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Prevention of the wortmannin-induced inhibition of phosphoinositide 3-kinase by sulfhydryl reducing agents

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Abstract:
The effects of the sulfhydryl reducing agents 2-mercaptoethanol and dithiothreitol on wortmannin-induced inhibition of phosphoinositide 3-kinase (PI3K) were studied in order to examine whether the sulfhydryl reducing agents directly affect the wortmannin inhibition of PI3K. These reducing agents are commonly used to stabilize enzyme structures by maintaining protein sulfhydryl groups in the reduced state. Preincubation of wortmannin with millimolar levels of 2-mercaptoethanol, a sulfhydryl derivative of ethanol, markedly prevented subsequent wortmannin-induced inhibition of PI3K. In contrast, ethanol, 2-mercaptoethanol lacking sulfhydryl group, and 2-(methylthio)ethanol, a methyl derivative of the sulfhydryl group of 2-mercaptoethanol, had little effect on the wortmannin-induced inhibition of PI3K, which suggests that the prevention of wortmannin-induced inhibition by 2-mercaptoethanol occurs through the sulfhydryl group of this agent. Moreover, dithiothreitol, a second sulfhydryl reducing agent, also markedly prevented wortmannin-induced inhibition of PI3K. These results indicate that the wortmannin-induced inhibition of PI3K is markedly prevented by millimolar concentrations of sulfhydryl reducing agents such as 2-mercaptoethanol and dithiothreitol in the medium, presumably by the binding of wortmannin to the agents.

Key words:
wortmannin, sulfhydryl reducing agent, phosphoinositide 3-kinase, PI3K, 2-mercaptoethanol, dithiothreitol

Abbreviations: DMSO – dimethyl sulfoxide, PI3K – phosphoinositide 3-kinase

Introduction

Phosphoinositide 3-kinase (PI3K) plays essential roles in the regulation of a variety of cell signaling networks such as cellular mobility, proliferation, differentiation, survival and metabolism [8, 16]. PI3K was at first recognized as a lipid kinase that catalyzed the phosphorylation of the hydroxy group on the third carbon of the inositol ring of phosphoinositides; it was later found to also possess protein kinase activity. Alterations in PI3K-dependent pathways have been implicated in diseases, such as cancer, inflammation, vascular restenosis and diabetes. As compounds that inhibit PI3K are potential tools in determining the precise role of this enzyme in cell signaling networks and are potential therapeutic drugs, such as anticancer drugs and anti-inflammatory agents, a number of compounds inhibiting this enzyme have been identified [9, 14].

Wortmannin, a hydrophobic fungal metabolite of the fungus *Talaromyces wortmanni*, was found to be a potent inhibitor (IC_{50} = approximately 3 nM) of PI3K [13]. Since then, this compound has been widely used as a powerful tool to examine the role of PI3K in cellular signaling [2]. However, this compound has also been shown to moderately inhibit other kinases such as DNA-dependent protein kinase [6], myosin light chain kinase [11] and phosphoinositide 4-kinase [10], which raises the possibility that some biological effects attributed to PI3K determined by the use of wortmannin may involve other enzymes. On the other hand, LY294002 is another commonly used PI3K inhibitor and has been shown to demonstrate different off-target inhibitory effects from wortmannin [17]. Therefore, the combination of two structurally unrelated inhibitors of PI3K, wortmannin and LY294002, has been widely used as a popular and powerful research tool to identify some of the physiological roles of the cellular signaling pathways that they inhibit. However, the apoptosis-inducing effects of wortmannin and LY294002 have been ascribed to their abilities to inhibit enzymes other than PI3K [9].

One of the other shortcomings of wortmannin is that it is known to be rather unstable in aqueous solution, and the compound is thought to interact with serum proteins and lose its ability of inhibit PI3K [7, 12]. We have previously reported that wortmannin rapidly bound to various amino compounds such as glutamine and tris(hydroxymethyl)aminomethane under physiological and standard experimental conditions and lost the ability to inhibit PI3K [5]. This result complicates the interpretation of the data used to identify the physiological roles of the cellular signaling pathways because the inhibitory potential of wortmannin is largely affected by the experimental conditions, such as the composition of the medium used. Two common sulfhydryl reducing agents, 2-mercaptoethanol and dithiothreitol, have been widely used to stabilize enzyme structures by maintaining protein sulfhydryl groups in the reduced state. In this paper, we have studied the effect of the sulfhydryl reducing agents 2-mercaptoethanol and dithiothreitol on the wortmannin-induced inhibition of PI3K to determine whether wortmannin activity persists under standard experimental conditions containing these sulfhydryl reducing agents because wortmannin is known to covalently bind to sulfhydryl groups as well as amino groups [3, 19].

Materials and Methods

Materials

Wortmannin and recombinant human PI3K p110γ were purchased from Kyowa Medex (Tokyo, Japan) and Jena Bioscience (Jena, Germany), respectively.

PI3K activity

PI3K activity was measured as described previously [5] according to the manufacturer's protocol with slight modifications. Recombinant human PI3K p110γ was used as the enzyme preparation to study the effects of the sulfhydryl reducing agents on wortmannin-induced PI3K inhibition. Different concentrations of wortmannin and vehicle (DMSO) were pretreated for 30 min with 100 µl of medium containing the indicated concentrations of various test compounds, such as 2-mercaptoethanol in 20 mM NaHEPES (pH 7.4). Aliquots (10 µl) of the pretreated media were mixed with 6 µl of reaction buffer containing 20 mM NaHEPES (pH 7.4), 3.3 mM $MgCl_2$, 83 mM NaCl and 33.3 µg/ml PI3K enzyme and then incubated for 20 min to allow the inhibition of PI3K by unreacted, free wortmannin. The PI3K reaction was initiated by the addition of 4 µl of 0.24 mM γ-[^{32}P]ATP (1 µCi) in 20 mM NaHEPES (pH 7.4). The final concentrations of the components in the 20-µl reactions were 20 mM NaHEPES (pH 7.4), 1 mM $MgCl_2$, 25 mM NaCl, 0.1 mg/ml phosphatidylinositol, 10 µg/ml PI3K, 48 µM ATP, and the indicated concentrations of the various test compounds. In all figures, the concentrations represent the final concentration of the given compound in the assay solution. After 10 min of incubation at 37°C, the reaction was terminated with 100 µl of 1 M HCl. Lipid was then extracted with 200 µl of chloroform/methanol (1:1). The radioactivity in the chloroform layer was measured by a liquid scintillation counter after the elimination of chloroform by evaporation. The reaction was linear with respect to time and enzyme concentration under the conditions used.

High-performance liquid chromatography

The identification of wortmannin and its relatives was performed using a Waters 2690 HPLC equipped with a Waters 996 photodiode array detector (PDA). Different concentrations of wortmannin and vehicle

(DMSO) were pretreated for 30 min with 100 µl of medium containing the indicated concentrations of various test compounds, such as 2-mercaptoethanol in 20 mM NaHEPES (pH 7.4). Analysis was carried out on a reverse-phase C-18 column (150 × 4.6 mm) with an acetonitrile/water gradient solution (acetonitrile percentage, 0–100%; flow rate, 1 ml/min). The elution profiles at 280 nm are shown in Figure 3. As described previously [5], hydrophilic compounds such as HEPES were easily eluted during the 0–4 min time period.

Statistics

All experiments were repeated at least four times. The data are expressed as the means ± SEM. Statistical calculations were performed using GraphPad Prism version 5.03 software (San Diego, CA, USA). A statistical analysis by two-way ANOVA was performed in the experiments in Figure 3, and this analysis revealed an extremely significant interaction between wortmannin and the sulfhydryl reducing agents 2-mercaptoethanol and dithiothreitol (both: $p < 0.0001$). Therefore, the statistical significance was assessed by one-way ANOVA followed by Tukey's post tests in the present study. In some cases described in the "Discussion", one-way ANOVA followed by post test for a linear trend were performed. Dose-response curves were generated by nonlinear regression analysis in GraphPad Prism.

Results

Wortmannin is a well-known inhibitor of PI3K and is widely used as a powerful tool to examine the role of PI3K in a variety of intracellular signaling pathways. In the present study, we examined whether the sulfhydryl reducing agents 2-mercaptoethanol and dithiothreitol, which are commonly used to maintain protein sulfhydryl groups in the reduced state, affect the wortmannin-induced inhibition of PI3K as an index of wortmannin activity.

Preincubation of wortmannin with 20 mM ethanol (10 mM final concentration in the PI3K assay solution) as described in "Materials and Methods" had little effect on wortmannin activity, and wortmannin normally inhibited PI3K in a concentration-dependent manner (Fig. 1). On the other hand, the same concentration of 2-mercaptoethanol without wortmannin had an effect on PI3K activity without wortmannin, but that effect was not significant as determined by one-way analysis of variance followed by Tukey's post tests. However, the preincubation of wortmannin with 2-mercaptoethanol, a sulfhydryl derivative of ethanol, markedly prevented the subsequent wortmannin-induced inhibition of PI3K. In contrast, 2-(methylthio)ethanol, the S-methyl derivative of 2-mercaptoethanol, had little effect on wortmannin activity, and wortmannin normally inhibited PI3K in a concentration-dependent manner. The effect of another sulfhydryl reducing agent dithiothreitol on the wortmannin-induced inhibition of PI3K was also examined. Dithiothreitol without wortmannin also had an effect on the activity of PI3K, but that effect was not significant. Dithiothreitol also markedly prevented the wortmannin-induced inhibition of PI3K as was seen with 2-mercaptoethanol.

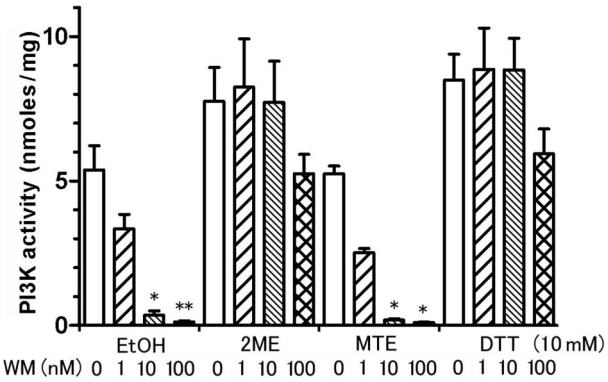

Fig. 1. Effects of different concentrations of wortmannin on PI3K activity in various media containing ethanol (EtOH), 2-mercaptoethanol (2ME), 2-(methylthio)ethanol (MTE) or dithiothreitol (DTT). Different concentrations (2, 20 and 200 nM) of wortmannin (WM) and vehicle (0.1% DMSO) were pretreated for 30 min with 20 mM ethanol, 2-mercaptoethanol, 2-(methylthio)ethanol or dithiothreitol to yield the final concentrations shown in Figure 1. The PI3K activities in the media were measured as described in detail in "Materials and Methods". Data are the means ± SEM (n = 4); * $p < 0.05$, ** $p < 0.01$, significantly different from the corresponding control (DMSO) without wortmannin

Figure 2 shows the effects of various concentrations of 2-mercaptoethanol and dithiothreitol on wortmannin activity. Although 2-mercaptoethanol without wortmannin had a slight effect on the activity of PI3K, the major effect of this compound was to prevent the inhibitory effect of wortmannin on PI3K in a concentration-dependent manner. This prevention by 2-mercaptoethanol was already observable at 6 mM

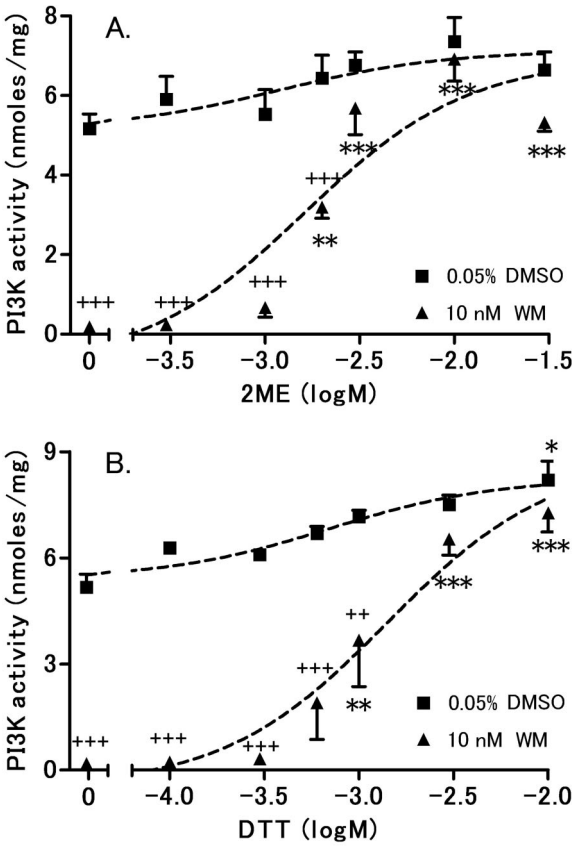

Fig. 2. Effect of wortmannin in different concentrations of 2-mercaptoethanol (2ME: **A**) or dithiothreitol (DTT: **B**) on PI3K activity. Wortmannin (20 nM) and vehicle (0.1% DMSO) were pretreated for 30 min with different concentrations of the indicated sulfhydryl reducing agent to yield the final concentrations shown in Figure 2. The PI3K activities in the media were measured as described in detail in "Materials and Methods". Data are the means ± SEM (n = 4); ** $p < 0.01$, *** $p < 0.001$, significantly different from the corresponding control without the sulfhydryl reducing agent 2-mercaptoethanol or dithiothreitol. ++ $p < 0.01$, +++ $p < 0.001$, significantly different from the corresponding control (DMSO) without wortmannin

(3 mM final concentration in the PI3K assay solution) and increased with increasing 2-mercaptoethanol concentrations until maximum inhibition was reached at 20–60 mM. Moreover, dithiothreitol also tended to increase PI3K activity but that increase was not significant except at the concentration of 20 mM. Dithiothreitol also showed similar concentration-dependent inhibition of wortmannin activity, which reached a maximum at 12–20 mM (Fig. 2B). These results indicate that 2-mercaptoethanol and dithiothreitol strongly prevent the inhibitory potential of wortmannin on PI3K. It should be noted that these concentrations of 2-mercaptoethanol and dithiothreitol are comparable to standard experiments using sulfhydryl reducing agent to stabilize enzyme structures.

Next, we attempted to study the mechanism by which the sulfhydryl reducing agents prevent the inhibitory potential of wortmannin on PI3K. HPLC analysis using a C-18 reversed-phase column was performed to examine whether wortmannin binds to the sulfhydryl reducing agents in the above mentioned preincubation medium. Unfortunately, 20 nM wortmannin, the concentration frequently used in the preincubation medium for the PI3K assay, was hardly detected by HPLC analysis as a clear peak. Therefore, the effects on the HPLC profile of various concentrations of the sulfhydryl reducing agents on 20 μM (instead of 20 nM) wortmannin were determined. Wortmannin alone showed a peak with a retention time of 8.44 min under the experimental conditions described in "Materials and Methods". In agreement with the above mentioned experiments on PI3K activity shown in Figure 2A, this peak decreased in a dose-dependent manner with increasing concentrations of 2-mercaptoethanol, and the peak (8.44 min) of wortmannin itself almost completely disappeared after treatment with 20 mM 2-mercaptoethanol. As shown in Figure 3, a new peak (7.31 min) that was small but clear and broad and had a different absorbance spectrum from wortmannin was observed when 20 μM wortmannin was incubated with 20 mM 2-mercaptoethanol. In contrast, ethanol, which lacks the sulfhydryl group of 2-mercaptoethanol, and 2-(methylthio)ethanol, which is the S-methyl derivative of 2-mercaptoethanol, had little effect on the elution profiles of wortmannin, even at a concentration as high as 100 mM. It is possible that the binding of wortmannin to 2-mercaptoethanol results from the sulfhydryl group of this agent. Additionally, dithiothreitol, another sulfhydryl reducing agent, dose-dependently induced a new peak (7.32 min), which had a different absorbance spectrum from wortmannin in the elution profile; the peak of wortmannin itself also decreased. These results suggest that 2-mercaptoethanol and dithiothreitol bind to wortmannin and induce a decrease in free wortmannin; this hypothesis is supported by our data on PI3K activity (Figs. 1 and 2).

Discussion

Preincubation of wortmannin with millimolar levels of 2-mercaptoethanol, which is the sulfhydryl deriva-

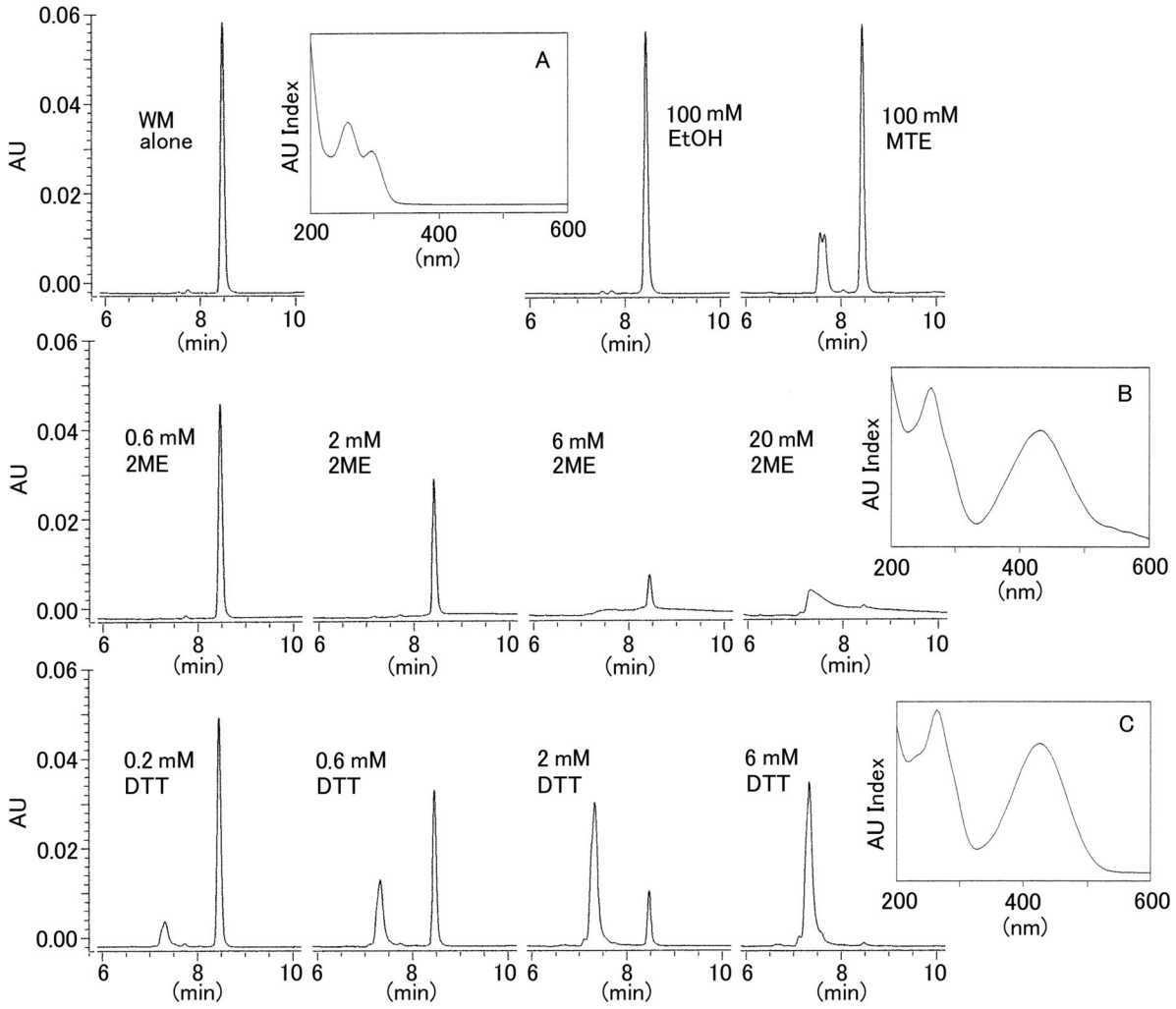

Fig. 3. HPLC profiles. Wortmannin (20 μM) was pretreated for 30 min in media containing the indicated concentrations of ethanol (EtOH), 2-(methylthio)ethanol (MTE) 2-mercaptoethanol (2ME), or dithiothreitol (DTT) and then subjected to HPLC analysis. The insets of the figures show the Spectral Index Plot (600–200 nm) of the peaks (Waters 996 PDA), which are free wortmannin (**A**) and the newly induced peaks (**B**, 20 mM 2ME; **C**, 6 mM DTT). The dual peak in the 100 mM MTE sample is from 2-(methylthio)ethanol itself

tive of ethanol, and dithiothreitol, which is also a sulfhydryl reducing agent, markedly prevented the subsequent wortmannin-induced inhibition of PI3K with a concomitant decrease in free wortmannin. In contrast, ethanol, 2-mercaptoethanol lacking sulfhydryl group, and 2-(methylthio)ethanol, the methyl derivative of the sulfhydryl group of 2-mercaptoethanol, had little effect on the wortmannin-induced inhibition of PI3K and free wortmannin concentration. These results suggest that wortmannin binds to the sulfhydryl group of 2-mercaptoethanol and dithiothreitol and loses its ability to inhibit PI3K. Based on our results, caution should be exercised in the experiments using wortmannin with sulfhydryl reducing agents.

Wortmannin forms a covalent complex with the lysine residue of the 110-kDa catalytic subunit of PI3K *via* the C20 site of the furan ring of this compound and irreversibly inhibits PI3K [15, 18, 20]. Many wortmannin derivatives have been extensively synthesized in drug discovery approaches targeting the PI3K/Akt pathway in cancer. During the course of such experiments, the C20 site of wortmannin is reported to chemically and covalently bind to a sulfhydryl group and to an amino group [3, 19]. Based on these reports and our results, it can be hypothesized that the inhibition of wortmannin activity by the tested sulfhydryl reducing agents is due to the binding of their sulfhydryl group to a wortmannin molecule.

However, the exact mechanism by which the sulfhydryl reducing agents inhibit wortmannin activity has not been studied.

It is widely accepted that wortmannin is unstable in aqueous solutions and interacts with serum proteins [7, 12]. Furthermore, wortmannin has been also reported to demonstrate a short half-life in cells [7]. One possible explanation is that wortmannin binds to intracellular proteins and loses its activity. Another possibility is that wortmannin interacts with natural intracellular sulfhydryl compounds such as glutathione because the intracellular glutathione concentration is at the millimolar level, which is comparable to the concentration of the sulfhydryl reducing agents used in our experiment. However, additional experiments are necessary to verify this hypothesis.

Baker and Knight have reported the use of the electropermeabilized cell technique to directly control the intracellular ionic composition [1]. Since then, various powerful techniques have been developed to directly control the intracellular environment. Digitonin and streptolysin-O selectively permeabilize the plasma membrane while leaving internal membranes including the ER intact. This permeabilization allows for the release of cytosolic proteins and can result in a decrease in various cellular functions [4]. Reintroduction of the cytosolic proteins can reconstitute cellular functions in the depleted cells. This depletion/reconstitution technique has been a powerful system to study in detail the roles of intracellular molecules in cellular events. Sulfhydryl reducing agents such as dithiothreitol are usually included in the medium to maintain conditions that mimic the cytosolic reducing environment. However, care should be taken in experiments using wortmannin because the activity of wortmannin is markedly decreased by sulfhydryl reducing agents such as dithiothreitol.

The sulfhydryl reducing agents 2-mercaptoethanol and dithiothreitol tended to potentiate the control of PI3K activity without wortmannin but did not always have a significant effect on PI3K activity as determined by one-way analysis of variance followed by Tukey's post tests (Figs. 1, 2). To clarify this result, the effects of the sulfhydryl reducing agents on the control of PI3K activity without wortmannin were statistically reassessed by one-way ANOVA followed by post test for a linear trend (GraphPad Prism). Both 2-mercaptoethanol and dithiothreitol at the concentrations used in Figure 2 were found to potentiate the control of PI3K activity without wortmannin in a significant linear trend ($p = 0.005$ for 2-mercaptoethanol and $p < 0.0001$ for dithiothreitol). Although further experiments are required to determine the exact potentiation mechanism, it is possible that the potentiation effects exerted by the sulfhydryl reducing agents are the result of a stabilization of enzyme structure by maintaining protein sulfhydryl groups in the reduced state. Therefore, we cannot completely exclude the minor contribution of the direct effect of these sulfhydryl reducing agents on the PI3K enzyme itself. However, it seems that the major effect of these sulfhydryl reducing agents is to prevent the wortmannin-induced inhibition of PI3K.

In conclusion, these results indicate that the ability of wortmannin to inhibit PI3K depends on the experimental conditions, such as medium composition, and is markedly decreased by the standard concentrations of the sulfhydryl reducing agents 2-mercaptoethanol and dithiothreitol, presumably through the binding of wortmannin to the agents. Wortmannin has been widely used as a popular and powerful research tool to identify some of the physiological roles of the cellular signaling pathways. However, care should be taken in experiments using wortmannin with sulfhydryl reducing agents because the concentrations of sulfhydryl reducing agents needed to suppress wortmannin activity were very similar to those usually used for stabilizing enzyme structures.

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