Phosphatidylinositol 3-OH Kinase Activity Is Not Required for Activation of Mitogen-activated Protein Kinase by Cytokines

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Hemopoietic cells respond to cytokines by initiating tyrosine phosphorylation of receptors and receptor-associated proteins, leading to the activation of numerous cytosolic and membrane associated enzymes, including phosphatidylinositol 3-OH kinase (PI 3-kinase). Recent reports have suggested that PI 3-kinase may serve as an upstream activator of mitogen-activated protein (MAP) kinase. After stimulation with interleukin-3 and granulocyte-macrophage colony-stimulating factor, we show here that inhibition of MAP kinase activity by two inhibitors of PI 3-kinase, wortmannin and LY-294002, does not correlate with their ability to inhibit PI 3-kinase or p70 S6 kinase phosphorylation. Complete inhibition of phosphatidylinositol 3,4,5-trisphosphate production occurred at approximately 100 nM WM or 25 μM LY-294002, but at these concentrations, WM significantly inhibited MAP kinase activation, while LY-294002 had virtually no effect on MAP kinase activity. Furthermore, WM does not inhibit phorbol ester-mediated MAP kinase activation, but LY-294002 does. Together these results suggest WM and LY-294002 are differentially inhibiting enzymes other than PI 3-kinase that function upstream of MAP kinase.

Investigations of signal transduction pathways have been aided tremendously by the availability of inhibitors of specific enzymes, allowing researchers to determine the importance of a particular enzyme when its activity is blocked. Certainly, the availability of the potent inhibitors of phosphatidylinositol 3-kinase (PI 3-kinase), wortmannin (1, 2) and LY-294002 (3), have been extremely useful in demonstrating the role of PI 3-kinase in signaling pathways (4–10). Recent studies from our laboratory (11), as well as that of Yao and Cooper (12), demonstrated the importance of PI 3-kinase as an upstream signal in the inhibition of apoptosis. We have focused on the pathways downstream of a number of cytokines, or hemopoietic growth factors, in our studies, and we were surprised to find that wortmannin and LY-294002 caused apoptosis in the presence of interleukin (IL)-3, IL-4, and Steel factor, but not in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF). Since the IL-3 and GM-CSF receptors share a common β subunit, and many studies have shown that they stimulate the same signal transduction pathways, it will be interesting to determine how GM-CSF is able to bypass the effects of the PI 3-kinase inhibitors. In any studies with inhibitors, we must also keep in mind the possibility that enzymes other than the desired target may be affected. Wortmannin and LY-294002 have been shown to have inhibitory effects on other kinases, albeit at higher concentrations than are required to inhibit PI 3-kinase (2, 3). Therefore, any effects with these inhibitors that is attributed to the role of PI 3-kinase in signaling pathways must be closely correlated with their potency in inhibiting that enzyme.

We have continued our investigation of the signaling network downstream of IL-3 and GM-CSF receptors to examine the role of the p21ras and MAP kinase pathways in inhibition of apoptosis. Consistent with our previous studies, we now show that the activation of MAP kinase by either IL-3 or GM-CSF is partially inhibited by the PI 3-kinase inhibitors. When comparing the inhibition observed following IL-3 or GM-CSF stimulation, it was apparent that each was similarly affected by the inhibitors. During the course of these studies, we noted that the ability of the inhibitors to block MAP kinase activation did not correlate with their reported effects on PI 3-kinase activation. Several reports have demonstrated an inhibition of MAP kinase activation by WM (4–6) and more recently, others have made the argument that PI 3-kinase may be contributing to activation of MAP kinase, again based on inhibitory effects of WM (13, 14). Careful analysis of our results suggests that the effects of WM and LY-294002 may be attributable to inhibition at a site upstream of MAP kinase that is distinct from PI 3-kinase.

EXPERIMENTAL PROCEDURES

Cell Lines and Reagents—MC-9 cells were grown at 37 °C and 5% CO₂ with humidity in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Intergen), 50 units/ml penicillin and 50 mg/ml streptomycin (Sigma), and 20 μg/ml amphotericin (18, 19). For use in assays, cells were washed free of cytokine with Hanks’ balanced salt solution and incubated in the above medium without cytokine for 3–5 h prior to assay. Alternatively, cells were incubated overnight with 1% WEHI-3-conditioned medium, supplemented with 5–10% WEHI-3-conditioned medium to examine the role of the p21ras and MAP kinase pathways in inhibition of apoptosis. Consistent with our previous studies, we now show that the activation of MAP kinase by either IL-3 or GM-CSF was partially inhibited by the PI 3-kinase inhibitors. When comparing the inhibition observed following IL-3 or GM-CSF stimulation, it was apparent that each was similarly affected by the inhibitors. During the course of these studies, we noted that the ability of the inhibitors to block MAP kinase activation did not correlate with their reported effects on PI 3-kinase activation. Several reports have demonstrated an inhibition of MAP kinase activation by WM (4–6) and more recently, others have made the argument that PI 3-kinase may be contributing to activation of MAP kinase, again based on inhibitory effects of WM (13, 14). Careful analysis of our results suggests that the effects of WM and LY-294002 may be attributable to inhibition at a site upstream of MAP kinase that is distinct from PI 3-kinase.
phenylmethylsulfonyl fluoride, 1 μM pepstatin, 0.5 μM leupeptin, and 10 μg/ml soybean trypsin inhibitor, immediately followed by removal of nuclei by centrifugation (20,000 × g, 1 min). Supernatants were incubated with 40 μg/ml anti-p44erk-1 antibody coupled to agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C for 4 h, with continuous mixing. Beads were washed three times with fresh solubilization buffer and once with kinase buffer (20 mM HEPES, pH 7.2, 5 mM MgCl₂, 1 mM EGTA, 5 mM 2-mercaptoethanol, 0.25 mM phenylmethylsulfonyl fluoride, 2 mM Na₃VO₄, 0.5 μM leupeptin). Beads were resuspended to 25 μl in kinase buffer containing 1 mg/ml MBP. Five μl of ATP solution (100 μM ATP and 1 μl of [γ-³²P]ATP in kinase buffer) was added followed by incubation for 15 min at 30°C. Reactions were stopped by addition of 30 μl of 2 × SDS sample buffer, followed by boiling for 2 min. Samples were separated by SDS-PAGE (15%), and proteins were transferred to nitrocellulose by semidry blotting. Phosphorylation of MBP was analyzed by autoradiography and measured by liquid scintillation counting of the excised bands. Immunoblotting was performed using anti-p44erk-1 (Santa Cruz Biotechnology) and detection by chemiluminescence using ECL detection reagents (Amersham) as recommended by the manufacturer. Alternatively, MAP kinase activity was measured by spotting 25 μl of the reaction volume onto 2 cm² sheets of P-81 filter paper (Whatman), followed by washing with various buffer volumes of 1% (v/v) phosphoric acid and measurement of associated radioactivity by liquid scintillation counting.

Measurement of Intracellular PI-3,4,5-P₃—Cells werestarved of cytokine overnight, then washed three times with fresh cell media RPMI-1640 and incubated the same medium with 0.5 μCi/ml [³²P]orthophosphate (ICN) for 2 h at 37°C. Cells were stimulated with cytokine in the presence or absence of inhibitors, then stopped by the addition of 3.5 ml of CHCl₃:acetone:methanol:acetic acid:water (4:6:17:15:2, v/v) and vortexed. Lipids were extracted as described previously (27), spotted onto anion-exchange silica gel plates, and chromatographed using a CHCl₃:acetone:methanol:acetic acid:water (4:6:17:15:2, v/v) solvent system (27). Lipids were visualized by autoradiography, and PI-3,4,5-P₃ was identified by co-migration with PI-3,4,5-P₃ standard (Amersham) as recommended by the manufacturer. Alternatively, MAP kinase activity was measured by spotting 25 μl of the reaction volume onto 2 cm² sheets of P-81 filter paper (Whatman), followed by washing with various buffer volumes of 1% (v/v) phosphoric acid and measurement of associated radioactivity by liquid scintillation counting. Values were normalized based on the relative radioactivity in each lane.

Confirmation of the identity of the above TLC spot as PI-3,4,5-P₃ was achieved by deacylation of the lipid, followed by HPLC separation of the water-soluble products using procedures and conditions similar to those described previously (27). Following a 10-min washing of the Partisil 10 SAX ion exchange column with water, a 20-min 0-0.25 M ammonium phosphate gradient was followed by a 50-min 0.25-1.0 M ammonium phosphate gradient. The peak of PI-3,4,5-P₃ radioactivity eluted between 0.05 and 0.1 M ammonium phosphate gradient. Internal standards of ADP and ATP were also co-chromatographed in each run. Phosphorylation of MBP following immunoprecipitation from cells detergent-solubilized as described under “Experimental Procedures.” Lysates were immunoprecipitated with anti-p44erk-1 coupled to agarose beads. Activity of washed immunoprecipitates was measured by the incorporation of [³²P] into myelin basic protein as determined by scintillation counting following fractionation by SDS-PAGE (15% polyacrylamide gel) and transfer to nitrocellulose. Results are the average ± standard deviation from four independent experiments. Values for cytokine-stimulated samples in the absence of inhibitors were typically 5-10-fold greater than unstimulated samples, which were in the range of 2000-4000 cpm.

RESULTS AND DISCUSSION

The finding that WM, a potent inhibitor of PI-3-kinase, leads to attenuation of MAP kinase activity suggests that PI-3-kinase may be an upstream activator of MAP kinase. To explore this possibility, we examined the effects of WM on MAP kinase activity in MC-9 cells stimulated with either IL-3 or GM-CSF (Fig. 1). Both activities were reduced equally, and maximally at approximately 100 nM WM. No greater reduction in activity was achieved at higher WM doses, and we found that WM itself did not inhibit MAP kinase activity in vitro at a concentration of 1 μM (data not shown). Fig. 1 also shows the effect of LY-294002 on MAP kinase activity in MC-9 cells. Again the maximal attenuation was equal between the two cytokines used, but to obtain a similar effect that was observed with WM, a dose of 100 μM LY-294002 was required. At a dose of 50 μM, a significantly less degree of attenuation was seen, and 25 μM LY-294002 gave no inhibition. Fig. 2 (A and B) shows representative experiments demonstrating in vitro p44erk-1 phosphorylation of MBP following immunoprecipitation from cells incubated with indicated concentrations of WM or LY-294002, respectively, prior to cytokine stimulation. p42erk-2 was also tested and showed similar results (data not shown). Fig. 2C compares an immunoprecipitated sample with cell extract alone or antibody alone, to verify the identity of the p44erk-1 and the IgG heavy chain.

Separation of MAP Kinase and PI 3-Kinase Activation

The initial paper describing LY-294002 (3) demonstrated an in vitro [³²P]orthophosphate-labeled cells pretreated with WM or LY-294002 and stimulated with cytokine. Total lipids were extracted and separated by thin-layer chromatography.
Separation of MAP Kinase and PI 3-Kinase Activation

**Table I**

| Inhibitor | Unstimulated GM-CSF | Fold stimulation |
|-----------|---------------------|-----------------|
| [LY-294002] (μM) | | |
| 0 | 2085 ± 196 | 11,052 ± 488 | 5.3 |
| 10 | 2109 ± 154 | 12,051 ± 1683 | 6.0 |
| 25 | 2009 ± 168 | 9655 ± 242 | 4.6 |
| 50 | 2403 ± 150 | 10,228 ± 583 | 4.9 |

| [Wortmannin] (nM) | | |
| 0 | 3655 ± 132 | 15,685 ± 800 | 4.3 |
| 10 | 4779 ± 744 | 12,751 ± 582 | 3.5 |
| 25 | 5348 ± 481 | 12,007 ± 953 | 3.3 |
| 50 | 4674 ± 90 | 9972 ± 96 | 2.7 |
| 100 | 5065 ± 141 | 7193 ± 345 | 2.0 |

*Compared to cpm in unstimulated cells in the absence of inhibitor.

**Fig. 2.** Representative experiment of MBP phosphorylation by anti-p44erk-1 immunoprecipitates and corresponding anti-p44erk-1 blot. A, kinase assay of MBP phosphorylation by immunoprecipitated p44erk-1 cell lysates from indicated conditions were separated on a 15% polyacrylamide gel, transferred to nitrocellulose, and exposed to film (upper panel). The membrane was then immuno-blotted with anti-p44erk-1 (lower panel). B, experiment was similar to that shown in A, but with the indicated concentrations of LY-294002. C, to confirm the identity of the p44erk-1 in the immunoprecipitates, whole cell extract (lane 1), antibody beads alone (lane 2), or immunoprecipitated extracts (lane 3) were immuno-blotted using the anti-Erk-1 antibody. This antibody detects both p44erk-1 and p42erk-2, but only p44erk-1 was immunoprecipitated by the anti-p44erk-1 beads. This panel also identifies the IgG heavy chain present in samples containing anti-p44erk-1 beads. Identical results were obtained when a different p44erk-1 antibody was used to immuno-blot (results not shown).

**Table II**

| Inhibitor | Fold increase in PI-3,4,5-P3 levels |
|-----------|----------------------------------|
| WM (0.01 μM) | 2.0 |
| WM (0.1 μM) | 5.0 |
| WM (1 μM) | 10.0 |

Values represent triplicate determinations of MAP kinase activity from filter paper binding assays as described under "Experimental Procedures." Cell treatments were carried out as described in Fig. 1.

**A**

GB/CM-3 produced small PI-3,4,5-P3 following stimulation with GM-CSF. While treatment of cells with LY-294002 at 25 μM resulted in no attenuation of GM-CSF-stimulated MAP kinase activity, this concentration of drug lowered PI-3,4,5-P3 levels to that of unstimulated cells. LY-294002 pretreatment with 50 μM lowered PI-3,4,5-P3 levels below that of untreated cells. WM at a concentration of 100 nM also lowered PI-3,4,5-P3 to that of untreated cells. These results confirm that the highest concentrations of drugs used for our experiments examining immunoprecipitated MAP kinase activity inhibit all cytokine-stimulated PI 3-kinase activity in these cells.

**B**

We took advantage of the recent reports that PI 3-kinase activity is required for the activation of the p70 S6 kinase (15–19), to examine the effect of the inhibitors on activation of p70 S6 kinase by cytokines. It has been well established that phosphorylation of multiple serine and threonine residues within the COOH-terminal auto-inhibitory domain of p70 S6 kinase are required for enzyme activation, and this modification leads to decreased mobility of the enzyme on SDS-PAGE (15–21). p70 S6 kinase activation following stimulation of MC-9 cells by IL-3 or GM-CSF was observed, as indicated by a decreased mobility (Fig. 4). Both WM (at 100 nM) and LY-294002 (at 25 μM) were able to block IL-3 and GM-CSF stimulated activation of p70 S6 kinase, as indicated by migration on SDS-PAGE. This was similar to the enzyme from control, untreated cells. It is important to note that LY-294002 was able to completely block this band shift of p70 S6 kinase at 25 μM, as expected based on its potency in inhibiting PI 3-kinase. However, as shown above, LY-294002 clearly had no effect on activation of MAP kinase at this concentration.

Finally, we tested the effects of either WM or LY-294002 on MAP kinase activity in cells stimulated with phorbol ester. In agreement with previous studies (8, 13), WM had no effect on MAP kinase activation by this mode of stimulation (Fig. 5). However, LY-294002 was able to inhibit phorbol ester-stimulated MAP kinase activation by approximately 50% when used at a concentration of 100 μM. To our knowledge, this is the first demonstration of an inhibitory effect by LY-294002 that is not observed when using low concentrations of WM that block PI 3-kinase activity. Based on this result, we predict that LY-294002, being a competitive inhibitor of the ATP binding site of PI 3-kinase, would also be able to block PI 3-kinase. However, as shown above, LY-294002 clearly had no effect on activation of MAP kinase at this concentration.

**C**

Several previous reports have direct implications on this study. Ferby et al. (8) show that in guinea pig neutrophils, WM leads to reduced activation of MAP kinase following PAF stimulation. LY-294002 was also tested, but in agreement with our study, doses higher than those required to inhibit PI 3-kinase were needed (IC50 100–200 μM). The authors did not come to a...
conclusion as to why these higher amounts of LY-294002 were needed. In another report by Karnitz and co-workers (13), PI 3-kinase activity was proposed to be upstream of MEK and MAP kinase activation in IL-2 stimulated CTLL-2 cells, again based upon the use of WM. From their results, it was clear that WM was acting on one or more components upstream of these enzymes, and distinct from p21ras and Raf kinase activity. However, experiments using LY-294002 were mentioned but not shown, and thus it is possible that higher concentrations of this compound were required, as we have found in our study. There have also been at least four other recent reports (31–34) in which PI 3-kinase has been reported to be acting upstream of MAP kinase or p21ras activation, largely on the basis of the inhibitory effect of WM. Clearly, those conclusions should be reconsidered.

Therefore, this study has provided strong evidence that cytokine-stimulated PI 3-kinase activity, leading to production of 3-phosphorylated inositol phospholipids, is not required for activation of MAP kinase in response to IL-3 and GM-CSF. This conclusion is based on a lack of correlation between the inhibitory effects of WM and LY-294002 in blocking PI 3-kinase activity compared to their effects on MAP kinase activation. It is also supported by previous studies showing that activation of PI 3-kinase is observed in the absence of p21ras and MAP

TABLE II

Inhibition of GM-CSF-stimulated PI-3,4,5-P3 production by wortmannin and LY-294002

| Inhibitor          | Condition | Fold stimulation* |
|--------------------|-----------|-------------------|
| [LY-294002] (μM)   | 0         | 1.00 ± 0.11       |
|                   | 0         | 1.00 ± 0.11       |
|                   | 10        | 1.77 ± 0.27       |
|                   | 10        | 1.72 ± 0.15       |
|                   | 25        | 1.09 ± 0.23       |
|                   | 50        | 0.94 ± 0.06       |
| [Wortmannin] (nm) | 0         | 1.00 ± 0.01       |
|                   | 0         | 1.00 ± 0.01       |
|                   | 10        | 2.92 ± 0.38       |
|                   | 10        | 1.83 ± 0.40       |
|                   | 25        | 1.42 ± 0.56       |
|                   | 50        | 1.36 ± 0.09       |
|                   | 100       | 1.16 ± 0.07       |

* Compared to unstimulated cells in the absence of inhibitor.

MAP kinase activation in IL-2 stimulated CTLL-2 cells, again based upon the use of WM. As well, 12-phorbol 13-myristate acetate-induced MEK or MAP kinase activity was not affected with WM. From their results, it was clear that WM was acting on one or more components upstream of these enzymes, and distinct from p21ras and Raf kinase activity. However, experiments using LY-294002 were mentioned but not shown, and thus it is possible that higher concentrations of this compound were required, as we have found in our study. There have also been at least four other recent reports (31–34) in which PI 3-kinase has been reported to be acting upstream of MAP kinase or p21ras activation, largely on the basis of the inhibitory effect of WM. Clearly, those conclusions should be reconsidered.

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**Fig. 3.** Representative results of thin layer chromatography procedure for separation of PI-3,4,5-P3 and identification of the separated spot by HPLC analysis. The PI-3,4,5-P3 standard was prepared by using immunoprecipitated PI 3-kinase in an in vitro reaction with [32P]ATP, using PI-4,5-P2 as substrate. Lipids were extracted as described under "Experimental Procedures" from cells that were either unstimulated, or stimulated with GM-CSF for 5 min following a 10-min preincubation in the absence or presence of 50 μM LY-294002. A, a representative sample separated by TLC as described under "Experimental Procedures"; the radioactivity in the PI-3,4,5-P3 spots were quantitated and normalized based on the relative radioactivity per lane, and used to generate the results in Table II. B, the spots separated by TLC of whole cell extracts from unstimulated (○) or GM-CSF-stimulated (●) cells were removed, deacylated, and chromatographed as described under "Experimental Procedures." C, the 32P-labeled products from the in vitro reaction described above also eluted at the identical time. The peaks from HPLC corresponded to the expected elution time for glycerol-P-IP3.

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|--------------------|-----------|-------------------|
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|                   | 50        | 1.36 ± 0.09       |
|                   | 100       | 1.16 ± 0.07       |

* Compared to unstimulated cells in the absence of inhibitor.
Separation of MAP Kinase and PI 3-Kinase Activation

Fig. 4. Phosphorylation of p70 S6 kinase by cytokines is inhibited by WM and LY-294002. MC-9 cells were prepared as described under "Experimental Procedures" and treated with the indicated concentrations of WM or LY-294002 for 10 min. Cells were then treated with medium alone, or medium containing GM-CSF or IL-3 as described in Fig. 1. Detergent-solubilized cells were fractionated by SDS-PAGE (12.5% polyacrylamide, 118:1 acrylamide:bisacrylamide ratio) and transferred to nitrocellulose. The membrane was then immunoblotted with anti-S6 kinase.

Fig. 5. MAP kinase activity is attenuated by LY-294002 but not WM following stimulation of cells with phorbol ester. MC-9 cells were treated with medium or medium containing indicated concentrations of WM or LY-294002 for 10 min and then with 100 nM phorbol dibutyrate or vehicle alone. MAP kinase assay was performed as in Fig. 1. Results are the average ± standard deviation of four independent experiments, and the average stimulation observed with phorbol ester was 3-fold greater than unstimulated samples.

In the case of IL-4-stimulated hemopoietic cells (22–27), these results do not agree with the results of Hu et al. (28), who showed that a constitutively active p110 construct could lead to activation of p21<sup>ras</sup> in NIH 3T3 cells or in Xenopus oocytes. In another study (29), overexpression of PI 3-kinase p85 subunit, acting in a dominant negative fashion, was thought to be acting upstream of p21<sup>ras</sup> and Raf activity. In light of our results, one might question whether these model systems, relying on artificial constructs, are truly indicative of the normal regulatory events that occur downstream of PI 3-kinase in response to growth factor activation. It is possible that drastic alterations in PI 3-kinase activity in cells over longer periods of time may have a multitude of diverse effects. However, our experiments cannot rule out the possibility that WM, but not low concentrations of LY-294002, is affecting some uncharacterized action of PI 3-kinase, independent of the lipid kinase activity, and this action normally contributes to activation of the MAP kinase pathway. In support of our conclusions regarding the inhibitory effects of WM, Cross et al. (30) recently described an inhibitory effect of WM on phospholipase A<sub>2</sub> activation that also appears to be independent of its ability to block PI 3-kinase. As in the latter study, we are uncertain where WM may be having its inhibitory effect. MAP kinase activation is known to involve a series of upstream activating enzymes, and both p21<sup>ras</sup>-dependent and -independent pathways have been described. WM may be acting on one of the upstream enzymes that only partially contributes to MAP kinase activation, since increasing concentrations of WM never cause more than 50% inhibition of MAP kinase activity. It will be important to test the effect of WM on the many kinases functioning upstream of MAP kinase, although a recent study (33) suggested that WM does not inhibit activation of MEK, MEKK, and Raf. There are also other enzymes that have been shown to be inhibited by WM, such as DNA-dependent protein kinase (35), which is related to PI 3-kinase, but the role of this enzyme in signaling pathways is not yet well defined.

One of our initial aims during the course of these studies was to define pathways downstream of cytokine receptors which prevent apoptosis. Our finding that GM-CSF, but not IL-3, provides a survival signal in the presence of either WM or LY-294002 implicated PI 3-kinase as a key molecule for the ability of some, but not all, cytokines to keep hemopoietic cells alive (11). Unlike MAP kinase attenuation following cytokine stimulation, this physiological function can be demonstrated at low concentrations of both WM and LY-294002 which effectively block PI 3-kinase activity. In addition, our findings reported here demonstrate that GM-CSF is not providing this signal through the MAP kinase pathway, since WM attenuates both GM-CSF- and IL-3-stimulated MAP kinase activity to the same extent.

The signaling pathways emanating from receptor activation are not necessarily linear, but must be considered a network of interwoven events. It is thus important to clarify the specific role of PI 3-kinase in differentiation, mitogenesis, and the prevention of apoptosis by characterizing the enzymes that it may be regulating, as well as the relative importance of the various inputs on the resulting physiological responses. The potent inhibitors of PI 3-kinase, WM and LY-294002, have been extremely useful in explaining the role of PI 3-kinase, particularly since they are structurally unrelated inhibitors of the enzyme. However, we have shown here that one must be careful to compare the effects of both inhibitors over a range of concentrations known to affect PI 3-kinase before making assumptions regarding downstream events thought to be regulated by this important signaling enzyme.

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