosphatase yet described, but more importantly is the first Ins(1,4,5)P_3 analogue with equal affinity as Ins(1,4,5)P_3 for 3-kinase.

On the basis of the observation that affinity for 5-phosphatase is increased following phosphorothioate substitution, we proposed that L-Ins(1,4,5)PS_3 would be a potent and selective 5-phosphatase inhibitor (Challiss *et al.*, 1991). As L-Ins(1,4,5)P_3 is recognized well by 5-phosphatase and poorly by both the Ins(1,4,5)P_3 receptor and 3-kinase, phosphorothioate substitution would be expected to decrease affinity for the Ins(1,4,5)P_3 receptor and 3-kinase, while greatly increasing affinity for 5-phosphatase. This has been verified in this study, L-Ins(1,4,5)PS_3 being identified as a potent and selective inhibitor of 5-phosphatase with 250-fold selectivity over inhibition of 3-kinase and an increase in affinity for 5-phosphatase of some 35-fold over L-Ins(1,4,5)P_3.

We have also synthesized Ins(1,3,5)PS_3, a trisphosphorothioate analogue of Ins(1,3,5)P_3. Ins(1,3,5)P_3 has been previously described as a competitive inhibitor of 5-phosphatase, with K_i = 45 μM, which interacts poorly with the Ins(1,4,5)P_3 receptor and 3-kinase (Polokoff *et al.*, 1988). As anticipated, Ins(1,3,5)PS_3, a *meso* compound, is a more potent inhibitor of 5-phosphatase than Ins(1,3,5)P_3, although its affinity for 3-kinase was also increased (Table 1). The selectivity of Ins(1,3,5)PS_3 for inhibition of 5-phosphatase is greater than that observed for L-Ins(1,4,5)PS_3.

Phosphorothioate substitution of inositol tetrakisphosphates appears not to have such a marked effect as observed with trisphosphates. So, while Ins(1,3,4,5)P_4 and DL-Ins(1,2,4,5)-P_4 are the inositol phosphates with highest affinity for 5-phosphatase {K_m[DL-Ins(1,3,4,5)P_4] = 1.4 μM, Polokoff *et al.* (1988); K_m[Ins(1,3,4,5)P_4] = 3.9 μM, K_i[DL-Ins(1,2,4,5)-P_4] = 2.9 μM, Mills *et al.* (1993)}, the phosphorothioate analogue of Ins(1,3,4,6)P_4 had only slightly higher affinity than the phosphate itself. These agents also interact with the Ins(1,4,5)P_3 receptor. However, methylation of the two free hydroxyl groups removed all affinity for the Ins(1,4,5)P_3 receptor and reduced affinity for 3-kinase, without greatly affecting the affinity for 5-phosphatase, thus identifying a rationale whereby the selectivity of potent 5-phosphatase inhibitors can be greatly increased. The introduction of larger groups, as seen by comparing DL-3,6-diBz-Ins(1,2,4,5)P_4 with DL-Ins(1,2,4,5)P_4 (Mills *et al.*, 1993), appears to decrease affinity for 5-phosphatase, suggesting steric interference by such groups with the enzyme.

However, the most selective inhibitor of 5-phosphatase yet described is L-*chiro*-Ins(1,4,6)PS_3. This analogue does not interact with the Ins(1,4,5)P_3 receptor nor with 3-kinase and inhibits 5-phosphatase competitively with K_i = 0.3 μM. L-*chiro*-Ins(1,4,6)PS_3 must, therefore, be seriously considered as the tool of choice when inhibition of 5-phosphatase is required in a broken cell or cell-free system.

One may speculate concerning the potential structural similarities between the potent inhibitors **6**–**9** in relation to their activity. L-*chiro*-Ins(2,3,5)PS_3 can clearly interact with 5-phosphatase in one of two ways: either by binding to the enzyme in the same fashion as Ins(1,4,5)P_3, but providing phosphorothioate groups in a 1,4,5-orientation together with an inverted 3-hydroxyl group, or by binding in the "inverted and rotated mode" of L-*chiro*-Ins(2,3,5)P_3 (Safrany *et al.*, 1992b) with both modified phosphorothioate and hydroxyl group orientations. It is, however, of greater interest to attempt to rationalize the activity of the non-Ca^{2+} mobilizing inhibitors **6**–**8** described here (Figure 3), since it is not clear how they might bind to the enzyme. We have already discussed the higher affinity of Ins(1,3,4,5)P_4 for 5-phosphatase with respect to Ins(1,4,5)P_3. We believe it is to be likely that, aside from enhancement of binding potency by phosphorothioate sub-

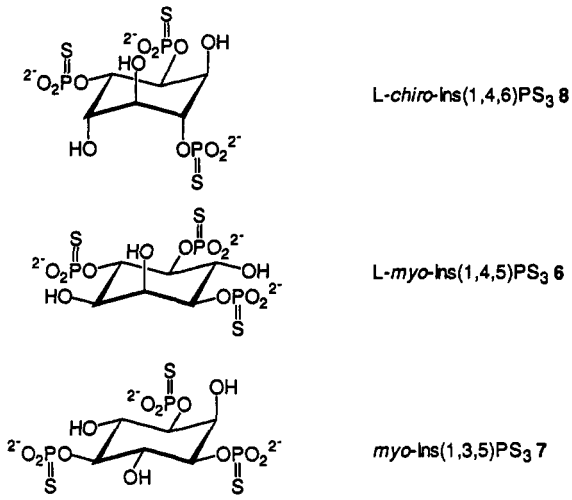

FIGURE 3: Structural relationships of selective 5-phosphatase inhibitors.

stitution, the higher affinity of L-Ins(1,4,5)PS_3 and Ins(1,3,5)-PS_3 may result from their expression of Ins(1,3,4,5)P_4-type structural motifs rather than those of Ins(1,4,5)P_3. Thus, by 120° rotation of the conformation shown in Figure 1 for L-Ins-(1,4,5)PS_3, the compound could bind with the three phosphorothioates in a pseudo 1,3,4-conformation, as shown in Figure 3, thus mimicking Ins(1,3,4)P_3, the intermediate product of Ins(1,3,4,5)P_4 hydrolysis. Similarly, Ins(1,3,5)-PS_3 provides three phosphorothioates in positions with correspond to three of the four phosphate groups of Ins(1,3,4,5)P_4. Apart from the lack of a 4-phosphate group, all other structural motifs are identical to those of Ins(1,3,4,5)P_4.

It is harder to rationalize the potent inhibitory activity of L-*chiro*-Ins(1,4,6)PS_3. In the conformation illustrated in Figure 1, where **8** possesses two axial phosphorothioate groups, it is not obvious how L-*chiro*-Ins(1,4,6)PS_3 can bind to 5-phosphatase. Molecular modeling, however (data not shown), shows that good overlay between the three phosphorothioates of L-*chiro*-Ins(1,4,6)PS_3 and the three phosphates of Ins(1,4,5)P_3 can be achieved if the inhibitor were to bind with the plane of its inositol ring orthogonal to that corresponding to a normally bound molecule of Ins(1,4,5)P_3. We consider, however, that a diaxial conformation for two of the three phosphorothioates of L-*chiro*-Ins(1,4,6)PS_3 is unlikely and that the molecule is more likely to be in the conformation shown in Figure 3 with the vicinal bisphosphorothioates diequatorial. Clearly, in this potential binding mode it is possible to visualize a similarity between L-*chiro*-Ins(1,4,6)-PS_3 and L-Ins(1,4,5)PS_3, and the reasons discussed above for L-Ins(1,4,5)PS_3 may become valid to explain the activity of L-*chiro*-Ins(1,4,6)PS_3. A clear difference is the axial 4-phosphorothioate [or pseudo 1-phosphorothioate compared to Ins-(1,4,5)P_3]. However, it is not difficult to imagine an axial phosphorothioate group being able to interact with a protein site which normally binds an equatorial phosphate, since to some extent, overlapping conformation space can be swept out. Similarly, the synthetic analogue D-*chiro*-inositol 1,3,4-trisphosphate, identical in all respects to Ins(1,4,5)P_3, but possessing an axial phosphate group in place of the equatorial 1-phosphate of Ins(1,4,5)P_3, shows only moderately reduced Ca^{2+}-releasing activity when compared to Ins(1,4,5)P_3 (Denis & Ballou, 1991).

We, like others (Polokoff *et al.*, 1988), have used 2,3-BPG as a 5-phosphatase inhibitor. However, as 2,3-BPG is also a very weak Ins(1,4,5)P_3 receptor antagonist and 3-kinase inhibitor (Guillemette *et al.*, 1990), its uses are limited. Recently, Fowler *et al.* (1993) have identified a noncompetitive inhibitor of 5-phosphatase, FLA 99, a disulfiram analogue, which inhibited the dephosphorylation of Ins(1,4,5)P_3 with "IC_{50}" = 50 μM and raised basal levels of Ca^{2+} in intact GH_3 cells, possibly by increasing resting levels of Ins(1,4,5)P_3. Thus, the potential of such inhibitors in assisting studies of the phosphoinositide signaling pathway is clear. These agents also inhibited cell proliferation; whether this is due to their properties as 5-phosphatase inhibitors is unknown and awaits resolution. In parallel, the infection of $CD4^+$ lymphocytes with the HIV virus, an event which has been identified as raising Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 levels (Nye & Pinching, 1990) by specifically perturbing 5-phosphatase (Nye *et al.*, 1992), is accompanied by a decline of these circulating $CD4^+$ cells (Fauci, 1993). It is, therefore, possible that the cytotoxic effects of HIV are due, in some manner, to its effects on inositol polyphosphate metabolism. We have already used L-Ins(1,4,5)PS_3 **6** and Ins(1,3,5)PS_3 **7** to modulate Ca^{2+}-release activity and metabolism of Ins(1,4,5)P_3 in permeabilized T-lymphocytes (Ward *et al.*, 1994).

The potent analogues described in this study all acted as competitive inhibitors, competing with Ins(1,4,5)P_3 for the active site of 5-phosphatase. Due to their highly polar nature, however, these agents are not expected to permeate the cell membrane. Thus, while they can be considered as useful agents in a cell-free or permeabilized cell preparation, this does not apply when studying inositol phosphate and phospholipid metabolism in intact cells, where it will be necessary to develop membrane-permeable analogues.

Agonist occupancy of a diverse group of cell-surface receptors is known to elicit cleavage of PtdIns(4,5)P_2. Less certain, however, is whether inositol bis- and monophosphates are produced solely as dephosphorylation products of Ins-(1,4,5)P_3 and its phosphorylated metabolite, Ins(1,3,4,5)P_4, or whether PtdIns(4)P and PtdIns are cleaved producing inositol bis- and monophosphates directly. We (Wojcikiewicz *et al.*, 1990a), like others (Imai & Gershengorn, 1986; Fisher *et al.*, 1990), have previously attempted to study the routes of phospholipid metabolism in intact and permeabilized cells. We propose that L-*chiro*-Ins(1,4,6)PS_3, being 19-fold more potent as a 5-phosphatase inhibitor than DL-Ins(1,4,5)P_3-5S used in a previous study (Wojcikiewicz *et al.*, 1990a) and not interacting with 3-kinase or the Ins(1,4,5)P_3 receptor, may be the current tool of choice to examine metabolism of inositol-containing phospholipids. While these studies, in the first instance, should be performed in permeabilized cells, it must be considered a priority to produce membrane-permeant precursors of these analogues if they are to be used in intact cells to identify the roles of PtdIns(4)P and PtdIns as direct precursors of inositol phosphates.

In summary, we have identified the partial agonist L-*chiro*-Ins(2,3,5)PS_3 as the most potent 5-phosphatase inhibitor yet described, but also as the most potent small molecule inhibitor of 3-kinase. We have also described primarily the properties of three novel, potent, and selective inhibitors of 5-phosphatase, Ins(1,3,5)PS_3, L-Ins(1,4,5)PS_3, and, in particular, L-*chiro*-Ins(1,4,6)PS_3, the last being more than an order of magnitude more potent than (1*R*,2*R*,4*R*)-cyclohexane-1,2,4-tris(methylenesulfonate), a recently reported selective 5-phosphatase inhibitor (Kozikowski *et al.*, 1993). These inhibitors possess a diverse range of phosphorothioate substitution patterns. Their potent activity underlines the marked lack of specificity of 5-phosphatase for binding inositol polyphosphates and analogues. While it is not certain precisely how these inhibitors bind the enzyme, they are nevertheless major leads toward

further refinement and the development of effective apolar inhibitors. The elucidation of their mode of action, as well as their use to unravel aspects of the polyphosphoinositide pathway of signal transduction, provide important further challenges.

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