Cancer Chemother Pharmacol (1994) 35: 71–79

ORIGINAL ARTICLE

G. Brunn · A. H. Fauq · S. Chow · A. P. Kozikowski
A. Gallegos · G. Powis

Cellular pharmacology of D-3-azido-3-deoxy-*myo*-inositol, an inhibitor of phosphatidylinositol signaling having antiproliferative activity

Received: 29 December 1993 / Accepted: 6 May 1994

Abstract D-3-Azido-3-deoxy-*myo*-inositol (3AMI) is an inhibitor of the growth of v-*sis*-transformed NIH 3T3 cells but not of wild-type NIH 3T3 cells, whose effects may be mediated through the phosphatidylinositol-3′-kinase pathway. We studied some properties of the cellular pharmacology of 3AMI using high-specific-activity [^{3}H]-3AMI. The uptake of [^{3}H]-3AMI by wild-type NIH 3T3 and v-*sis* NIH 3T3 cells was similar. [^{3}H]-3AMI was a substrate for phosphatidylinositol synthetase, with the maximal velocity (V_{max}) being 1.0 nmol min^{-1} mg^{-1} and the Michaelis constant (K_m) being 23 m*M*. Corresponding values obtained for [^{3}H]-*myo*-inositol as a substrate were 5.5 nmol min^{-1} mg^{-1} and 3.2 m*M*. [^{3}H]-3AMI was incorporated into the cellular inositol lipids of v-*sis* NIH 3T3 cells to a similar extent as that observed for [^{3}H]-*myo*-inositol but was not incorporated into the inositol lipids of wild-type NIH 3T3 cells. The [^{3}H]-3AMI incorporated by the v-*sis* NIH 3T3 cells was present in the phosphatidylinositol and phosphatidylinositol phosphate fractions but not in bisphosphorylated phosphatidylinositol. *myo*-Inositol antagonized the growth-inhibitory effects of 3AMI. The v-*sis* NIH 3T3 cells were found to be more sensitive than the wild-type NIH 3T3 cells to growth inhibition (without 3AMI) caused by the removal of *myo*-inositol from the medium. The results of the study suggest that 3AMI is an antimetabolite of *myo*-inositol. The relative sensitivity of v-*sis* NIH 3T3 and some other cells to 3AMI may be a reflection of increased *myo*-inositol requirements for the growth of these cells as compared with wild-type NIH 3T3 cells.

G. Brunn
Mayo Clinic and Foundation, Rochester, Minnesota, USA

A. H. Fauq · A. P. Kozikowski
Mayo Clinic, Jacksonville, Florida, USA

S. Chow
Karolinska Institutet, Stockholm, Sweden

A. Gallegos · G. Powis (✉)
Arizona Cancer Center, University of Arizona, 1515 North Campbell Avenue, Tucson, AZ 85724, USA

Key words 3AMI · Phosphatidylinositol · Inositol phosphates · Signaling

Abbreviations 3AMI, D-3-azido-3-deoxy-*myo*-inositol; 3AmMI, D-3-amino-3-deoxy-*myo*-inositol; PIPLC, phosphoinositide-selective phospholipase C; PtdIns(4,5)P_2, phosphatidylinositol(4,5)bisphosphate; Ins(1,4,5)P_3, *myo*-inositol(1,4,5)trisphosphate; Ins(1,3,4,5)P_4, *myo*-inositol(1,3,4,5)tetrakisphosphate; PKC, protein kinase C; PtdIns3 K, phosphatidylinositol-3′-kinase; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; CDP-diglyceride, cytidine dipalmitoyl diphosphodiglyceride; DMEM, Dulbecco's modified Eagle's medium; PBS, Dulbecco's phosphate-buffered saline; HCS, heat-inactivated calf serum; IC_{50}, concentration required to cause 50% inhibition; PtdIns synthetase; phosphatidylinositol synthetase; DAG, diacylglycerol

Introduction

The cyclohexitol sugar *myo*-inositol occupies a key position in intracellular signaling pathways that mediate the effects of growth factors and mitogens on cell proliferation. Mitogenic peptides such as bombesin and vasopressin bind to cell-surface receptors [35, 41] and activate a guanine nucleotide-binding, (G) protein-regulated, phosphoinositide-selective phospholipase C (PIPLC-β) [45], causing the hydrolysis of phosphatidylinositol(4,5)-bisphosphate [PtdIns(4,5)P_2] to inositol (1,4,5)trisphosphate [Ins(1,4,5)P_3] and diacylglycerol (DAG) [6, 10, 24]. Growth-factor-receptor protein tyrosine kinases activate another PIPLC, PIPLC-γ, that also hydrolyzes PtdIns(4,5)P_2 [27]. Ins(1,4,5)P_3 releases Ca^{2+} from non mitochondrial intracellular stores, causing an increase in the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$), whereas DAG activates a Ca^{2+}- and phospholipid-dependent protein kinase C (PKC) [5, 36]. A subsequent series of events, probably involving phosphorylation of nuclear transcription factors by PKC, results in increased DNA synthesis [7].

$Ins(1,4,5)P_3$ can undergo further metabolism by inositol-3-kinase to give inositol(1,3,4,5)tetrakisphosphate [$Ins(1,3,4,5)P_4$] [20]. The role of Ins(1,3,4,5) in Ca^{2+} signaling remains controversial [29, 38, 44], but it may modulate Ca^{2+} mobilization by $(1,4,5)P_3$ [17] and facilitate the refilling of internal Ca^{2+} stores by external Ca^{2+} [20, 21], thus permitting the continued release of Ca^{2+} by $Ins(1,4,5)P_3$.

A second *myo*-inositol signaling pathway involves phosphorylation of the D-3 position of the *myo*-inositol ring of phosphatidylinositols (PtdIns) by phosphatidylinositol 3′-kinase (type 1 phosphatidylinositol kinase, PtdIns3K). PtdIns3K is phosphorylated by and associates with a number of growth-factor-receptor protein tyrosine kinases, including the platelet-derived growth factor (PDGF) receptor and the epidermal growth factor (EGF) receptor [4, 23] as well as oncogene protein tyrosine kinases [9, 16]. The PtdIns-3-phosphates formed are not substrates for hydrolysis by known PIPLCs and, thus, do not contribute to the inositol phosphate signaling pathway [42]. Evidence for the importance of PtdIns3K in mitogenesis comes from situations in which mutated tyrosine kinases fail to associate with and activate PtdIns3K. Polyoma middle-T mutants that associate with and activate $pp60^{c\text{-}src}$ tyrosine kinase but fail to activate PtdIns3K are nontransforming [23, 48]. Cells transfected with mutated PDGF receptors that retain protein tyrosine kinase activity but do not associate with or activate PtdIns3K fail to show a mitogenic response to PDGF, unlike cells transfected with the wild-type PDGF receptor [12]. The function of PtdIns-3-phosphates in mitogenesis is not known but they may be involved in cytoskeletal reorganization [14].

On the basis of the evidence that phosphorylation at the D-3 position of *myo*-inositol is important for intracellular signaling by mitogens and, perhaps, for cell transformation, we synthesized a series of D-3-deoxy-3-substituted *myo*-inositol analogues that inhibited the proliferation of wild-type and transformed NIH 3T3 cells in culture [25, 26, 39]. The most active of these analogues was 3AMI. We have now synthesized high-specific-activity [^{3}H]-3AMI and report on its cellular pharmacology.

Materials and methods

Compounds

3AMI was synthesized as previously described [39]. [^{3}H]-3AMI was synthesized according to the procedure described by Kozikowski et al. [25] and had a specific activity of 6 Ci/mmol. [2-^{3}H]-*myo*-Inositol (specific activity, 20 Ci/mmol) and [^{14}C-(U)]-glucose-6-phosphate (specific activity, 300 mCi/mmol) were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, Mo.). [^{3}H]-PtdIns, [^{3}H]-PtdIns(4)P and [^{3}H]-PtdIns(4,5)P_2 standards for chromatography were purchased from Amersham Radiochemicals (Downers Grove, Ill.). Cytidine dipalmitoyl diphosphodigylceride (CDP-diglyceride) was purchased from Sedary Research Laboratories (London, Ontario, Canada). *myo*-Inositol, arg^8-vasopressin, and bradykinin were purchased from Sigma Chemical Co. (St. Louis, Mo.). PDGF (β-chain homodimer) was obtained from Bachem Inc. (Torrance, Calif.) and aequorin was supplied by Friday Harbor Laboratories (Friday Harbor, Wash.). Both Dulbecco's modified Eagle's medium (DMEM) containing 40 μ*M* *myo*-inositol and *myo*-inositol-free DMEM were purchased from Grand Island Biological Co. (Grand Island, N.Y.).

Cell lines

Wild-type mouse NIH 3T3 cells were obtained from the American Type Culture Collection (Rockville, Md.). v-*sis* NIH 3T3 cells and v-*erb* B NIH 3T3 cells [32] were provided by Dr. D. S. Aaronson (National Cancer Institute, Bethesda, Md.), and *mas* NIH 3T3 cells [49] were provided by Dr. D. Young (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.).

Cell-growth assays

Cells were maintained in bulk culture in DMEM supplemented with 10% heat-inactivated calf serum (HCS) and were passaged using 0.05% trypsin and 0.5 m*M* ethylenediaminetetraacetic acid (EDTA). Cell-growth assays were performed by plating 5×10^3 cells in 1.6-cm-diameter culture wells in 0.5 ml DMEM with 10% HCS and allowing the cells to attach to the culture surface for 24 h. The medium was removed and the cells were washed first with Dulbecco's phosphate-buffered saline (PBS) and then with DMEM with or without *myo*-inositol, as appropriate; finally, DMEM with or without *myo*-inositol and containing 10% dialyzed HCS as well as the *myo*-inositol analogues was added. After 3 days, attached cells were harvested with 0.05% trypsin and 0.5 mM EDTA and then counted using an automated cell counter (Coulter, Hialea, Fla.). The mean concentration of *myo*-inositol analogue required to cause 50% growth inhibition in treated cells as compared with nontreated cells (IC_{50}) was calculated from nonlinear least-squares regression analysis of the cell-proliferation concentration data [3]. The time course of the inhibition of cell growth was measured by plating 10^3–10^4 cells in 1.6-cm-diameter culture wells in DMEM with or without *myo*-inositol and containing 10% dialyzed HCS with or without 100 μ*M* 3AMI. Cells were harvested and counted every day for 7 days. At day 3, free-floating cells in the medium were collected, washed three times with DMEM, and replated in DMEM (with *myo*-inositol) and 10% dialyzed HCS. Cells were measured every day for an additional 4 days. All incubations were conducted in quadruplicate.

Biochemical measurements

The uptake of [^{3}H]-*myo*-inositol and [^{3}H]-3AMI by wild-type NIH 3T3 and v-*sis* NIH 3T3 cells over 2 h was measured as previously described [31]. The phosphatidylinositol synthetase (PtdIns synthetase)-catalyzed incorporation of *myo*-inositol, 3AMI, D-3-deoxy-3-fluoro-*myo*-inositol, and D-3-deoxy-3-amino-*myo*-inositol (3AmMI) into PtdIns was measured as described by Moyer et al. [33] using partly purified rat-brain PtdIns synthetase [40]. The incorporation of [^{3}H]-*myo*-inositol and [^{3}H]-3AMI into cellular phospholipids was measured by growing 5×10^5 wild-type NIH 3T3 or v-*sis* NIH 3T3 cells in 60-mm diameter culture dishes in *myo*-inositol-free DMEM and 10% dialyzed HCS together with 1 μCi (0.17 μ*M*) [^{3}H]-*myo*-inositol/ml or 1 μCi [^{3}H]-3AMI/ml for 48 h. The cells were harvested with 0.05% trypsin and 0.5 m*M* EDTA and extracted with chloroform:methanol:HCl (80:40:1, by vol.). The organic layer was removed and dried under N_2 and the residue was dissolved in 100 μl chloroform. An aliquot of the chloroform solution was counted by liquid scintillation and the remainder was applied to a silica-gel thin-layer chromatography (TLC) plate (Whatman LK6D, Maidstone, UK), which was developed using a solvent system of chloroform:methanol:H_2O:ammonium hydroxide (50:50:10:5, by vol.). The radioactivity on the plates was measured using a radio-TLC scanner (Model RS, Packard Instruments, Downers Grove, Ill.) and compared with standards of [^{3}H]-PtdIns, [^{3}H]-PtdIns(4)P, and [^{3}H]-PtdIns(4,5)P_2 run under the same conditions. The formation of [^{3}H]-*myo*-inositol phosphates was measured in wild-type NIH 3T3 cells and v-*sis* NIH 3T3 cells grown with 1 μCi (0.17 μ*M*) [^{3}H]-*myo*-inositol/ml or 1 μCi [^{3}H]-3AMI/ml for 48 h as

described above. The cells were washed four times with *myo*-inositol-free DMEM, incubated in 2 ml *myo*-inositol-free DMEM containing 15 m*M* LiCl for 10 min at 37 °C, and then stimulated with 3.3×10^{-9} *M* PDGF for 10 min at 37 °C. The incubation was terminated by the addition of 2 ml 10% perchloric acid at 4 °C. *myo*-Inositol phosphates (i.e., *myo*-inositol mono-, bis-, and trisphosphates) were isolated by anion-exchange chromatography as previously described [1].

The de novo synthesis of *myo*-inositol by wild-type NIH 3T3 cells and v-*sis* transformed NIH 3T3 cells was measured by a modification of the method of Li et al. [28] as the conversion of [^{14}C]-glucose-6-phosphate to [^{14}C]-*myo*-inositol-1-phosphate by *myo*-inositol-1-phosphate synthase, which is the rate-limiting step in *myo*-inositol biosynthesis [15]. Briefly, saponin-permeabilized cells were incubated for up to 1 h at 37 °C with 12 μ*M* [^{14}C]-glucose-6-phosphate, 0.8 m*M* nicotinamide adenine dinucleotide (NAD^+), and 5 m*M* LiCl. [^{14}C]-Glucose-6-phosphate and [^{14}C]-*myo*-inositol-1-phosphate were separated by high-performance liquid chromatography (HPLC) using a 25-cm, 7-μm, mixed-mode RP-18/anion-exchange column (Alltech Associates, Deerfield, Ill.) and 20 m*M* $(NH_4)H_2PO_4$ as the eluant, run at a flow rate of 1 ml/min and detected with a radioactive flow detector (Flo-One Beta, Packard Instruments, Downers Grove, Ill.). The retention times found for [^{14}C]-*myo*-inositol-1-phosphate and [^{14}C]-glucose-6-phosphate with this HPLC system were 9.8 and 11.1 min, respectively. The limit of detection of the assay under the conditions employed was calculated to be 6 pmol *myo*-inositol-1-phosphate formed per 10^6 cells per hour.

Changes in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) were measured using the Ca^{2+}-sensitive photoprotein aequorin. Wild-type NIH 3T3 cells and v-*sis* NIH 3T3 cells were loaded with aequorin by a low-Ca^{2+} centrifugation technique as previously described [37]. In all, 10^6 cells were plated in 35-mm culture dishes containing 2 ml DMEM with 10% dialyzed HCS. After incubation overnight to allow the cells to attach to the surface of the culture dish, the medium was replaced with DMEM without HCS for 3 h. Some cells were grown for 2 days with 100 μ*M* 3AMI in *myo*-inositol-free DMEM with 10% dialyzed HCS, loaded with aequorin, and then exposed to the same medium with 100 μ*M* 3AMI overnight prior to the measurement of $[Ca^{2+}]_i$. The culture dish was placed in a 37 °C thermostated holder over a photomultiplier tube, and the surface of the medium was flushed with a humidified atmosphere of 5% CO_2: 95% air. Arg^8-vasopressin (10^{-7} *M*) and bradykinin (2×10^{-7} *M*), were used to produce maximal increases in $[Ca^{2+}]_i$. At the end of the study, the cells were lysed with a solution of 1% Triton X-100 and 5 m*M* $CaCl_2$ and the total light signal was integrated. An estimate of $[Ca^{2+}]_i$ was obtained using the calibration method for aequorin previously reported by Allen and Blinks [2].

Results

Cell growth inhibition by myo-inositol deprivation and 3AMI

The growth of wild-type NIH 3T3 cells was unaffected by 3AMI concentrations of up to 33 m*M* (the highest concentration tested), with or without *myo*-inositol in the medium. In contrast, 3AMI inhibited the growth of v-*sis* NIH 3T3 cells with an IC_{50} of 53 μ*M* without *myo*-inositol in the medium. The results confirm our previous observation that in the absence of *myo*-inositol, v-*sis* NIH 3T3 cells are extremely sensitive to growth inhibition by 3-deoxy-3-substituted *myo*-inositols as compared with a wild-type NIH 3T3 cell line [39]. We therefore used the two cell lines to compare the cellular pharmacology of 3AMI. *myo*-Inositol reversed the growth-inhibitory effects of 3AMI on v-*sis* NIH 3T3 cells with an IC_{50} for *myo*-inositol of 2 μ*M*.

The time course of the growth inhibition of NIH 3T3 cells and v-*sis* NIH 3T3 cells by 3AMI is shown in Fig. 1 and is compared with the effects of *myo*-inositol deprivation on cell growth. The growth curve generated for wild-type NIH 3T3 cells without *myo*-inositol in the medium (Fig. 1A) was the same as that generated for wild-type cells in medium containing 40 μ*M* *myo*-inositol (results not shown), demonstrating that *myo*-inositol is not necessary for the growth of these cells over the period studied. In contrast, the growth of v-*sis* NIH 3T3 cells required *myo*-inositol in the medium. Without *myo*-inositol, the number of attached cells began to decline slowly after 3 days. About 50% of the total v-*sis* NIH 3T3 cells that detached in *myo*-inositol free medium could be collected at day 3, and when replated in *myo*-inositol-containing medium they proceeded to grow normally. The growth of wild-type NIH 3T3 cells over 7 days was not inhibited by 100 μ*M* 3AMI, either in the absence of *myo*-inositol (Fig. 1A) or in its presence (results not shown). In the presence of 100 μ*M* 3AMI, the number of attached v-*sis* NIH 3T3 cells declined drastically after only 2 days (Fig. 1B). Less than 10% of the detached cells could be collected from the medium at day 3. However, when these cells were replated in 3AMI-free medium with *myo*-inositol they grew at a normal rate.

Fig. 1 A, B Cell-growth inhibition caused by *myo*-inositol deprivation and by 3AMI. **A** Approximately 10^3 wild-type NIH 3T3 cells (○) or v-*sis* NIH 3T3 cells (●) were grown in *myo*-inositol-free DMEM and 10% dialyzed HCS. Adherent cells were counted every day. At day 3 the floating v-*sis* NIH 3T3 cells were collected and replated in the same medium containing 40 μ*M* *myo*-inositol (▽). **B** Approximately 10^4 wild-type NIH 3T3 cells (○) or v-*sis* NIH 3T3 cells (●) were grown in *myo*-inositol-free DMEM containing 100 μ*M* 3AMI and 10% dialyzed HCS. Adherent cells were counted every 3 days. At day 3 the floating v-*sis* NIH 3T3 cells were collected, washed, and replated in same medium without 3AMI and with 40 μ*M* *myo*-inositol (▽). More cells were plated in **B** than in **A** so as to obtain sufficient floating cells to replate. Each point represents the mean value for triplicate determinations

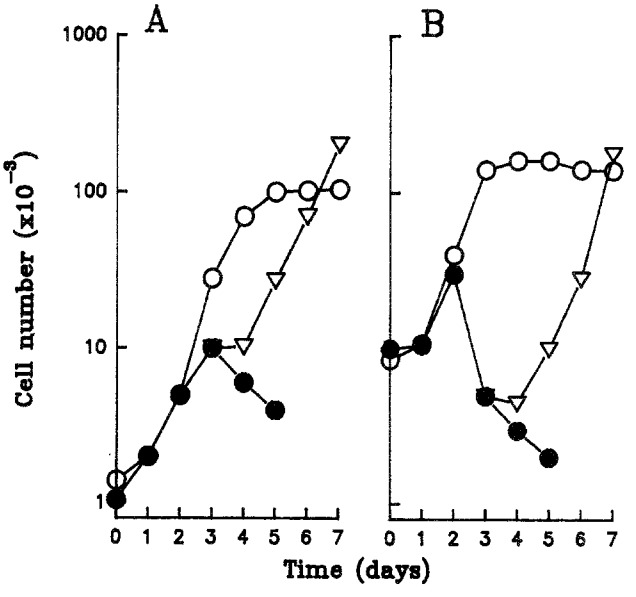

Table 1. Effect of LiCl on the cytotoxicity of D-3-substituted-3-deoxy *myo*-inositol analogues

	Without *myo*-inositol		With *myo*-inositol	
	NIH 3T3 IC_{50} (m*M*)	v-*sis* NIH 3T3 IC_{50} (m*M*)	NIH 3T3 IC_{50} (m*M*)	v-*sis* NIH 3T3 IC_{50} (m*M*)
3AMI	NT	0.08 ± 0.01	NT	NT
3AMI + 5 m*M* LiCl	ND	0.11 ± 0.00	ND	NT
3AmMI	0.13 ± 0.01	2.29 ± 0.25	4.54 ± 0.77	3.00 ± 0.30
3AmMI + 5 m*M* LiCl	0.10 ± 0.02	0.96 ± 0.11*	5.91 ± 1.07	3.18 ± 0.30

Cell growth was measured as described Materials and methods using 5 m*M* LiCl, a concentration that caused < 10% growth inhibition, and 3AMI or 3AmMI in various combinations with or without *myo*-inositol in the medium. Data represent IC_{50} values (± SD)
NT, Not toxic (< 10% inhibition of cell growth at 10 m*M*, the highest concentration tested); ND, not determined
* $P < 0.05$ versus the value obtained without LiCl

For further exploration of the relationship between the cell-growth inhibition produced by *myo*-inositol deprivation and by 3AMI, other NIH 3T3 cell lines were studied. We had previously observed that v-*erb* B NIH 3T3 cells were relatively resistant to 3AMI (IC_{50}, 29 m*M*), whereas *mas* NIH 3T3 cells were more sensitive (IC_{50}, 5 m*M*) [39]. These cells were studied for their *myo*-inositol requirement. The growth of v-*erb* B cells was not affected by the absence of *myo*-inositol in the medium for 7 days, whereas the growth of *mas* NIH 3T3 cells was inhibited by 57% (results not shown). Taken together, the results of these studies suggest that the effects of 3AMI in inhibiting cell growth are functionally like those of *myo*-inositol deprivation.

Synthesis of *myo*-inositol

Attempts were made to measure rates of [^{14}C]-*myo*-inositol-1-phosphate synthesis from [^{14}C]-glucose-6-phosphate in saponin-permeabilized wild-type NIH 3T3 cells and v-*sis* NIH 3T3 cells. However, we could not detect [^{14}C]-*myo*-inositol-1-phosphate synthesis by either cell line. The calculated limit of detectability for [^{14}C]-*myo*-inositol-1-phosphate synthesis in our assay was 6 pmol 10^{-6} cells h^{-1}. This is well below the levels of *myo*-inositol-1-phosphate biosynthesis (100 pmol/10^6 cells per 20 min) reported for retinal capillary pericytes using the same procedure [28].

Effects of LiCl on cell-growth inhibition

Because we could not detect *myo*-inositol biosynthesis by wild-type NIH 3T3 cells or v-*sis* NIH 3T3 cells, we studied the possibility that salvage or reutilization of *myo*-inositol formed from PtdIns would allow wild-type NIH 3T3 cells but not v-*sis* NIH 3T3 cells to grow in the absence of *myo*-inositol. To do this we studied the effects of LiCl, an inhibitor of *myo*-inositol phosphate phosphatases [34], on the cell-growth inhibition induced by 3AMI and by another 3-substituted *myo*-inositol, 3AmMI. We found no difference in the LiCl IC_{50} values (± SD) that inhibited the growth of wild-type NIH 3T3 cells (IC_{50}, 22.0 ± 2.1 μ*M* without *myo*-inositol in the medium; 20.0 ± 5.3 m*M* with *myo*-inositol) or v-*sis* NIH 3T3 cells (IC_{50}, 19.1 ± 3.2 m*M* without *myo*-inositol; 25.0 ± 4.7 m*M* with *myo*-inositol). A concentration of LiCl that caused less than 10% growth inhibition, 5 m*M*, was chosen for combination with the *myo*-inositol analogues. The growth inhibition induced by 3AMI or 3AmMI in either wild-type NIH 3T3 cells or v-*sis* NIH 3T3 cells was unaffected by this concentration of LiCl, with or without *myo*-inositol, except for a small potentiation in the cell-growth produced inhibition by 3AmMI in v-*sis* NIH 3T3 cells without *myo*-inositol (Table 1). Overall, the studies with LiCl do not support a role for *myo*-inositol salvage as a factor in the *myo*-inositol requirement for cell-growth or for differences in the growth inhibition induced by 3AMI or 3AmMI between wild-type NIH 3T3 cells and v-*sis* NIH 3T3 cells.

Cellular uptake of *myo*-inositol

The uptake of [^{3}H]-3AMI by wild-type NIH 3T3 cells exhibited a maximal rate (V_{max}) of 2.6 nmol 10^{-6} cell h^{-1} and a Michaelis constant (K_m) of 0.42 m*M*. We have previously reported that 3AMI is a competitive inhibitor of [^{3}H]-*myo*-inositol uptake by wild-type NIH 3T3 cells, with its inhibition constant (K_i) being 0.35 m*M* [39]. The uptake of [^{3}H]-*myo*-inositol by wild-type NIH 3T3 cells had a V_{max} of 2.8 nmol 10^{-6} cell h^{-1} and a K_m of 69 μ*M*. These values are similar to those previously reported for *myo*-inositol uptake by NIH 3T3 cells [43]. Uptake of [^{3}H]-3AMI by v-*sis* NIH 3T3 cells exhibited a V_{max} of 1.8 nmol 10^{-6} cell h^{-1} and a K_m of 0.35 m*M*, and the uptake of [^{3}H]-*myo*-inositol showed a V_{max} of 2.3 nmol 10^{-6} cell h^{-1} and a K_m of 53 μ*M*.

PtdIns synthetase

The ability of [^{3}H]-3AMI to act as a substrate for partly purified PtdIns synthetase with CDP-diglyceride as an acceptor is shown in Fig. 2. The V_{max} was 1.0 nmol min^{-1} mg^{-1} and the K_m, 23.0 m*M*. With [^{3}H]-*myo*-inositol as a substrate, the V_{max} was 5.5 nmol min^{-1} mg^{-1} and the K_m, 3.2 m*M*. The values obtained for *myo*-inositol as the

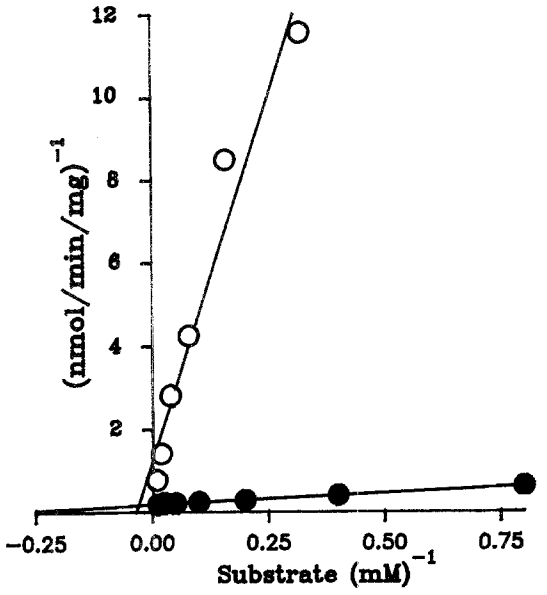

Fig. 2 3AMI and *myo*-inositol as substrates for PtdIns synthetase: Lineweaver-Burk plots of the incorporation of [^{3}H]-3AMI (○) and [^{3}H]-*myo*-inositol (●) into phospholipid when cells were incubated at various concentrations with rat-brain PtdIns synthetase and CDP-diglyceride for 30 min at 37 °C

substrate for PtdIns synthetase are similar to those previously reported [33]. We also studied the ability of some nonradiolabeled D-3-substituted 3-deoxy-*myo*-inositol analogues to act as inhibitors of PtdIns synthetase with [^{3}H]-*myo*-inositol serving as the substrate. The K_i values (together with the type of inhibition) were: D-3-fluoro-3-deoxy-*myo*-inositol, 37.2 m*M* (competitive); 3AmMI, 24.6 m*M* (noncompetitive); and 3AMI, 31.2 m*M* (noncompetitive).

Incorporation into cellular phospholipids

The incorporation of [^{3}H]-*myo*-inositol and [^{3}H]-3AMI into cellular phospholipids of wild-type and v-*sis* NIH 3T3 cells is shown in Table 2. Incorporation of [^{3}H]-*myo*-inositol into cellular phospholipids of wild-type NIH 3T3 cells was approximately twice that of v-*sis* NIH 3T3 cells. [^{3}H]-3AMI was incorporated into the cellular phospholipids of v-*sis* NIH 3T3 cells to a similar extent as that found for [^{3}H]-*myo*-inositol but was hardly incorporated into wild-type NIH 3T3 cells.

TLC analysis of the phospholipids is shown in Fig. 3. The majority of the radioactivity from [^{3}H]-*myo*-inositol chromatographed with PtdIns and lesser amounts, with PtdIns phosphates. The percentages of total radioactivity chromatographing with the standards in wild-type NIH 3T3 cells were PtdIns, 74%; PtdIns(4)P, 5%; PtdIns(4,5)P_2, 2%; and other peaks, 19%; those in v-*sis* NIH 3T3 cells were PtdIns, 85%; PtdIns(4)P, 6%; PtdIns(4,5)P_2, 3%; and other peaks, 6%. These values are similar to those reported by other workers for [^{3}H]-*myo*-inositol incorporation into PtdIns of fibroblasts [43]. [^{3}H]-3AMI, which was poorly incorporated into PtdIns by wild-type NIH 3T3 cells, had percentages of total radioactivity chromatographing with the standards as follows: PtdIns, 50%; PtdIns(4)P, 32%; PtdIns(4,5)P_2, 4%; and other peaks, 14%. In v-*sis* NIH 3T3 cells the percentages were: PtdIns, 91%; PtdIns(4)P, 8%; PtdIns(4,5)P_2, 0; and other peaks, 1%. Thus, it appears that although [^{3}H]-3AMI can be incorporated into PtdIns and PtdIns monophosphate by v-*sis* NIH 3T3 cells, it may not undergo further phosphorylation to PtdIns bisphosphates.

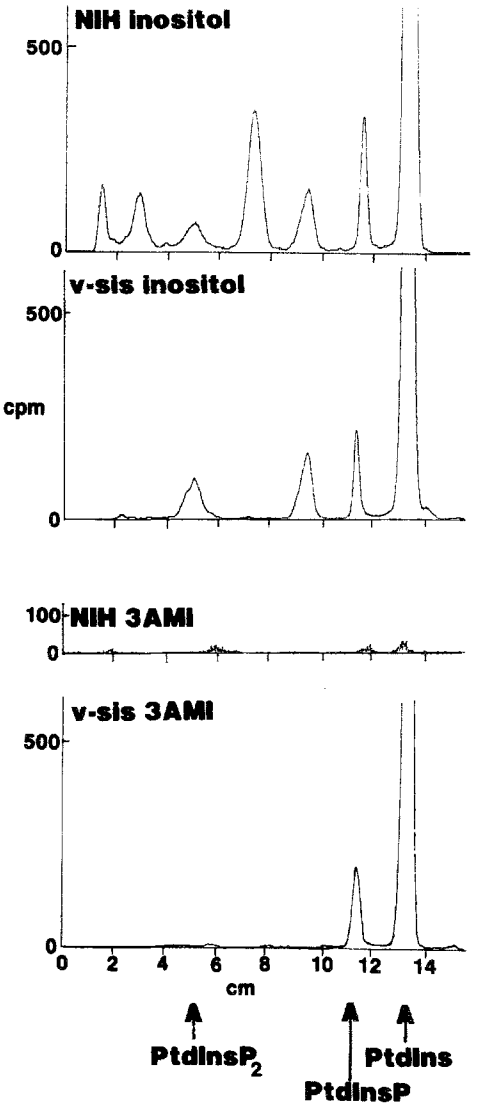

Fig. 3 Incorporation of [^{3}H]-*myo*-inositol and [^{3}H]-3AMI into phosphatidylinositols (PtdIns) of wild-type NIH 3T3 cells (*NIH*) and v-*sis* NIH 3T3 cells (v-*sis*). Cells were grown with 1 μCi [^{3}H]-*myo*-inositol or [^{3}H]-3AMI in *myo*-inositol-free DMEM and 10% dialyzed HCS for 48 h. Phospholipids were extracted from 2 × 10^6 cells with chloroform:methanol:HCl (80:40:1, by vol.) and subjected to TLC on silica gel with a solvent system of chloroform:methanol:H_2O:ammonium hydroxide (50:50:10:5, by vol.). Radioactivity was quantified using a radio-TLC scanner. The position of standards are shown by the *arrows*

Table 2. Incorporation of 3AMI into phosphatidylinositol and inositol phosphates of NIH 3T3 and v-*sis* NIH cells

Compound	Cell line	[^{3}H]-Phospholipid (pmol/10^6 cells)	[^{3}H]-Inositol phosphates	
			Without PDGF (fmol/10^6 cells)	With PDGF (fmol/10^6 cells)
myo-Inositol	NIH 3T3	8.00±0.36	198 ±57	581 ± 10*
	v-*sis* NIH 3T3	4.15±0.21	420 ±30	560 ±160
3AMI	NIH 3T3	0.29±0.02	6.8± 0.4	6.1± 0.8
	v-*sis* NIH 3T3	3.33±0.06	6.3± 4.0	6.2± 1.5

Wild-type NIH 3T3 and v-*sis* NIH 3T3 cells were grown with 1 μCi [^{3}H]-AMI/ml or 1 μCi [^{3}H]-*myo*-inositol/ml for 2 days. The incorporation of radioactivity into cellular phospholipids as well as the basal and (3.3×10^9 *M*) PDGF-stimulated inositol phosphate formation were measured. Data represent mean values for 3 separate determinations (±SE)
* $P<0.01$ versus the nonstimulated value

Table 3. Effect of 3AMI on mitogen-stimulated $[Ca^{2+}]_i$ responses by NIH 3T3 and v-*sis* NIH 3T3 cells

Cell line	Without 100 μ*M* 3AMI		With 100 μ*M* 3AMI	
	Vasopressin $[Ca^{2+}]_i$ (μ*M*)	Bradykinin $[Ca^{2+}]_i$ (μ*M*)	Vasopressin $[Ca^{2+}]_i$ (μ*M*)	Bradykinin $[Ca^{2+}]_i$ (μ*M*)
NIH 3T3	0.55±0.10	0.56±0.04	0.50±0.16	0.58±0.06
v-*sis* NIH 3T3	0.72±0.13	1.20±0.15	0.37±0.30	1.33±0.17

Wild-type NIH 3T3 and v-*sis* NIH 3T3 cells were grown in *myo*-inositol-free DMEM with 10% HCS, with or without 100 μ*M* 3AMI for 3 days. At 24 h prior to the study, the cells were harvested, loaded with the Ca^{2+}-sensitive photoprotein aequorin, and allowed to reattach to the tissue-culture plates. Both control and drug-treated cells were exposed to the appropriate medium without HCS for 3 h prior to the addition of 10^{-7} *M* arg^8-vasopressin and 2×10^{-7} *M* bradykinin. Data are expresssed as the maximal increase in $[Ca^{2+}]$ and represent mean values for 3–5 determinations (±SE). The resting $[Ca^{2+}]_i$ in the cells was 0.1 μ*M*

Formation of inositol phosphates

The formation of [^{3}H]-*myo*-inositol phosphates by wild-type NIH 3T3 cells and v-*sis* NIH 3T3 cells grown with [^{3}H]-*myo*-inositol or [^{3}H]-3AMI is shown in Table 2. PDGF stimulated [^{3}H]-inositol phosphate formation 2.9-fold in wild-type NIH 3T3 cells. The resting level of [^{3}H]-inositol phosphate formation in v-*sis* NIH 3T3 cells was over 2-fold that of wild-type NIH 3T3 cells and there was only a small, nonsignificant stimulation of [^{3}H]-inositol phosphate formation by PDGF. The v-*sis* NIH 3T3 cells are known to produce an autocrine PDGF-like factor [18] and to have low levels of PDGF receptors [19]. The resting levels of [^{3}H]-inositol phosphate formation were very low in both wild-type NIH 3T3 cells and v-*sis* NIH 3T3 cells exposed to [^{3}H]-3AMI, being less than 5% of those found for [^{3}H]-*myo*-inositol, and were not stimulated by PDGF.

Ca^{2+} signaling

Wild-type NIH 3T3 cells or v-*sis* NIH 3T3 cells grown in 100 μ*M* 3AMI for 3 days showed no effect in terms of the peak Ca^{2+} responses of the surviving cells to vasopressin and bradykinin (Table 3).

Discussion

We have previously reported that 3AMI is the most active of a series of growth-inhibitory D-3-substituted 3-deoxy-*myo*-inositol analogues [26, 39]. 3AMI selectively inhibits the growth of v-*sis* transformed NIH 3T3 cells, showing over a 1000-fold increased potency in the absence of exogenous *myo*-inositol for growth inhibition of v-*sis* NIH 3T3 cells as compared with wild-type NIH 3T3 cells. Providing a mechanistic basis for this remarkable selectivity was the reason for our study. To facilitate the investigation we synthesized high-specific-activity [^{3}H]-3AMI starting from the naturally occurring compound quebrachitol [25].

3AMI appears to be acting as a *myo*-inositol antimetabolite in inhibiting cell-growth. The effect of 3AMI on v-*sis* NIH 3T3 cell-growth was antagonized by *myo*-inositol with an IC_{50} of 2 μ*M*. This is considerably lower than the concentration of *myo*-inositol in serum, which is around 40 μ*M* [46]. Microinjection or scrape-loading of monodeoxyfluoro-*myo*-inositols into cells has shown that only the 3-fluoro- and 5-fluoro isomers inhibit serum-stimulated cell proliferation [11]. It has been suggested that cells that do not require *myo*-inositol for growth may be capable of synthesizing *myo*-inositol de novo [47]. However, we could not detect *myo*-inositol synthesis by either wild-type NIH 3T3 cells or v-*sis* NIH 3T3 cells. We also considered the possibility that wild-type NIH 3T3 cells might have a more efficient salvage pathway than v-*sis* NIH 3T3 cells for the reutilization of *myo*-inositol produced by the breakdown of PtdIns [6]. We used LiCl to inhibit *myo*-inositol phosphate phosphatases [34] but could not find any evidence of

differences in the salvage of *myo*-inositol as a factor in the *myo*-inositol requirement for growth between wild-type NIH 3T3 cells and v-*sis* NIH 3T3 cells. Despite our inability to demonstrate differences in *myo*-inositol synthesis or *myo*-inositol salvage between the cell types, the v-*sis* NIH 3T3 cells that showed the greatest sensitivity to 3AMI were also those that required *myo*-inositol for growth. It therefore remains possible that v-*sis* NIH 3T3 cells are sensitive to 3AMI because they have low levels of endogenous *myo*-inositol, whereas wild-type NIH 3T3 cells have higher levels of endogenous *myo*-inositol, which counteracts the effects of 3AMI as a *myo*-inositol antimetabolite.

Studies using [^{3}H]-3AMI showed that it was incorporated into the cellular phospholipids of v-*sis* NIH 3T3 cells to about the same extent as was [^{3}H]-*myo*-inositol. However, wild-type NIH 3T3 cells incorporated [^{3}H]-3AMI to less than 5% of the extent of [^{3}H]-*myo*-inositol incorporation. This difference could not be explained by the absence of an uptake mechanism for [^{3}H]-3AMI in the wild-type NIH 3T3 cells, which had a K_m value about 10-fold that found for *myo*-inositol, but was the same as that observed in v-*sis* NIH 3T3 cells, which incorporated [^{3}H]-3AMI into cellular phospholipids. Additionally, the difference could not be explained by the inability of [^{3}H]-3AMI to act as a substrate for PtdIns synthetase. This finding was important to establish because on the basis of work with 5-deoxy-5-substituted *myo*-inositols, Moyer et al. [33] had suggested, that PtdIns synthetase had strict structural requirements for *myo*-inositols as substrates and, thus, it might not have tolerated 3-deoxy-3-substituted *myo*-inositols. Johnson et al. [22] have reported that 3-modified *myo*-inositols are substrates for PtdIns synthetase. We found that the K_m of 3AMI for rat-brain PtdIns synthetase was about 10-fold that of *myo*-inositol. Unless mouse PtdIns kinase has properties very different from those of the rat enzyme, the inability of 3AMI to be incorporated into wild-type NIH 3T3 cell phospholipids is unlikely to lie at the level of PtdIns synthetase. Another explanation, although one that we could not confirm (see above), is that wild-type NIH 3T3 cells have sufficient *myo*-inositol, produced through either synthesis or salvage, to compete with 3AMI for incorporation into phospholipids.

Although 3AMI was incorporated into the PtdIns fraction of v-*sis* NIH 3T3 cells and could be phosphorylated to give PtdIns monophosphate, the PtdIns monophosphate was not further phosphorylated to PtdIns bisphosphate. Cosulich et al. [11] have recently reported that [^{3}H]-3-deoxy-3-fluoro-*myo*-inositol incorporated into the PtdIns of electropermeabilized thymoctyes also fails to form detectable PtdIns bisphosphates. This may explain why there was no formation of inositol phosphates in v-*sis* NIH 3T3 cells labeled with [^{3}H]-3AMI, either under resting conditions or on stimulation with PDGF. PDGF causes inositol phosphate formation through activation of PIPLC-γ [27]. The major substrate for PIPLC is PtdIns(4,5)P_2, and PtdIns monophosphates do not appear to be substrates for PIPLC under physiological conditions [30]. The inability of 3AMI to form PtdIns phosphates that lead to the production of inositol phosphates is probably attributable to the observation that the PtdIns is not phosphorylated past the monophosphate stage and thus fails to give rise to PtdIns phosphates that are substrates for PIPLC.

In wild-type NIH 3T3 cells and v-*sis* NIH 3T3 cells grown with 3AMI for 3 days, there was no alteration in the $[Ca^{2+}]_i$ responses to bradykinin and vasopressin. Both agonists are thought to cause an increase in $[Ca^{2+}]_i$ due to inositol phosphate formation through activation of PIPLC [13, 35]. It might be expected that $[Ca^{2+}]_i$ signaling would be decreased in the v-*sis* NIH 3T3 cells since there was no formation of inositol phosphates. It is possible that the increase in $[Ca^{2+}]_i$ caused by these agents is not due to the formation of inositol phosphates. There is evidence that bradykinin, at least, causes an increase in $[Ca^{2+}]_i$ through the release of arachidonic acid [8]. Alternatively, inositol phosphate production may not be affected by exposure of cells to 3AMI, despite its incorporation into PtdIns. Another possibility is that the cells surviving a 3-day exposure to 3AMI may be those that have unaffected inositol phosphate production and, thus, normal Ca^{2+} signaling. The majority of the cells were lost by this period of 3AMI treatment and could not be studied for their $[Ca^{2+}]_i$, response.

The mechanism of growth inhibition by 3AMI is unknown. The block of inositol phosphate and DAG formation through decreased PtdIns hydrolysis in 3AMI treated cells might be expected to lead to inhibition of cell-growth. It is known that blockage of increases in PKC activity in fibroblasts due to DAG produced in response to growth factors such as PDGF can prevent increased DNA synthesis [13]. However, the overall importance of the inositol phosphate and DAG signaling pathway to mitogenesis remains unclear at this time [12].

An alternative target for inhibition by 3AMI is the PtdIns3K pathway. 3-Substituted PtdIns and PtdIns monophosphates formed from 3AMI would not be substrates for PtdIns3K and might antagonize the effects of PtdIns-3-phosphates. The mechanisms by which PtdIns-3-phosphates cause mitogenesis is not known but may involve reorganization of the cytoskeleton. Cellular proliferation is known to be closely associated with changes in the actin network of the cytoskeleton, and a correlation between actin polymerization and the formation of PtdIns(3,4,5)P_3 has been reported [14]. It is noteworthy that we observed signs of changes in the cytoskeleton in 3AMI-treated fibroblasts, which became rounded and detached from the cell-culture surface. We also have preliminary evidence that a PtdIns prepared from 3-deoxy-3-fluoro-*myo*-inositol is an inhibitor of PtdIns3K (G. Powis and M. Berggren, unpublished observations). Thus, as well as acting as antimetabolites of PtdIns-3-phosphates, PtdIns derived 3-AMI might inhibit PtdIns3K.

In summary, we have confirmed that 3AMI exhibits a remarkable selectivity for growth inhibition of v-*sis* NIH 3T3 cells as compared with wild-type NIH 3T3 cells. 3AMI appears to be acting as an antimetabolite of *myo*-inositol. Wild-type NIH 3T3 cells can take up 3AMI but do not form PtdIns from 3AMI although it is a substrate for PtdIns synthetase. This could explain the observed lack of growth inhibition of wild-type NIH 3T3 cells by 3AMI. 3AMI is

taken up by v-*sis* NIH 3T3 cells and is incorporated into PtdIns and PtdIns monophosphate but does not form PtdIns bisphosphates. The block in the formation of inositol phosphates as well as a block in the formation of PtdIns-3-phosphates could explain the observed inhibition of cell growth by 3AMI in v-*sis* NIH 3T3 cells.

Acknowledgement This work was supported by NIH grant CA 61015.

References

1. Abraham RT, Ho SN, Barna TJ, Mckean DJ (1987) Transmembrane signaling during interleukin 1-dependent T-cell activation. J Biol Chem 22: 2719
2. Allen DG, Blinks JR (1979) The interpretation of light signals from aequorin-injected skeletal and cardiac muscle cells: a new method of calibration. In: Ashley CC, Campbell AK (eds) Detection and measurement of free Ca^{2+} in cells. Elsevier-Holland, Amsterdam, p 159
3. Alley MC, Powis G, Appel PL, Kooistra KL, Lieber MM (1984) Activation and inactivation of cancer chemotherapeutic agents by rat hepatocytes cocultured with human tumor cell lines. Cancer Res 44: 549
4. Auger KR, Serunian LA, Soltoff SP, Libby P, Cantley LC (1989) PDGF-dependent tyrosine phosphorylation stimulates production of novel polyphosphoinositides in intact cells. Cell 57: 167
5. Berridge MJ (1983) Rapid accumulation of inositol trisphosphate reveals that agonists hydrolyse polyphosphoinositides instead of phosphatidylinositol. Biochem J 212: 849
6. Berridge MJ, Irvine RF (1989) Inositol phosphates and cell signaling. Nature 341: 197
7. Bohman D (1990) Transcription factor phosphorylation: a link between signal transduction and the regulation of gene expression. Cancer Cells 2: 337
8. Burch RM, Axelrod J (1987) Dissociation of bradykinin-induced prostaglandin formation from phosphatidylinositol turnover in Swiss 3T3 fibroblasts: evidence for G protein regulation of phospholipase A_2. Proc Natl Acad Sci USA 84: 6374
9. Cantley LC, Auger KR, Carpenter C, Duckworth B, Graziani A, Kapeller R, Soltoff S (1991) Oncogenes and signal transduction. Cell 64: 281
10. Cook SJ, Wakelam MJO (1989) Analyses of the water soluble products of phosphatidylcholine breakdown by ion-exchange chromatography. Biochem J 263: 581
11. Cosulich SC, Offer J, Smith GA, Hesketh R, Metcalfe JC (1993) Effects of fluorinated inositols on the proliferation of Swiss 3T3 fibroblasts. Biochem J 292: 719
12. Coughlin SR, Escobedo JA, Williams LT (1989) Role of phosphatidylinositol kinase in PDGF receptor signal transduction. Science 243: 1191
13. Dengler R, Faubner A, Müller-Esterl W, Roscher AA (1990) [Hyp^3]-bradykinin and [Hyp^3]-Lys bradykinin interact with B2-bradykinin receptors and stimulate inositol phosphate production in cultured human fibroblasts. FEBS Lett 262: 111
14. Eberle M, Traynor-Kaplan AE, Sklar LA, Norgauer J (1990) Is there a relationship between phosphatidylinositol trisphosphate and F-actin polymerization in human neutrophils? J Biol Chem 265: 16725
15. Eisenberg F Jr, Parthasarathy R (1987) Measurement of biosynthesis of *myo*-inositol from glucose 6-phosphate. Methods Enzymol 141: 127
16. Fukui Y, Kornbluth S, Jong S-M, Wang L-H, Hanafusa H (1989) Phosphatidylinositol kinase type I activity associates with various oncogene products. Oncogene Res 4: 283
17. Hansen CA, Mah S, Williamson JR (1986) Formation and metabolism of inositol 1,3,4,5-tetrakisphosphate in liver. J Biol Chem 261: 8100
18. Heldin C-H, Westermark B (1990) Platelet-derived growth factor: mechanism of action and possible in vivo function. Cell Regul 1: 555
19. Huang SS, Huang JS (1988) Rapid turnover of the platelet-derived growth factor receptor in *sis*-transformed cells and reversal by suramin. J Biol Chem 263: 12608
20. Irvine RF, Moore RM (1986) Microinjection of inositol 1,3,4,5-tetrakisphosphate activates sea urchin eggs by a mechanism dependent on external Ca^{2+}. Biochem J 240: 917
21. Irvine RF, Moore RM (1987) Inositol(1,4,5)tetrakisphosphate-induced activation of sea urchin eggs. Biochem Biophys Res Commun 146: 284
22. Johnson SC, Dahl J, Shih T-L, Schedler DJA, Anderson L, Benjamin TL, Baker DC (1993) Synthesis and evaluation of 3-modified 1D-*myo*-inositols as inhibitors and substrates of phosphatidylinositol synthase and inhibitors of *myo*-inositol uptake by cells. J Med Chem 36: 3628
23. Kaplan DR, Whitman M, Schaffhausen B, Pallas DC, White M, Cantley L, Roberts TM (1987) Common elements in growth factor stimulation and oncogenic transformation: 85 kd phosphoprotein and phosphatidylinositol kinase activity. Cell 50: 1021
24. Kikkawa U, Nishizuka Y (1986) The role of protein kinase C in transmembrane signaling. Annu Rev Cell Biol 2: 149
25. Kozikowski AP, Fauq AH, Aksoy IA, Seewald MJ, Powis G (1990) Synthesis of the first optically pure, fluorinated inositol 1,4,5-trisphosphate of *myo*-inositol stereochemistry and its effect on Ca^{2+} release in Swiss 3T3 cells. J Am Chem Soc 112: 7403
26. Kozikowski AP, Fauq AH, Powis G, Melder DC (1990) Efficient synthetic routes to fluorinated isosteres of inositol and their effects on cellular growth. J Am Chem Soc 112: 4528
27. Kriz R, Lin L-L, Sultzman L, Ellis C, Heldin C-H, Pawson T, Knopf J Phospholipase C isozymes: structural and functional similarities. Proto-oncogenes in cell development. Ciba Foundation Symposium. 150: 112
28. Li W, Zhou Q, Qin M, Tao L, Lous M, Hu T (1991) Reduced absolute rate of *myo*-inositol biosynthesis of cultured bovine retinal capillary pericytes in high glucose. Exp Cell Res 52: 569
29. Llano I, Marty A, Tanguy J (1987) Dependence of intracellular effects of GTP-2S and inositol trisphosphate on cell membrane potential and on external Ca^{2+} ions. Pflugers Arch 409: 499
30. Majerus PW, Ross TS, Cunningham TW, Caldwell KK, Jefferson AB, Bansal VS (1990) Recent insights in phosphatidylinositol signaling. Cell 63: 459
31. Markovits J, Linassier C, Fosse P, Couprie J, Pierre J, Jacquemin-Sablon A, Saucier JM, Le-Pecq JB, Larsen AK (1989) Inhibitory effects of the tyrosine kinase inhibitor genistein on mammalian DNA topoisomerase II. Cancer Res 49: 5111
32. Melchiori A, Carlone S, Allavena G, Aresu O, Parods S, Aarronson SA, Albinii A (1990) Invasiveness and chemotactic activity of oncogene transformed NIH/3T3 cells. Anticancer Res 10: 37
33. Moyer JD, Reizes O, Ahir S, Jiang C, Malinowski N, Baker DC (1989) Substrate properties of analogs of *myo*-inositol. Mol Pharmacol 33: 683
34. Nahorski SR, Potter BVL (1989) Lithium and the phosphoinositide cycle: an example of uncompetitive inhibition and its pharmacological consequences. Trends Pharmacol Sci 10: 139
35. Nånberg E, Rozengurt E (1988) Temporal relationship between inositol polyphosphate formation and increases in cytosolic Ca^{2+} in quiescent 3T3 cells stimulated by platelet-derived growth factor, bombesin and vasopressin. EMBO J 9: 2741
36. Nishizuka Y (1988) The molecular heterogeneity of protein kinase C and its implications for cellular regulation. Nature 334: 661
37. Olsen R, Santone K, Melder D, Oakes SG, Abraham R, Powis G (1988) An increase in intracellular free Ca^{2+} associated with serum free growth stimulation of Swiss 3T3 fibroblasts by epidermal growth factor in the presence of bradykinin. J Biol Chem 263: 18 030
38. Penner R, Matthews G, Neher E (1988) Regulation of calcium influx by second messengers in rat mast cells. Nature 334: 499

39. Powis G, Aksoy IA, Melder DC, Aksoy S, Eichinger H, Fauq AH, Kozikowski AP (1991) D-3-Deoxy-3-substituted *myo*-inositol analogues as inhibitors of cell-growth. Cancer Chemother Pharmacol 29: 95
40. Rao RJ, Strickland KP (1974) On the solubility, stability and partial purification of CDP-diacyl-*sn*-glycerol: inositol transferase from rat brain. Biochim Biophys Acta 348: 306
41. Rozengurt E (1989) Signal transduction pathways in mitogenesis. Br Med Bull 45: 515
42. Serunian LA, Haber MT, Fukui T, Kim JW, Rhee SG, Lowenstein JM, Cantley LC (1989) Polyphosphoinositides produced by phosphatidylinositol 3-kinase are poor substrates for phospholipases C from rat liver and bovine brain. J Biol Chem 264: 17 809
43. Sinha BK, Katki AG, Batist B, Cowan KH, Myers CE (1987) Adriamycin-stimulated hydroxyl radical formation in human breast tumor cells. Biochem Pharmacol 36: 793
44. Slack BE, Ball JE, Benos DJ (1986) Inositol-1,4,5-trisphosphate injection mimics fertilization potentials in sea urchin eggs. Am J Physiol 250: C340
45. Taylor SJ, Chae HZ, Rhee SG, Exton JH (1991) Activation of the β1 isozyme of phospholipase C by α subunits of the G_q class of G proteins. Nature 350: 516
46. Tietz NW, Finley PR, Pruden E (1988) Clinical guide to laboratory tests. Saunders, New York
47. Whiting PH, Palmano KP, Hawthorne JN (1979) Enzymes of *myo*-inositol and inositol lipid metabolism in rats with streptozotocin-induced diabetes. Biochem J 179: 549
48. Whitman M, Kaplan DR, Schaffhausen B, Cantley L, Roberts TM (1985) Association of phosphatidylinositol kinase activity with polyoma middle-T competent transformation. Nature 315: 239
49. Young D, Waitches G, Birchmeier C, Fasano O, Wigler M (1986) Isolation and characterization of a new cellular oncogene encoding a protein with multiple potential transmembrane domains. Cell 45: 711