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D-3-Deoxy-3-substituted *myo*-inositol analogues as inhibitors of cell growth

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Summary. A number of unnatural D-3-deoxy-3-substituted *myo*-inositols were synthesized and their effects on the growth of wild-type NIH 3T3 cells and oncogene-transformed NIH 3T3 cells were studied. The compounds were found to exhibit a diversity of growth-inhibitory activities and showed selectivity in inhibiting the growth of some transformed cells as compared with wild-type cells. Remarkably, D-3-deoxy-3-azido-*myo*-inositol exhibited potent growth-inhibitory effects toward v-*sis*-transformed NIH 3T3 cells but had little effect on the growth of wild-type cells. The growth-inhibitory effects of the *myo*-inositol analogues were antagonized by *myo*-inositol. Since [^{3}H]-3-deoxy-3-fluoro-myo-inositol was shown to be taken up by cells and incorporated into cellular phospholipids, we suggest that these unnatural *myo*-inositol analogues may act as antimetabolites in the phosphatidylinositol intracellular signalling pathways. Because cells transformed by oncogenes often exhibit elevated phosphatidylinositol turnover, the inhibition of signalling pathways that mediate oncogene action could offer new opportunities for controlling the growth of cancer cells.

Introduction

A major advance in our understanding of the control of cell function by agents that act on cell-surface receptors, including hormones, neurotransmitters, and growth factors, has been the identification of intracellular signalling pathways [6, 31, 55, 59]. The activation of a number of these pathways and their interaction probably determines the specificity of the cell response to different external stimuli. *myo*-Inositol occupies a unique position in the formation of second messengers that mediate the effects of growth factors [5, 47, 57] and oncogenes [16, 36, 57] on cell proliferation and transformation. Cells must either synthesize *myo*-inositol from glucose or obtain it from the extracellular environment [14, 19] and form phosphatidylinositols (PtdIns). Ligand activation of certain growth-factor receptors and transformation of cells by some oncogenes have been demonstrated to increase the turnover of PtdIns and the formation of *myo*-inositol-derived second messengers [5, 36, 57].

When bound to their ligands, several growth-factor and mitogen receptors activate PtdIns phospholipase C either by a mechanism involving tyrosine phosphorylation of the enzyme by the protein tyrosine kinase activity of the ligand-activated receptor [38, 49] or by a putative guanine nucleotide binding (G) protein mechanism [21]. PtdIns phospholipase C hydrolyzes phosphatidylinositol-4,5-bisphosphate [PtdIns(4,5)P_2] to form inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] and diacylglycerol [6]. Ins(1,4,5)P_3 releases Ca^{2+} from nonmitochondrial stores to increase the cytoplasmic free Ca^{2+} concentration $[Ca^{2+}]_i$, whereas diacylglycerol is an activator of protein kinase C [31]. Taken together, the increase in cytoplasmic free Ca^{2+} concentration and the increased activity of protein kinase C leads to a sequence of events that culminate in DNA synthesis and cell proliferation [57]. Other inositol phosphates in addition to Ins(1,4,5)P_3 are formed in the cell, and their functions are under investigation [6, 26]. Phosphorylation of Ins(1,4,5)P_3 by a specific 3-kinase gives inositol 1,3,4,5-tetrakisphosphate [Ins(1,3,4,5)P_4] [25], which may act synergistically with Ins(1,4,5)P_3 in the activation of Ca^{2+}-mediated responses in several systems [6, 24].

Recently, another PtdIns signalling pathway has been identified and linked to the action of some growth factors and oncogenes. Phosphatidylinositol-3′-kinase (also designated type I-phosphatidylinositol kinase) is found associated with a number of protein tyrosine kinases, including the ligand-activated receptors for insulin, platelet-

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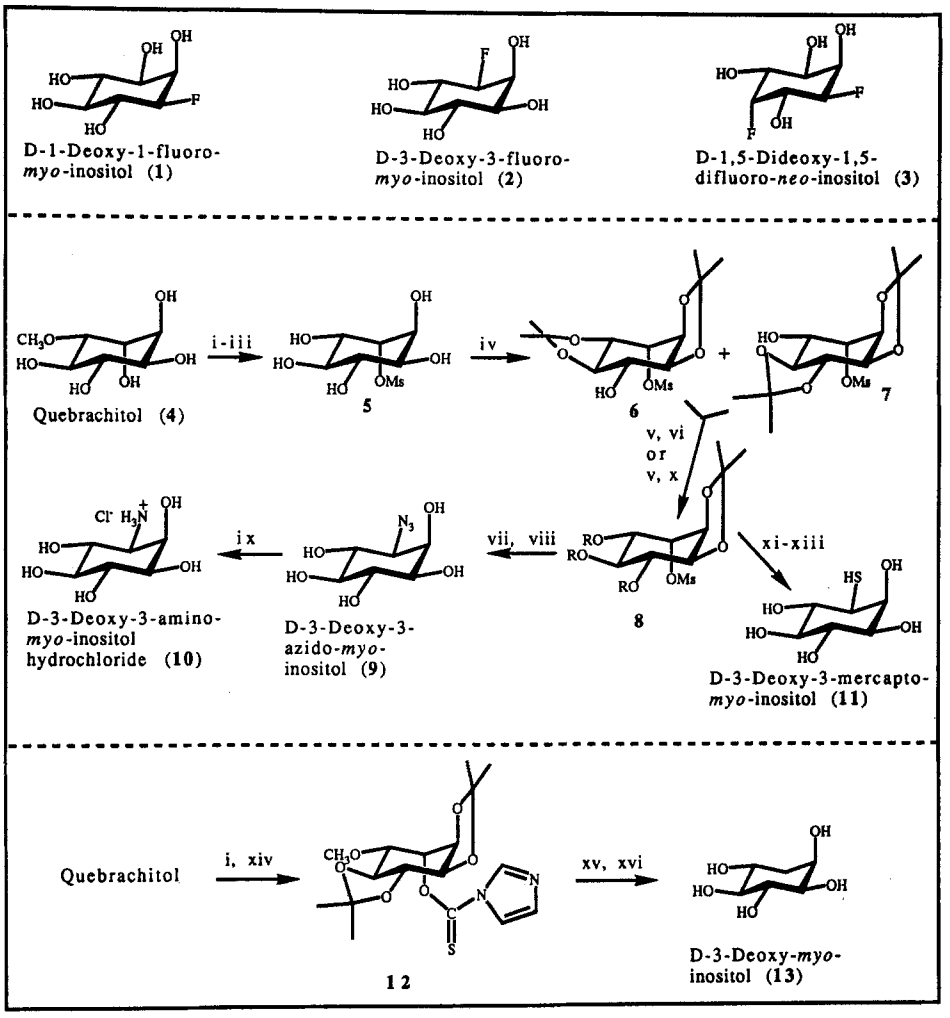

Fig. 1. Structures of *myo*-inositol isosteres used in the present study and schemes for their synthesis. Reagents and reactions conditons follow: *i*, $H_2C = C(OCH_3)CH_3$, camphorsulfonic acid (CSA), $(CH_3)_2NCHO$ (DMF), 65° C, 6 h (75%); *ii*, $MeSO_2Cl$, Et_3N, CH_2Cl_2, 0° C, 20 min (98%); *iii*, BBr_3, CH_2Cl_2, 23° C (86%); *iv*, $H_2C = C(OCH_3)CH_3$, CSA, DMF, 60° C (90%), 6 : 7 = 1 : 1; *v*, 95% aqueous CH_3CO_2H, room temperature, 18 h (91%); *vi*, $H_2C = CHOCH_2CH_3$, pyridinium-*p*-toluenesulfonate, CH_2Cl_2, 23° C (89%), R = -CH(CH$_3$)OCH$_2$CH$_3$; *vii*, NaN_3, hexamethylphosphoramide (HMPA), 115° C, 14 h (47%); *viii*, CH_3CO_2H, H_2O, THF (2 : 1 : 1), 80° C, 12 h (85%); *ix*, H_2, PtO_2, 0.4 *N* HCl, 23° C, 14 h (92%); *x*, Ac_2O, pyridine, 23° C (98%), R = –C(O)CH$_3$; *xi*, $(CH_3)_2NC(S)SNa \cdot 2H_2O$, HMPA, 80° C, 10 h (93%); xii, $LiAlH_4$, Et_2O, 23° C (88%); *xiii*, CF_3CO_2H, H_2O, 90° C, 1 h (99%); *xiv*, 1,1′-thiocarbonyldiimidazole, THF, reflux, 5 h (82%); *xv*, Bu_3SnH, toluene, reflux (92%); *xvi*, BBr_3, CH_2Cl_2, 23° C (83%)

derived growth factor (PDGF), epidermal growth factor (EGF), and colony-stimulating factor 1 (CSF-1); the protooncogene products $p60^{c\text{-}src}$ and $p59^{c\text{-}fyn}$; and the oncogene products $p60^{v\text{-}src}$, $p62^{v\text{-}yes}$, $P68^{gag\text{-}ros}$, $P130^{gag\text{-}fps}$, $P47^{gag\text{-}crk}$, and polyoma middle-T-antigen-activated $p60^{c\text{-}src}$ [3, 7, 9, 10, 15, 17, 18, 29, 48, 51, 58]. The enzyme phosphorylates the D-3 position of the *myo*-inositol ring of Ptdlns to yield a class of Ptdlns-3′-phosphates that are not substrates for hydrolysis by Ptdlns phospholipase C [35, 50] and therefore appear to exert their signalling action independently of the inositol phosphate pathway. PDGF receptor mutants that do not associate with phosphatidylinositol-3′-kinase do not exhibit a mitogenic response to PDGF [15], and mutants of middle T antigen with which the enzyme does not associate are nontransforming [18]. Some nontransforming oncogene mutants also associate with the activated enzyme [12, 17], and activated phosphatidylinositol-3′-kinase appears to be necessary but not sufficient for cell transformation. The relative contributions of the inositol phosphate and the phosphatidylinositol-3′-kinase signalling pathways in mediating the effects of growth factors and oncogenes on cell proliferation and transformation remain to be determined [22, 54].

As part of a continuing effort to understand the mechanisms of *myo*-inositol-based intracellular signalling in relation to cell proliferation, we synthesized a variety of substituted isosteres of *myo*-inositol that might act as antimetabolites of *myo*-inositol-derived second messengers. We concentrated on *myo*-inositol analogues substituted at the D-3 position because of the apparent importance of this position in signal transduction for growth factors and oncogenes that lead to cell proliferation and transformation. Such *myo*-inositol isosteres could act either by blocking the formation of certain Ptdlns and inositol phosphates or by forming fraudulent analogues. The present report describes the growth-inhibitory properties and the possible mechanism of action of five different isosteres of *myo*-inositol, each containing a group other than hydroxyl at the D-3 position, on wild-type NIH 3T3 cells and oncogene-transformed NIH 3T3 cells.

Materials and methods

Compounds. The chemical routes to the analogues D-1-deoxy-1-fluoro-*myo*-inositol (compound 1), D-3-deoxy-3-fluoro-*myo*-inositol (compound 2), and D-1,5-dideoxy-1,5-difluoro-*neo*-inositol (compound 3) have been reported elsewhere [32, 34]. The methods used to prepare D-3-azido-3-deoxy-*myo*-inositol (compound 9), D-3-amino-3-deoxy-*myo*-inositol (compound 10), D-3-deoxy-3-mercapto-*myo*-inositol (compound 11), and D-3-deoxy-*myo*-inositol (or L-viburnitol, compound 13) are presented in Fig. 1 [8, 42, 44, 46]. Quebrachitol, a by-product of the rubber tree industry, was used as the homochiral building block for the

preparation of the latter four compounds. Protection/deprotection protocols were required for the synthesis of compounds 9–11 and 13, whereas such additional steps were not needed in the preparation of compound 2. All of the analogues synthesized were shown to be free of detectable impurities as determined by [^{1}H]–[^{13}C], and [^{19}F]-NMR (nuclear magnetic resonance) and by a comparison of their optical rotations and other physical data with those reported in the literature. [^{3}H]-D-3-Deoxy-3-fluoro-*myo*-inositol (sp. act., 800 μCi/mmol) was prepared by a catalytic exchange process using [^{3}H]$_2$O and Ranay nickel [40]. [2-^{3}H]-*myo*-inositol (sp. act., 20 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, Mo.). *myo*-Inositol, cardiolipin, phosphatidylinositol, phosphatidylserine, phosphatidylcholine, and prostaglandin $F_{2\gamma}$ ($PGF_{2\gamma}$) were purchased from Sigma Chemical Co. (St. Louis, Mo.). PDGF, β-chain homodimer, was obtained from Bachem Inc. (Torrance, Calif.) and EGF from Calbiochem (Irvine, Calif.). Dulbecco's modified Eagle's medium (DMEM) and *myo*-inositol-free DMEM were purchased from Gibco (Grand Island, N. Y.).

Cell lines: Wild-type NIH 3T3 cells were obtained from the American Type Culture Collection (Rockville, Md.). The oncogene-expressing NIH 3T3 cell lines used included, v-*sis* [4], provided by Dr. D. S. Aaronson (National Cancer Institute, Bethesda, Md.); v-*src* and c-*src* [28], provided by Dr. N. Maihle (Mayo Clinic); v-*erb*B and H-*ras* [39], provided by Dr. D. S. Aaronson; *mas* [61], provided by Dr. D. Young (Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.); and v-*mos* [37], provided by Dr. H. Grunicke (University of Innsbruck, Austria).

Cell-growth assay. Wild-type NIH 3T3 cells and oncogene-expressing NIH 3T3 cells were maintained in bulk culture in DMEM supplemented with 10% heat-inactivated calf serum and were passaged using 0.05% trypsin and 0.5 mM ethylenediaminetetraacetic acid (EDTA). For cell-growth assays, the cells were plated at a density of 5×10^3 cells in 1.6-cm-diameter culture wells in 0.5 ml DMEM containing 10% heat-inactivated calf serum and were allowed to attach to the surface of the well for 24 h. The medium was then replaced with fresh medium containing the *myo*-inositol analogues. In studies in which *myo*-inositol was omitted from the medium, *myo*-inositol-free DMEM and dialyzed, heat-inactivated calf serum were used, which did not adversely affect cell growth over 3 days. Unless otherwise stated, adherent cells were harvested after 3 days and were counted using an automated cell counter (Coulter, Hialeah, Fla.). Inhibition of cell growth caused by serial concentrations of analogues was expressed as a percentage of the number of nontreated cells at the end of the 3-day incubation period. Incubations were conducted in quadruplicate. The mean concentration of analogue required to cause 50% inhibition of cell growth (IC_{50}) ± SE was calculated from nonlinear least-squares regression analysis of the cell-proliferation concentration data [2]. For studies investigating the time course of cell-growth inhibition, cells were plated in the same manner and then harvested and counted daily for 6 days.

myo-*Inositol uptake and incorporation into cellular lipids.* Cells were plated at a density of 2×10^5 cells/1.6-cm culture well and were grown for 24 h in DMEM supplemented with 10% heat-inactivated calf serum. The cells were then washed with 4 × 1 ml *myo*-inositol-free DMEM and incubated in the same medium containing either 0.02–1 mM [^{3}H]-*myo*-inositol together with different concentrations of the analogues or 0.01–5 mM [^{3}H]-D-3-deoxy-3-fluoro-*myo*-inositol for 2 h at 37°C. The cells were then washed with 6 × 1 ml Dulbecco's phosphate-buffered saline (PBS) containing 1% bovine serum albumin, digested with 1 ml 75% methanol for 1 h, and counted by liquid scintillation. For studies investigating the incorporation of radioactivity into cellular lipids, cells were incubated for up to 5 days with *myo*-inositol-free DMEM and 10% dialyzed, heat-inactivated calf serum containing 1 mM (0.8 μCi/ml) [^{3}H]-*myo*-inositol or 1 mM (0.8 μCi/ml) [^{3}H]-D-3-deoxy-3-fluoro-*myo*-inositol. The cells were then washed with 6 × 1 ml PBS, harvested with trypsin and EDTA, and counted. An aliquot of cells was taken for liquid scintillation counting and the remainder were extracted with 3 × 2 ml chloroform:methanol (2:1, v/v). The combined organic extract was washed with 5 × 5 ml 0.9% NaCl until no further counts were incorporated into the aqueous layer, and the organic extract was dried under N_2. The residue was dissolved in a small volume of chloroform prior to liquid scintillation counting.

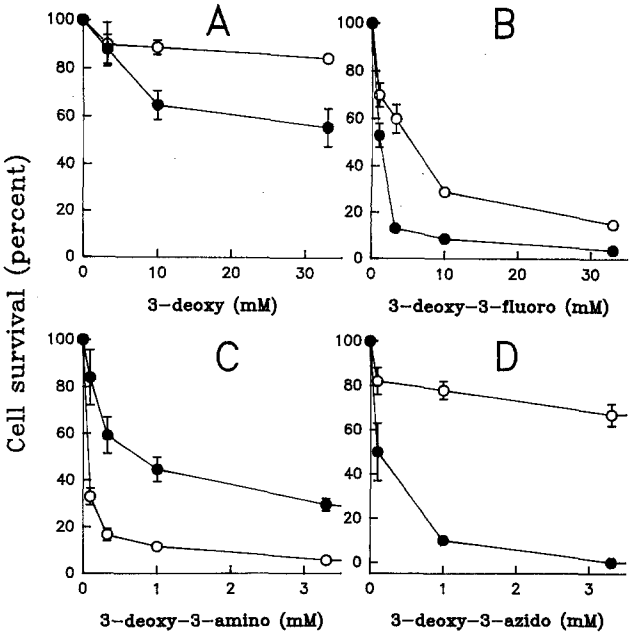

Fig. 2 A–D. Concentration-response curves showing inhibition of NIH 3T3 cell growth by D-3-modified-*myo*-inositol analogues. Wild-type NIH 3T3 cells (○) or v-*sis*-transformed NIH 3T3 cells (●) were plated at a density of 5×10^3 cells/1.6-cm-diameter culture well in DMEM containing 10% heat-inactivated calf serum and were allowed to attach to the surface of the well for 24 h. The medium was replaced with *myo*-inositol-free DMEM containing 10% dialyzed, heat-inactivated calf serum and *myo*-inositol analogues at the concentrations shown. After 3 days, adherent cells were harvested with trypsin and counted using an automated cell counter. Inhibition of cell growth after 3 days' exposure to the analogues was expressed as a percentage of the number of nontreated cells. Each point represents the mean value for quadruplicate determinations and bars indicate the SD. The analogues used included **A** D-3-deoxy-*myo*-inositol, **B** D-3-deoxy-3-fluoro-*myo*-inositol, **C** D-3-deoxy-3-amino-*myo*-inositol hydrochloride, and **D** D-3-deoxy-3-azido-*myo*-inositol. Each plot is representative of at least 3 separate studies.

Analysis of cellular phospholipids. Wild-type NIH 3T3 cells or v-*sis*-transformed NIH 3T3 cells were grown for 24 h in *myo*-inositol-free DMEM supplemented with 10% dialyzed, heat-inactivated calf serum and 1 mM (0.8 μCi/ml) [^{3}H]-D-3-deoxy-3-fluoro-*myo*-inositol. Total cell lipids were extracted as described above and 500 μg lipid was taken for the analysis of phospholipids by high-performance liquid chromatography (HPLC) on a 25-cm, Si-50, 5-μm LiChroCart column (Merck, Darmstadt, FRG) using an eluent consisting of acetonitrile with 0.05% phosphoric acid run for 5 min at a flow rate of 1 ml/min, followed by a curvilinear gradient over 40 min to 0.2% phosphoric acid in acetonitrile and then 0.2% phosphoric acid in methanol for 15 min. Detection was carried out using UV absorption at 205 nm and radiochromatogram flow detection (Flo One Beta, Packard Instruments, Downers Grove, Ill.).

Measurement of Ca^{2+} concentration. Changes in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) were measured using the Ca^{2+}-sensitive photoprotein aequorin. Wild-type NIH 3T3 cells were loaded with aequorin by a low-Ca^{2+} centrifugation technique as previously described [43] and 10^6 cells were plated in 35-mm culture dishes containing 2 ml DMEM supplemented with 10% heat-inactivated calf serum. After incubation overnight, during which time the cells attached to the surface of the culture dish, the medium was replaced with DMEM in the absence of calf serum for 3 h. Some cells were grown for 2 days with 10 mM D-3-deoxy-3-fluoro-*myo*-inositol in *myo*-inositol-free DMEM supplemented with 10% dialyzed, heat-inactivated calf serum, loaded with aequorin, and then exposed to the same medium overnight prior to the measurement of $[Ca^{2+}]_i$. The culture dish was placed in a 37°C thermostatted holder over a photomultiplier tube, the surface of the medium was flushed with

Table 1. Growth inhibition of NIH 3T3 and v-*sis*-transformed NIH 3T3 cells by *myo*-inositol analogues

	NIH 3T3 cell IC_{50} (mM)	v-*sis* NIH 3T3 cell IC_{50} (mM)
Without *myo*-inositol:		
D-3-deoxy-*myo*-inositol	NT[a]	43.2 ±8.4[b]
D-3-deoxy-3-fluoro-*myo*-inositol	7 ± 1.3	1.1 ±0.6*
D-3-deoxy-3-mercapto-*myo*-inositol[c]	10.8 ± 3	4 ±0.9*
D-3-deoxy-3-amino-*myo*-inositol	0.07 ± 0.01	1.1 ±0.2*
D-3-deoxy-3-azido-*myo*-inositol	51.7 ± 5.6[b]	0.04 ±0.01*
D-1-deoxy-1-fluoro-*myo*-inositol	21.4 ± 2.4	8.7 ±0.6*
D-1,5-dideoxy-1,5-difluoro-*neo*-inositol	NT[a]	NT[a]
With *myo*-inositol:		
D-3-deoxy-3-fluoro-*myo*-inositol	48.3 ±12.3[b, **]	NT[a, **]
D-3-deoxy-3-amino-*myo*-inositol	0.53 ± 0.07**	0.59 ±0.08**
D-3-deoxy-3-azido-*myo*-inositol	18.7 ± 4.1**	12.8 ±2.1**

Cell growth was measured by the increase in cell number measured over 3 days in cells that were continuously exposed to a range of concentrations of either *myo*-inositol analogue in *myo*-inositol-free DMEM containing 10% dialyzed, heat-inactivated calf serum or *myo*-inositol-containing DMEM supplemented with 10% nondialyzed, heat-inactivated calf serum as described in Fig. 2. Values were expressed as the mean concentration of analogue that was required to cause 50% inhibition of cell growth ± SE (IC_{50}) as calculated from nonlinear least-squares regression analysis of the cell proliferation-concentration response data

[a] NT, Nontoxic, with <20% growth inhibition being obtained at 33 mM, which was the highest concentration studied

[b] Estimated from regression analysis of the data beyond 33 mM

[c] Studies were conducted in the presence of 0.1 mM dithiothreitol to limit oxidation of the analogue; at this concentration, dithiothreitol was nontoxic to the cells

* $P < 0.05$ vs the value obtained for wild-type cells

** $P < 0.05$ vs the value obtained in the absence of *myo*-inositol

humidified 5% CO_2: 95% air, and mitogens were added. The concentrations of mitogens used to increase $[Ca^{2+}]_i$ were 3.3×10^{-9} M PDGF, 10^{-5} M $PGF_{2\gamma}$, and 5×10^{-9} M EGF, which were shown in preliminary studies to produce maximal $[Ca^{2+}]_i$ responses in NIH 3T3 cells. At the end of the study, the cells were lysed with a solution of 1% Triton X-100 and 5 mM $CaCl_2$ and the total light signal was integrated. The mitogen-induced $[Ca^{2+}]_i$ responses were integrated and normalized by dividing them by the total light signal obtained after the cells had been lysed. An estimate of $[Ca^{2+}]_i$ was obtained using the calibration method for aequorin previously reported by Allen and Blinks [1].

Results

Cell-growth inhibition

The *myo*-inositol analogues were investigated for their effects on the growth of wild-type NIH 3T3 cells [27] and v-*sis*-transformed NIH 3T3 cells [4]. We initially chose to study v-*sis*-transformed cells because autocrine growth stimulation by the v-*sis* oncogene requires the interaction of its protein product, which is similar to and functionally equivalent to PDGF, with PDGF receptors inside or on the cell surface [11, 20, 30]. Such transformed cells are known to exhibit constitutively activated phosphatidylinositol-3′-kinase activity as compared with the nontransformed cells [29] and might thus be expected to show an increased sensitivity to agents that block the phosphatidylinositol-3′-kinase signalling pathway.

The diverse growth-inhibitory activity of the D-3-*myo*-inositol analogues is shown in Fig. 2, and the IC_{50} values are given in Table 1. It should be noted that even at high concentrations (50 mM), *myo*-inositol did not inhibit the growth of wild-type or v-*sis*-transformed NIH 3T3 cells (results not shown). D-3-Deoxy-*myo*-inositol produced no inhibition of the serum-dependent proliferation of wild-type NIH 3T3 cells and showed only weak activity against v-*sis*-transformed NIH 3T3 cells. D-3-Deoxy-3-fluoro-*myo*-inositol was more effective in inhibiting the proliferation of wild-type NIH 3T3 cells (IC_{50} = 7 mM) and exhibited substantially more activity against v-*sis*-transformed NIH 3T3 cells (IC_{50} = 1.1 mM). Inhibition of the growth of both wild-type and v-*sis*-transformed cells by D-3-deoxy-3-fluoro-*myo*-inositol was found to require at least 2 days of continuous exposure to the agent (Fig. 3). Although D-3-deoxy-3-fluoro-*myo*-inositol represents an easily accessible analogue, its growth-inhibitory activity was relatively modest.

In a search for other, more potent analogues, we investigated substitution of the D-3-hydroxy group of *myo*-inositol by thiol, amino and azido groups. D-3-Deoxy-3-mercapto-*myo*-inositol was found to display growth-inhibitory activity similar to that exhibited by the the 3-fluoro analogue. However, D-3-deoxy-3-amino-*myo*-inositol was considerably more potent in inhibiting the growth of wild-type NIH 3T3 cells (IC_{50} = 0.07 mM), although it exhibited only moderate activity against v-*sis*-transformed NIH 3T3 cells (IC_{50} = 1.1 mM). Most interestingly, D-3-deoxy-3-azido-*myo*-inositol, a compound that served as the synthetic precursor for D-3-deoxy-3-amino-*myo*-inositol, proved to be a potent inhibitor of the growth of v-*sis*-transformed NIH 3T3 cells (IC_{50} = 0.04 mM) but a very weak inhibitor of the growth of wild-type NIH 3T3 cells (IC_{50} = 51.7 mM). This indicates a >1,200-fold selectivity of D-3-deoxy-3-azido-*myo*-inositol for the v-*sis* transformed cells as compared with the wild-type cells. The growth-inhibitory activity of some *myo*-inositol analogues substituted at other positions was also studied. D-1-Deoxy-1-fluoro-*myo*-inositol and D-1,5-dideoxy-1,5-difluoro-

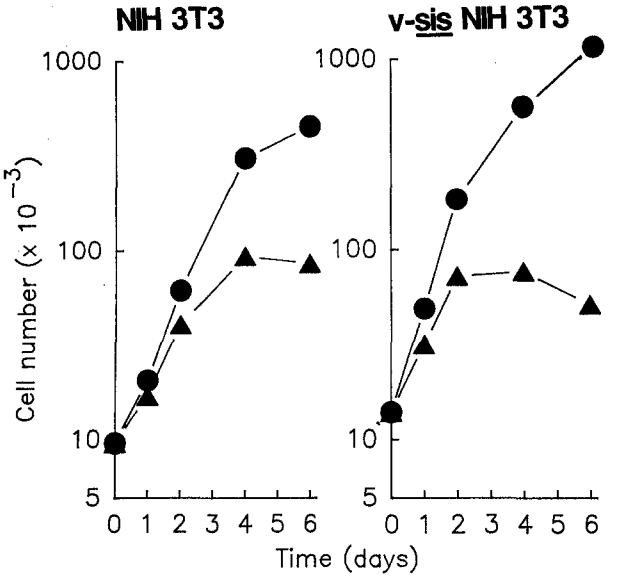

Fig. 3. Time course of cell-growth inhibition of NIH 3T3 and v-*sis* NIH 3T3 cells by 33 mM D-3-deoxy-3-fluoro-*myo*-inositol. Cells were grown in *myo*-inositol-free DMEM supplemented with 10% heat-inactivated calf serum (●) or in the same medium supplemented with 33 mM D-3-deoxy-3-fluoro-*myo*-inositol (▲)

neo-inositol were considerably less potent in inhibiting cell growth than was D-3-deoxy-3-fluoro-*myo*-inositol.

Effect of myo-inositol

The growth-inhibition studies described herein initially used medium that was *myo*-inositol-deficient. It could be expected that *myo*-inositol would compete with the *myo*-inositol analogues either for uptake into the cell or for incorporation into phosphatidylinositol, thus decreasing the growth-inhibitory activity of the analogues. Studies were therefore conducted in culture medium containing 40 μM *myo*-inositol, which is similar to the concentration found in human serum [53]. The addition of *myo*-inositol at this concentration did not alter the growth rate of wild-type or v-*sis*-transformed NIH 3T3 cells over 3 days. In the *myo*-inositol-containing medium, inhibition of the growth of both wild-type and v-*sis*-transformed NIH 3T3 cells by D-3-deoxy-3-fluoro-*myo*-inositol and that of v-*sis*-transformed NIH 3T3 cells by D-3-deoxy-3-azido-*myo*-inositol was considerably decreased (Table 1). Surprisingly, inhibition of the growth of wild-type NIH 3T3 cells by D-3-deoxy-3-azido-*myo*-inositol showed potentiation in this medium. Inhibition of the growth of both cell types by D-3-deoxy-3-amino-*myo*-inositol was only moderately suppressed by the *myo*-inositol. The IC_{50} of *myo*-inositol for the reversal of growth inhibition by D-3-deoxy-3-amino-*myo*-inositol was 6.4±0.6 μM in wild-type NIH 3T3 cells and 12.5±3.2 μM in v-*sis*-transformed NIH 3T3 cells.

Inhibition of the growth of other oncogene-expressing cells

The ability of D-3-deoxy-3-fluoro-*myo*-inositol and D-3-deoxy-3-azido-*myo*-inositol to inhibit the growth of a variety of oncogene-expressing NIH 3T3 cells in the presence and absence of *myo*-inositol was studied (Table 2). There was no apparent correlation between the extent of cell-growth inhibition by the *myo*-inositol analogues and the approximate cell-doubling times of the different cell lines, which were 8–12 h (v-*sis*, v-*src*, c-*src*), 15–20 h (wild-type, H-*ras*), and 22–27 h (v-*mos*, v-*erb*B, *mas*). The ability of 40 μM *myo*-inositol to reverse growth inhibition by the analogues was most marked in v-*sis*-, H-*ras*-, v-*erb*B-, and v-*mos*-expressing NIH 3T3 cells. In the presence of very high (10 mM) concentrations of *myo*-inositol, the growth inhibition caused by D-3-deoxy-3-fluoro-*myo*-inositol and D-3-deoxy-3-azido-*myo*-inositol in *mas*-expressing NIH 3T3 cells was also reversed (results not shown).

Uptake of myo-inositol

Studies into the mechanism of action of the analogues focused first on their uptake and then on their inhibition of *myo*-inositol uptake. The uptake of [^{3}H]-*myo*-inositol by wild-type NIH 3T3 cells showed a mean K_m value of

Table 2. Growth inhibition of oncogene-expressing NIH 3T3 cells by *myo*-inositol analogues

	D-3-deoxy-3-fluoro-*myo*-inositol		D-3-deoxy-3-azido-*myo*-inositol	
	– *myo*-inositol IC_{50}(mM)	+ *myo*-inositol IC_{50} (mM)	– *myo*-inositol IC_{50} (mM)	+ *myo*-inositol IC_{50} (mM)
v-*src*	20.2±4.2	16.8±3.7	NT[a]	24.9±3.2*
c-*src*	19.3±3.8	19.6±2.9	40.7±10.9[b]	25.1±5.3*
H-*ras*	2.9±0.4	24 ±2.1*	4.1± 1.3	21.8±4.1*
v-*erb*B	37 ±1.5[b]	62.3±9.2[b],*	20.2± 5.4	29.2±5.1*
mas	3.5±0.6	4.6±0.5	13.9± 2.9	4.7±0.3*
v-*mos*	13.7±5.1	59.2±4*	8.9± 2.8	30.4±4*

Growth was measured as described in Table 1 in *myo*-inositol-free DMEM supplemented with 10% dialyzed, heat-inactivated calf serum (– *myo*-inositol) or in DMEM with 40 μM *myo*-inositol supplemented with 10% heat-inactivated calf serum (+ *myo*-inositol), and the IC_{50} values (± SE) were calculated

[a] NT, Nontoxic, with <20% growth inhibition being obtained at 33 mM, which was the highest concentration tested

[b] Estimated from regression analysis of the data beyond 33 mM

* P <0.05 vs the value obtained in the absence of *myo*-inositol

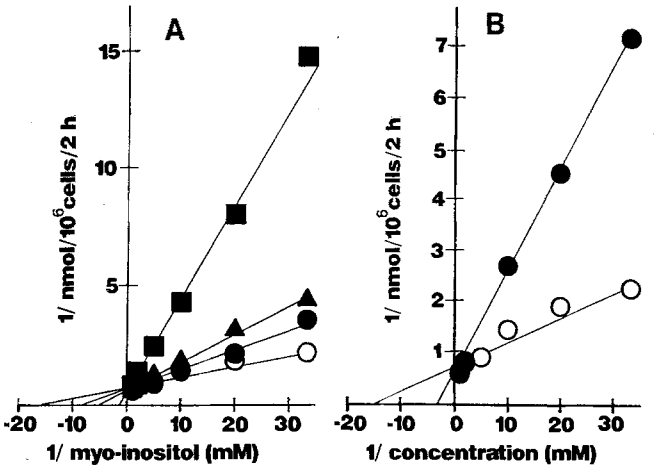

Fig. 4 A, B. Uptake of *myo*-inositol and D-3-deoxy-3-fluoro-*myo*-inositol by wild-type NIH 3T3 cells. **A** Lineweaver-Burk plot of the uptake of [^{3}H]-*myo*-inositol over 2 h in the absence (○) and the presence of 0.5 (●), 1 (▲), and 3 mM D-3-deoxy-3-fluoro-*myo*-inositol (■). **B** Lineweaver-Burk plot of the uptake of [^{3}H]-*myo*-inositol (○) and [^{3}H]-D-3-deoxy-3-fluoro-*myo*-inositol (●)

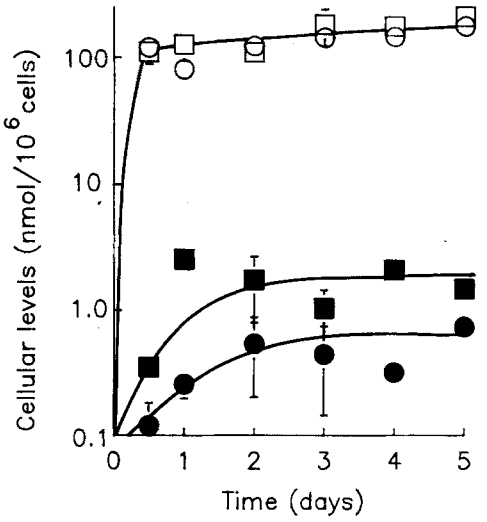

Fig. 5. Incorporation of radioactivity from [^{3}H]-*myo*-inositol or [^{3}H]-D-3-deoxy-3-fluoro-*myo*-inositol into NIH 3T3 cells. Cells were incubated in *myo*-inositol-free DMEM containing 10% dialyzed, heat-inactivated calf serum with 1 mM [^{3}H]-*myo*-inositol (0.8 μCi/ml; ○, ●) or 1 mM [^{3}H]-D-3-deoxy-3-fluoro-*myo*-inositol (0.8 μCi/ml; □, ■). The total radioactivity present in the cells (*open symbols*) or the radioactivity extracted into chloroform:methanol (2:1, v/v; *closed symbols*) was measured. Points represent the mean values for 3 determinations and bars indicate the SD

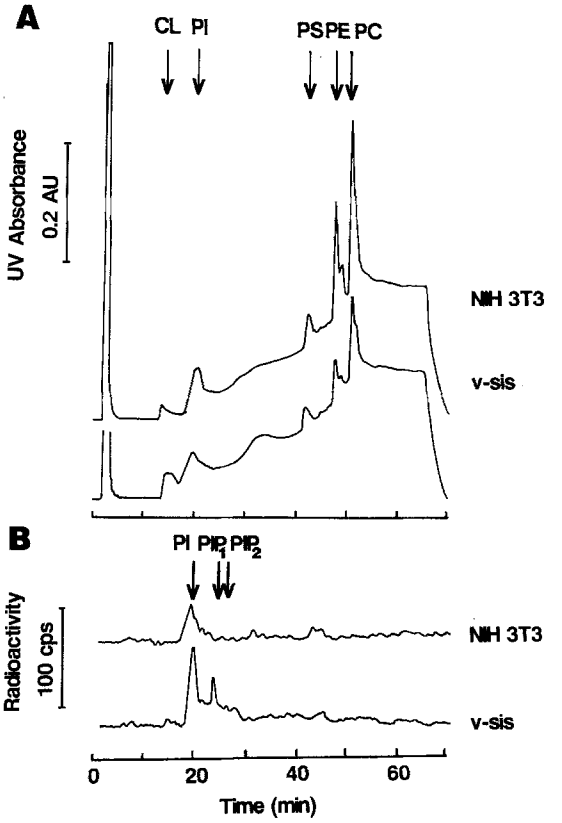

Fig. 6 A, B. HPLC of lipid extracts from NIH 3T3 cells grown in the presence of [^{3}H]-D-3-deoxy-3-fluoro-*myo*-inositol. Wild-type NIH 3T3 cells or v-*sis*-transformed NIH 3T3 cells were grown for 24 h in *myo*-inositol-free DMEM supplemented with 10% dialyzed heat-inactivated calf serum containing 1 mM [^{3}H]-D-3-deoxy-3-fluoro-*myo*-inositol (0.8 μCi/ml). Total cell lipids were extracted into chloroform:methanol (2:1, v/v) and 500 μg cellular lipid was taken for HPLC analysis as described in Materials and methods. **A** Detection by UV absorption at 205 nm. *Arrows* show the positions of phospholipid standards. *CL*, Cardiolipin; *PI*, phosphatidylinositol; *PS*, phosphatidylserine; *PC*, phosphatidylcholine. **B** Detection by radiochromatogram flow detector with correction for quenching. *Arrows* show the positions of [^{3}H]-radiolabeled standards. *PI*, Phosphatidylinositol; *PIP*, phosphatidylinositol phosphate; *PIP*$_2$, phosphatidylinositol-4,5-bisphosphate

68 ± 5 μM (n = 5) and a mean V_{max} value of 2.75 ± 0.26 nmol 10^{-6} cells h^{-1} (Fig. 4). These values are similar to those previously reported for *myo*-inositol uptake by NIH 3T3 cells [52]. [^{3}H]-D-3-deoxy-3-fluoro-*myo*-inositol uptake exhibited a K_m value of 0.55 mM and a V_{max} value of 2.94 nmol 10^{-6} cells 2 h^{-1}. To obtain an estimate of the relative affinity for cellular uptake of the other D-3-substituted analogues, competitive inhibition studies of the uptake of [^{3}H]-*myo*-inositol were conducted. The K_i values were: D-3-deoxy-*myo*-inositol, 4.67 ± 0.19 mM; D-3-deoxy-3-fluoro-*myo*-inositol, 0.63 ± 0.08 mM; D-3-deoxy-3-azido-*myo*-inositol, 0.35 ± 0.07 mM; and D-3-deoxy-3-amino-*myo*-inositol, 0.28 ± 0.02 mM.

Incorporation into phospholipids

For investigations of the incorporation of the *myo*-inositol analogue into cellular phospholipids, NIH 3T3 cells were incubated with medium containing [^{3}H]-*myo*-inositol or [^{3}H]-D-3-deoxy-3-fluoro-*myo*-inositol (Fig. 5). For both compounds, the incorporation of total radioactivity into cells reached a maximum at approximately 12 h as expressed relative to cell number and remained constant for at least 5 days. The incorporation of radioactivity into cellular phospholipids was about 1% of that of total radioactivity for [^{3}H]-*myo*-inositol but was approximately twice this value for [^{3}H]-D-3-deoxy-3-fluoro-*myo*-inositol. An

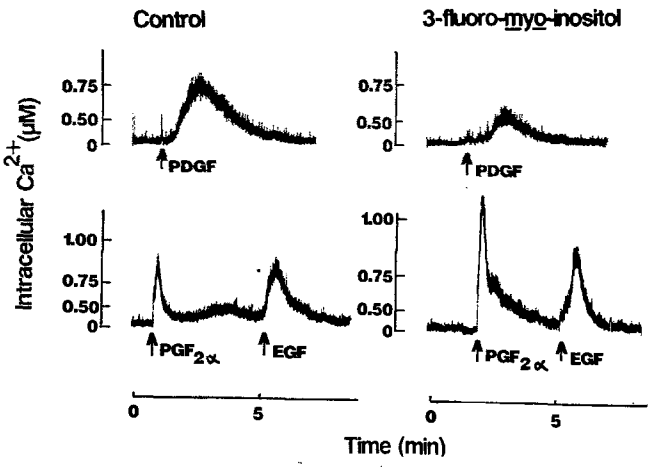

Fig. 7. Effect of D-3-deoxy-3-fluoro-*myo*-inositol on $[Ca^{2+}]_i$ responses in NIH 3T3 cells. Cells were grown in *myo*-inositol-free DMEM supplemented with 10% dialyzed, heat-inactivated calf serum and 33 mM D-3-deoxy-3-fluoro-*myo*-inositol for 3 days. Control NIH 3T3 cells were grown in DMEM supplemented with 10% heat-inactivated calf serum. At 24 h prior to study, the cells were harvested, loaded with aequorin, and allowed to reattach to tissue-culture plates. Both control and analogue-treated cells were exposed to the appropriate medium for 3 h prior to the addition of 3.3×10^{-9} M PDGF and of 10^{-5} M $PGF_{2\gamma}$ followed by 5.4×10^{-9} M EGF. Mitogen additions are indicated by *arrows*

increased in the incorporation of D-3-deoxy-3-fluoro-*myo*-inositol might be expected if downstream metabolism of the PtdIns were blocked by substitution of the D-3 position of the *myo*-inositol ring. HPLC studies of the cellular phospholipids revealed that the radioactivity from [^{3}H]-D-3-deoxy-3-fluoro-*myo*-inositol was contained exclusively in the peaks due to PtdIns in both the wild-type and the v-*sis*-transformed NIH 3T3 cells (Fig. 6). Because of synthetic limitations, the amount of tritium incorporated into the [^{3}H]-D-3-deoxy-3-fluoro-*myo*-inositol was insufficient to determine whether the analogue was incorporated into specific PtdIns or into inositol phosphates. As expected, the incorporation of 0.5 μM [^{3}H]-*myo*-inositol into cellular phospholipids of NIH 3T3 cells was inhibited in the presence of 10 mM D-3-deoxy-3-fluoro-*myo*-inositol (results not shown).

Effect on Ca^{2+} signalling

In an attempt to determine which *myo*-inositol signalling pathways were being inhibited by the analogues, NIH 3T3 cells were grown in the presence of 33 mM D-3-deoxy-3-fluoro-*myo*-inositol for 3 days and the changes in $[Ca^{2+}]_i$ in response to PDGF, $PGF_{2\gamma}$, and EGF treatment were compared with those observed in control cells (Fig. 7). The mean peak $[Ca^{2+}]_i$ responses of untreated and D-3-deoxy-3-fluoro-*myo*-inositol-treated cells ($n = 3$), respectively, were 0.89 ± 0.04 and 0.63 ± 0.04 μM for PDGF ($P < 0.05$), 1.04 ± 0.5 and 1.31 ± 0.21 μM for $PGF_{2\gamma}$ ($P < 0.05$), and 0.93 ± 0.11 and 0.74 ± 0.13 μM for $PGF_{2\gamma}$ followed by EGF ($P < 0.05$). From these results, it appears that exposure of cells to D-3-deoxy-3-fluoro-*myo*-inositol does not result in a decrease in $[Ca^{2+}]_i$ responses to mitogens.

Discussion

Using novel synthetic methods, we synthesized a series of D-3-substituted *myo*-inositol analogues that exhibit growth-inhibitory activity toward a panel of oncogene-transformed NIH 3T3 cells. In the absence of *myo*-inositol, three types of growth-inhibition responses to the analogues were apparent: (1) relative insensitivity (IC_{50}, ≥ 20 mM), viz., wild-type, v-*src*-, c-*src*-, and v-*erb*B-expressing NIH 3T3 cells; (2) moderate sensitivity (IC_{50}, 1–20 mM), viz., H-*ras*-, *mas*-, and v-*mos*-expressing cells; and (3) high sensitivity (IC_{50}, <1 mM), viz., v-*sis*-transformed cells. Exceptions to these generalizations were the moderate and high sensitivity of the wild-type cells to D-3-deoxy-3-fluoro-*myo*-inositol and D-3-deoxy-3-amino-*myo*-inositol, respectively. The most active analogue synthesized was D-3-deoxy-3-azido-*myo*-inositol, which exhibited a >1,200-fold selectivity in its inhibition of the growth of v-*sis*-transformed cells as compared with wild-type NIH 3T3 cells.

In 1963, Eagle and McCasland [13] reported on studies investigating the ability of a series of unnatural inositol analogues, including some halogenated *chiro*- and *neo*-inositols, to stimulate the growth of a *myo*-inositol-requiring KB carcinoma cell line in *myo*-inositol-free medium. None of the analogues substituted for *myo*-inositol in stimulating cell growth, and at a 500:1 ratio to *myo*-inositol, they did not inhibit cell growth. The synthesis of 2-, 4-, and 5-halogen-substituted *myo*-inositol analogues, mostly as racemates, was recently reported by Moyer et al. [40, 41]. The only 3-substituted analogue synthesized was 3-deoxy-*myo*-inositol, and the growth-inhibitory activity of the analogues was not reported. The present article is the first report on the synthesis and antiproliferative activity of D-3-deoxy-3-substituted *myo*-inositol analogues. *myo*-Inositol partly reversed growth inhibition by the analogues. This effect might be expected if the analogues were acting as antimetabolites or competitors of *myo*-inositol. That the effect occurred at physiological levels of *myo*-inositol, which are around 40 μM in human serum [53], and largely abolished the selectivity for transformed cells is disappointing; thus it is not likely that these analogues per se will exhibit useful antiproliferative activity in vivo.

A number of mechanisms might account for the growth-inhibitory activity of the 3-substituted *myo*-inositol analogues. The first would involve the inhibition of *myo*-inositol uptake. *myo*-Inositol can be either synthesized by a cell from glucose or taken up from the extracellular medium [14, 19]. The uptake of *myo*-inositol occurs by a saturable, Na^+-dependent, and partially energy-dependent process [60]. D-3-Deoxy-3-fluoro-*myo*-inositol was found to be taken up via the same process as *myo*-inositol in NIH 3T3 cells, with the affinity for the former being about one-tenth of that for the latter, and was a competitive inhibitor of *myo*-inositol uptake. D-3-Deoxy-3-fluoro-*myo*-inositol, D-3-deoxy-3-amino-*myo*-inositol, and D-3-deoxy-3-azido-*myo*-inositol analogues were equally effective inhibitors of *myo*-inositol uptake by cells but exhibited a >700-fold difference in growth-inhibitory activity. Therefore, it appears unlikely that the inhibition of *myo*-inositol uptake

plays a major role in the inhibition of cell growth by these analogues.

The work of Moyer et al. [40, 41] using 2- and 4-modified *myo*-inositols suggested that these analogues could not serve as substrates for Ptdlns synthetase, although several 5-modified analogues were incorporated into Ptdlns. The present study demonstrated that [^{3}H]-D-3-deoxy-3-fluoro-*myo*-inositol is incorporated into the Ptdlns fraction of cellular lipids of both wild-type and v-*sis*-transformed NIH 3T3 cells. This incorporation was greater than that of [^{3}H]-*myo*-inositol at similar concentrations. These results suggest that D-3-deoxy-3-fluoro-*myo*-inositol is a substrate for Ptdlns synthetase.

Because of the relatively low specific activity of [^{3}H]-D-3-deoxy-3-fluoro-*myo*-inositol, we could not determine whether 3-substituted Ins(1,4,5)P_3 was formed in analogue-exposed cells; however, we did obtain indirect evidence of the formation of 3-substituted Ins(1,4,5,)P_3. We have previously reported that D-3-deoxy-3-fluoro-Ins(1,4,5)P_3 is as effective as Ins(1,4,5)P_3 in releasing Ca^{2+} from intracellular stores [33]. In the present study, we found that cells grown in the presence of cytostatic concentrations of D-3-deoxy-3-fluoro-*myo*-inositol exhibit normal $[Ca^{2+}]_i$ signalling in response to a variety of mitogens. This suggests either that sufficient *myo*-inositol is synthesized by the cell to maintain the cellular pool of Ptdlns(4,5)P_2 from which Ins(1,4,5)P_3 is formed by the action of Ptdlns phospholipase C or that the 3-fluoro-Ptdlns(4,5)P_2 formed from D-3-deoxy-3-fluoro-*myo*-inositol serves as a substrate for Ptdlns phospholipase C. Cells exhibiting active *myo*-inositol transport usually display relatively minor *myo*-inositol biosynthesis [56]. We therefore believe that it is most likely that 3-substituted Ptdlns are indeed formed in the cells via hydrolysis by Ptdlns phospholipase C, thus enabling normal $[Ca^{2+}]_i$ signalling. If this is the case, it is interesting that 3′-fluoro-Ptdlns(4,5)P_2 appears to be a substrate for Ptdlns phospholipase C, since it has been reported that 3′-phosphorylated Ptdlns are not substrates for Ptdlns phospholipase C [35, 50]. Substitution of Ins(1,4,5)P_3 at the 3 position would prevent the formation of Ins(1,3,4,5)P_4. The role of Ins(1,3,4,5)P_4 formed from Ins(1,4,5)P_3 in $[Ca^{2+}]_i$ signalling has been the subject of intense speculation [23, 45], but its contribution to the regulation of cell growth is presently unknown.

We believe that the most probable mechanism underlying the inhibition of cell proliferation by the *myo*-inositol analogues involves antagonism of the formation of 3′-phosphorylated Ptdlns through phosphatidylinositol-3′-kinase. However, this idea currently remains speculative. We had hoped to obtain an indication as to whether the phosphatidylinositol-3′-kinase pathway was involved in the activity of the analogues by studying a panel of oncogene-expressing NIH 3T3 cells. There did not appear to be a correlation between the inhibition of cell growth by the analogues and the reported association of increased phosphatidylinositol-3′-kinase activity in the v-*sis*- and v-*src*-transformed cells. However, as little is known concerning the phosphatidylinositol-3′-kinase activity in other oncogene-expressing cells, an interpretation of the present results on this basis would be premature.

In summary, we conclude that D-3-substituted *myo*-inositol isosteres owe at least part of their growth-inhibitory action to their ability to enter the Ptdlns synthetic pathway, whereby they produce modified phospholipids that act directly to block Ptdlns signalling and/or to elicit the production of fraudulent second messengers. The fraudulent second messengers might include inositol phosphates or Ptdlns that are blocked at the D-3 position of the *myo*-inositol ring. Inhibition of Ptdlns turnover, specifically turnover linked to cell proliferation such as that involving the phosphatidylinositol-3′-phosphate pathway, may be one means of controlling cell growth. Although additional experiments are clearly needed to elucidate the precise mechanisms of action involved, the present study provides the first direct evidence that unnatural *myo*-inositol can exhibit a selectivity of action in blocking cellular growth, whereby members of this series of compounds offer new opportunities for the enhancement of our understanding of the role of intracellular signalling in the control of cell growth and for the subsequent development of cell-selective antiproliferative agents.

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