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A comparison of demethoxyviridin and wortmannin as inhibitors of phosphatidylinositol 3-kinase

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Abstract

The mammalian Ptdlns 3-kinase is shown to be inhibited by low nanomolar concentrations of demethoxyviridin, an antifungal agent structurally related to wortmannin. The inhibitory potency of both compounds could be observed in purified Ptdlns 3-kinase whether or not the regulatory subunit (p85α) was present, suggesting that the inhibitors bind to the catalytic subunit (p110) of the Ptdlns 3-kinase. These inhibitors also show similar potency against the intrinsic p85-phosphorylating activity of the p110-kinase. However, the structurally related Ptdlns 3-kinase from *Saccharomyces cerevisae* (Vps34p) is not inhibited by either compound. Both inhibitors target the mammalian Ptdlns 3-kinase in vitro and in vivo, implying that these compounds should be useful in suppressing Ptdlns 3-kinase in mammalian systems. The inhibitors did not affect the mammalian Ptdlns 4-kinase, but they are able to inhibit a membrane-associated Ptdlns 4-kinase from *Schizosacchromyces pombe*.

Key words: Demethoxyviridin; Wortmannin; Lipid kinase; Phosphatidylinositol 3-kinase

1. Introduction

The phosphatidylinositol 3-kinase (Ptdlns 3-kinase) seems to play an important role in growth factor mediated cell transformation and mitogenesis (reviewed in [1–6]. The enzyme exists as a heterodimer, which contains a regulatory subunit (p85) and a catalytic subunit (p110). The regulatory subunit (p85) is responsible for the association of the complex with other signal transduction elements a process mediated by either the intrinsic SH2 or SH3 domains [7–10]. The catalytic subunit (p110) shows homology to the *VPS34* gene product (Vps34p) from *Saccharomyces cerevisae*, which is involved in protein sorting [11,12]. The Vps34p has been shown recently to be a Ptdlns 3-kinase [13].

Recent evidence has shown that wortmannin can inhibit the mammalian Ptdlns 3-kinase [14,15]. This antifungal compound [16] has diverse effects in mammalian cells [17,18] and has been employed as a potent inhibitor of neutrophil activation [19], where it blocks phospholipase C and phospholipase D activation. Studies from this laboratory have shown that wortmannin does not significantly affect partially purified mammalian phospholipase D [20] consistent with the notion that wortmannin targets some upstream signalling component [19]. In line with the effect of wortmannin on the Ptdlns 3-kinase, it has been reported to affect Ptdlns metabolism but not at the level of the Ptdlns 4-kinase, Ptdlns 4-phosphate 5-kinase nor diacylglycerol kinase [21]. Like wortmannin the structurally related but distinct compound demethoxyviridin (DMV) has potent effects on neutrophil activation that resemble those induced by wortmannin [19]. While DMV has some inhibitory effects upon partially purified phospholipase D, these are seen in the mM range and are not consistent with the efficacy of the compound in vivo [20].

If Ptdlns 3-kinase is indeed the physiological target of wortmannin, DMV may well act at the same target enzyme. In this paper we compare the ability of wortmannin and DMV to inhibit complexed and free catalytic forms of the Ptdlns 3-kinase as well as the related Vps34p from *S. cerevisae*. The data show that the mammalian enzyme is indeed a target for DMV as it is for wortmannin.

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Abbreviations: DMV: demethoxyviridin; Ptdlns: phosphatidylinositol; MLCK: myosin light chain kinase

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2. Materials and methods

2.1. Material and cells

Maintenance, culture, transformation of yeast (*Schizosacchromyces pombe*) and the construction of the expression plasmids were as described [22,23]. Insect cells (Sf9) and viruses were maintained as described earlier [10]. Wortmannin and all lipids were obtained from Sigma, [γ-^{32}P]ATP was from Amersham. Wortmannin was also generously provided by Dr. R. Movva, Sandoz, Basel, Switzerland. Demethoxyviridin (DMV) was a kind gift of Dr. J. Hanson, Sussex University, Brighton, UK.

2.2. Ptdlns 3-kinase assay

The lipid (Ptdlns) was dried in a nitrogen stream or under vacuum and then sonicated in water on ice for 10–20 s at optimum power using 2.0 ml of water/mg lipid. The reaction mix contained 10 μl of enzyme preparation, 5 μl of lipid-solution, 5 μl of 15 mM $MgCl_2$, 20 mM EGTA-Na_2 (pH 7.4), 15 μl of water. The assay was started by adding 5 μl of 10 mM ATP (pH 7.4), containing [γ-^{32}P]ATP (2–5 Ci/mmol ATP). For the determination of the competing effects of ATP or Ptdlns on the inhibitory activity the assay was started by adding the enzyme preparation. The enzyme used was in buffer A (50 mM β-glycerol-phosphate, 20 mM NaF, 10 mM benzamidine, 2 mM EDTA, 1 mM dithiothreitol, pH 7.4) containing 160 mM to 320 mM NaCl which was then diluted 4-fold in the assay mix. The inhibitors were added at the indicated concentrations. The incubation was stopped by adding 0.1 ml 1 N HCl to the assay (40 μl), followed by 0.4 ml $CHCl_3$/MeOH (2:1). The lower phase was removed, washed twice using each time 0.2 ml 1 N HCl/MeOH (1:1). The phosphorylated lipids were either separated by thin layer chromatography and the corresponding lipid-spots scraped out and counted or the organic phase was counted directly.

2.3. Purification of Ptdlns 3-kinase from Sf9-cells

The Ptdlns 3-kinase was purified by a procedure to be described elsewhere (R. Woscholski et al., manuscript in preparation). Briefly, the baculovirus expressed complex of p85α and p110 or the p110-subunit alone [11] were chromatographed on Q-Sepharose, S-Sepharose, Heparin–Sepharose, Sephacryl S-300 and finally Heparin–Sepharose. The complex (p85α/p110) was about 90% pure, the catalytic subunit (p110) was about 30% pure as judged by sodium dodecylsulfate-polyacrylamide electrophoresis and protein staining (data not shown). The immobilized mammalian Ptdlns 3-kinase complex was generated by binding purified complex on beads, crosslinked to the phospho-peptide DY(P)VPMLG, as described before [7,9].

2.4. Preparation of the particulate fraction from yeast expressing the mammalian Ptdlns 3-kinase or the VPS34-Ptdlns 3-kinase

The catalytic subunit (p110) of the mammalian Ptdlns 3-kinase or the VPS34-Ptdlns 3-kinase were expressed in *S. pombe* [22]. Yeast particulate fractions containing either p110 or Vps34p [22] were employed for testing the inhibitors using the assay described above. The extracted lipids were separated using a borate system [24]. The Ptdlns-3-phosphate spots were identified by comigration with a [^{32}P]Ptdlns-3-phosphate standard.

2.5. Protein kinase assay

Purified Ptdlns 3-kinase was incubated for 30 min at 37°C in the presence of 10 mM $MnCl_2$ and 2.5 μM ATP (2 Ci/μmol ATP). The reaction was stopped with Laemmli sample buffer and separated by sodium dodecylsulfate gel electrophoresis and Coomassie blue stained [25]. The incorporated [^{32}P]phosphate was visualised by autoradiography, cut out and counted.

For the immobilised Ptdlns 3-kinase, the enzyme (10-fold higher amounts than above) was treated with or without the inhibitor as indicated (25 or 750 nM) for 20 min at 37°C (see above). The matrix (30 μl) was then washed two times with 1 ml buffer and then incubated in the presence of 10 mM $MnCl_2$ and 2.5 μM ATP (2 Ci/μmol ATP) for 30 min. The immobilised Ptdlns 3-kinase was then eluted using 0.1 ml Laemmli buffer at 50°C (1 h), separated by sodium dodecylsulfate gel electrophoresis and Coomassie blue stained.

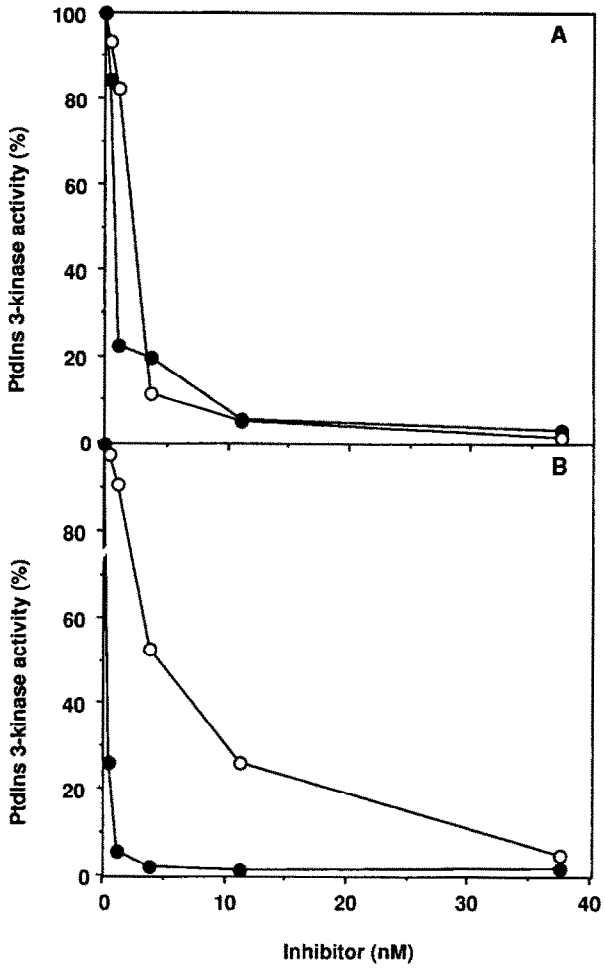

Fig. 1. Determination of the inhibitory potency of DMV and wortmannin. Purified mammalian Ptdlns 3-kinase, either as a complex of p110 and p85α (Fig. 1A) or as the free catalytic subunit p110 only (Fig. 1B) were incubated with wortmannin (open circles) or DMV (closed circles) at the stated concentrations and immediately subjected to a Ptdlns 3-kinase assay. The Ptdlns 3-kinase activity is given as the percentage of the uninhibited kinase activity (100%).

3. Results and discussion

3.1. The effect of demethoxyviridin and wortmannin on purified Ptdlns 3-kinase

Mammalian p85α- and p110-subunits of the Ptdlns 3-kinase were expressed in insect cells using the baculovirus system [10,11]. The Ptdlns 3-kinase (the free catalytic subunit p110 and the complex p85α/p110) from this source were purified, and then employed to optimize the assay conditions (R. Woscholski et al., manuscript in preparation). Using this optimized assay DMV and wortmannin were investigated for their ability to inhibit the Ptdlns 3-kinase activity. As shown in Fig. 1A, both compounds were able to inhibit heterodimeric Ptdlns 3-kinase activity at nanomolar concentrations. These inhibitors seem to be approximately equipotent; the IC-50 values for DMV and wortmannin were determined using a double-reciprocal plot and were 3.4 nM and 7.1 nM,

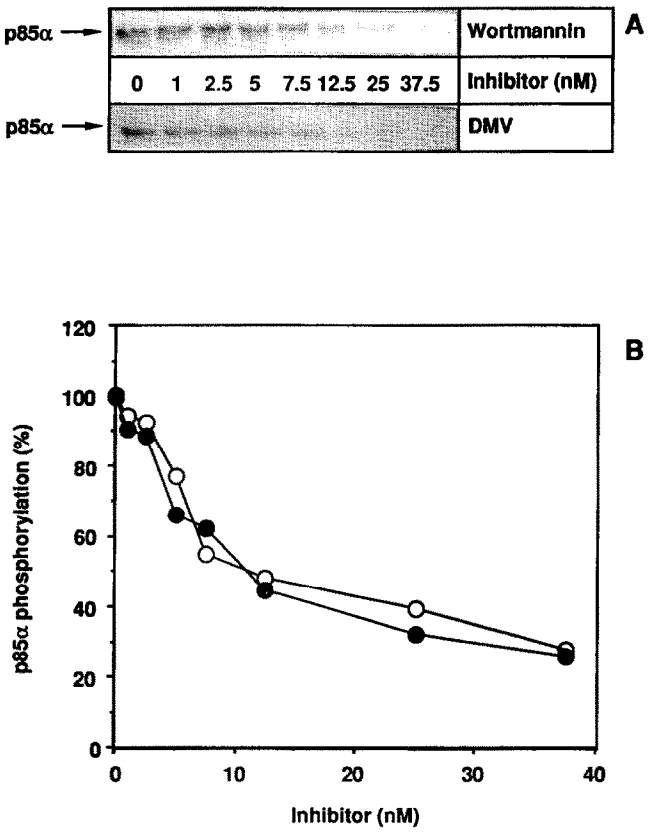

Fig. 2. Effect of the lipid kinase inhibitors on the intrinsic protein kinase activity of the mammalian Ptdlns 3-kinase. Purified Ptdlns 3-kinase activity was assayed in the presence of DMV (closed circles) or wortmannin (open circles) using the indicated concentrations. The phosphorylated proteins were separated by sodium dodecylsulfate gel electrophoresis and then subjected to autoradiography (Fig. 2A). The corresponding p85α-bands were cut out and counted (Fig. 2B). The Ptdlns 3-kinase activity is given as the percentage of the uninhibited kinase activity (100%).

respectively, which is in agreement with recently reported data using wortmannin and anti-p85α immunoprecipitates [14,15]. Our data confirm that wortmannin is a potent inhibitor of the mammalian Ptdlns 3-kinase. Furthermore, the structurally related compound DMV is as potent as wortmannin, suggesting that both compounds share structural elements important for the binding and inhibition of the mammalian Ptdlns 3-kinase.

In order to investigate which subunit is targeted by the inhibitor, we tested the Ptdlns 3-kinase activity of the free catalytic subunit p110 in the presence or absence of these inhibitors. As shown in Fig. 1B, both inhibitors were able to target the free catalytic subunit (p110). In contrast to the data obtained for the heterodimeric Ptdlns 3-kinase complex (see Fig. 1A), the inhibitors seem to be different with respect to their inhibitory potency; the IC-50 values were calculated as 0.13 nM for DMV and 6.2 nM for wortmannin based upon reciprocal plots. Thus DMV specifically displays a greater potency than wortmannin for the free catalytic subunit when compared to the complex. However, both compounds inhibit the Ptdlns 3-kinase regardless of the presence of the regulatory subunit (p85α), suggesting that both inhibitors bind to the catalytic subunit (p110) of the Ptdlns 3-kinase.

Recently it has been shown that the mammalian Ptdlns 3-kinase is associated with a protein kinase, which can phosphorylate the regulatory subunit of the enzyme [26,27]. The protein kinase activity seems to be intrinsic to the p110 subunit suggesting that the catalytic subunit is able to phosphorylate both the p85-subunit and the Ptdlns-lipids acting as a dual specificity kinase [26]. We therefore investigated the effect of the inhibitors on the intrinsic protein kinase activity by using purified Ptdlns 3-kinase (p100/p85α), which was incubated with the indicated concentrations of inhibitors (Fig. 2). Both compounds blocked the manganese-dependent protein kinase activity at concentrations higher than 30 nM. The estimated IC-50 values for DMV and wortmannin were determined using a double-reciprocal plot (not shown) as 6.5 nM and 17.1 nM, respectively. Thus, the lipid and protein kinase activities of the Ptdlns 3-kinase possess a similar sensitivity with respect to the tested inhibitors. These data support the concept that a common catalytic site is involved in both lipid and protein kinase activity [27].

3.2. *Characterisation of the inhibitory activity*

Since it has been shown that wortmannin could bind in an irreversible fashion to the myosin light chain kinase (MLCK) and more recently the Ptdlns 3-kinase [29] thereby inhibiting these kinase activities, we investigated if DMV behaves in a similar fashion. Immobilized mammalian Ptdlns 3-kinase, was incubated with 25 nM inhibitor for 20 min at 37°C. The immobilised Ptdlns 3-kinase was removed and extensively washed (see section 2) and subjected to a lipid and protein kinase assay. The lipid and protein kinase activities were inhibited regardless of

Table 1
Characterisation of the inhibitory activity

Ptdlns (μM)	ATP (μM)	Ptdlns 3-kinase activity (%)	
		DMV	Wortmannin
25	125	22.5	58.3
250	125	14.3	39.1
25	1250	45.2	70.0
250	1250	34.5	61.5

The purified Ptdlns 3-kinase (p110/p85α) were assayed in the presence of the indicated concentrations of ATP, Ptdlns and 75 nM Inhibitor. The lower concentrations of the substrates used were choosen to be approximately equivalent to the corresponding K_m values (R. Woscholski et al., manuscript in preparation). The assay was started by adding the enzyme, allowing equal opportunity for the inhibitor and the competing substrates to bind. The determined lipid kinase activity is given as a percentage of the uninhibited lipid kinase activity (100%) under similar substrate conditions.

the washing procedure employed, suggesting that DMV could not be washed away (data not shown). We therefore conclude that a very slow dissociation or an irreversible covalent binding of the inhibitors is responsible for this observation, which is in agreement with earlier reported data for wortmannin using MLCK [28] or Ptdlns 3-kinase [29].

In order to test the nature of the inhibition, we determined whether the inhibition by DMV or wortmannin could be protected with either the lipid substrate Ptdlns or with ATP. As shown in Table 1, the inhibition seems to be unaffected by the Ptdlns-concentrations used. Increasing the ATP-concentration 10-fold slightly weakened the inhibitory activity of DMV. This effect was even less obvious when wortmannin was used. A slight effect of ATP on MLCK-inhibition, using wortmannin only, has been reported [28]. Since ATP and Ptdlns did not have a profound effect on the Ptdlns 3-kinase-inhibition and these inhibitors bind in an irreversible fashion, we conclude that neither inhibitor is competitive with respect to Ptdlns or ATP.

Wortmannin and DMV both have been shown to be unstable in aqueous solutions with respect to their antifungicidal action [18,30]. We therefore investigated the stability of both compounds in aqueous solutions with respect to their inhibitory potency for Ptdlns 3-kinase. Aqueous solutions of DMV or wortmannin (30 μM and 25 μM, respectively) were stored at either 37°C or on ice in the presence or absence of 20 mM Tris-HCl (pH 7.4). As shown in Table 2, both inhibitors were subject to destruction at neutral pH, whereas the unbuffered aqueous solutions of wortmannin and DMV (pH 5.5–6.0) were much more stable. However, the data indicate that wortmannin is more stable to destruction than DMV, since wortmannin, in contrast to DMV, is not destroyed when stored at 4°C at pH 7.4.

Wortmannin has been shown to be subject to a hydrolytic opening of the furan ring which destroys its inhibitory potency when it is tested on the phagocytosis- induced respiratory burst in neutrophils [18]. Since the DMV structure shares essential carbonyl groups and the furan ring with wortmannin [18], it is tempting to suggest

Table 2
Stability of the inhibitors in 20 mM Tris-HCl (pH 7.4)

Inhibitor	Temperature (°C)	Ptdlns 3-kinase activity (%)
DMV	0	49.9
DMV	37	84.2
Wortmannin	0	0.6
Wortmannin	37	89.2

The inhibitors (30 μM) were stored at the indicated temperature for 3 h in the presence of 20 mM Tris-HCl (pH 7.4). The inhibitors (750 nM) were then introduced into a Ptdlns 3-kinase assay. The determined activity is given as a percentage of the uninhibited lipid kinase activity (100%). Storage in water alone (pH 5.5–6.0) had no effect on the stability of the inhibitors (data not shown).

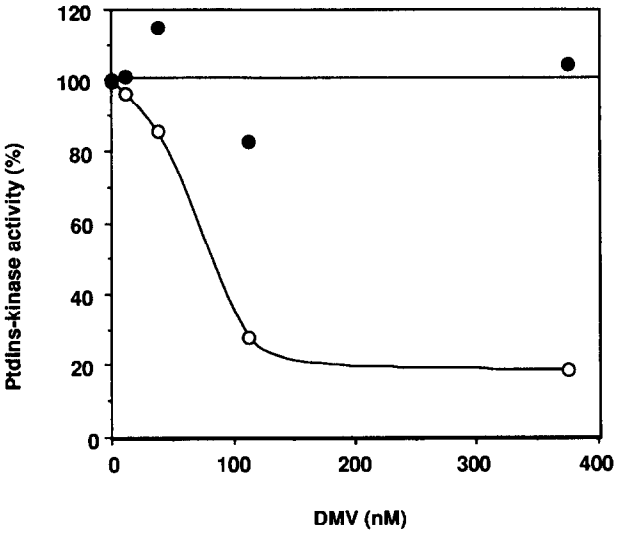

Fig. 3. Effect of the inhibitors on Vps34p-Ptdlns 3-kinase. Yeast (*S. pombe*) particulate fractions containing the Vps34p (*S. cerevisae*) were incubated with the indicated concentrations of DMV and then immediately subjected to a Ptdlns 3-kinase assay. The extracted lipids were then separated using the borate system. Ptdlns-3-phosphate-spots and Ptdlns-4-phosphate-spots were visualised by autoradiography, scraped out and counted. The Ptdlns 3-kinase activity of the Vps34p (closed circles) and the endogenous Ptdlns 4-kinase activity (*S. pombe*) of the yeast membrane (open circles) are expressed as a percentage of the lipid kinase activity observed in the absence of DMV.

that indeed DMV and wortmannin rely on this structural element for their action as inhibitors of the respiratory burst response [18], MLCK [28] and the mammalian Ptdlns 3-kinase [15]. Our data indicate that DMV, the more potent inhibitor (see Fig. 1), is also the one more vulnerable to nucleophilic destruction in water. An explanation for this difference might be that DMV is missing an oxygen in the neighbourhood of the important C-3 carbonyl group [18], which could therefore result in a strengthening of the nucleophilic character of the carbonyl group facilitating the nucleophilic addition at C-20 in the furan ring. Thus, DMV should be more reactive as an alkylating agent [18], resulting in both increased sensitivity to hydrolytic destruction and to increased potency in inhibition of the mammalian Ptdlns 3-kinase.

3.3. *The inhibitors have no affect on the Vps34p-lipid kinase*

It has been shown recently that the product of the VPS34 gene from *S. cerevisae* is a Ptdlns 3-kinase, closely related in sequence over the C-terminal half of the protein to the mammalian catalytic subunit (p110) of the Ptdlns 3-kinase [13,22]. We therefore compared the effect of these inhibitors on this yeast enzyme in parallel to the mammalian Ptdlns 3-kinase using a yeast expression system [22].

As shown in Fig. 3, the Vps34p-Ptdlns 3-kinase was not subject to inhibition by DMV using concentrations

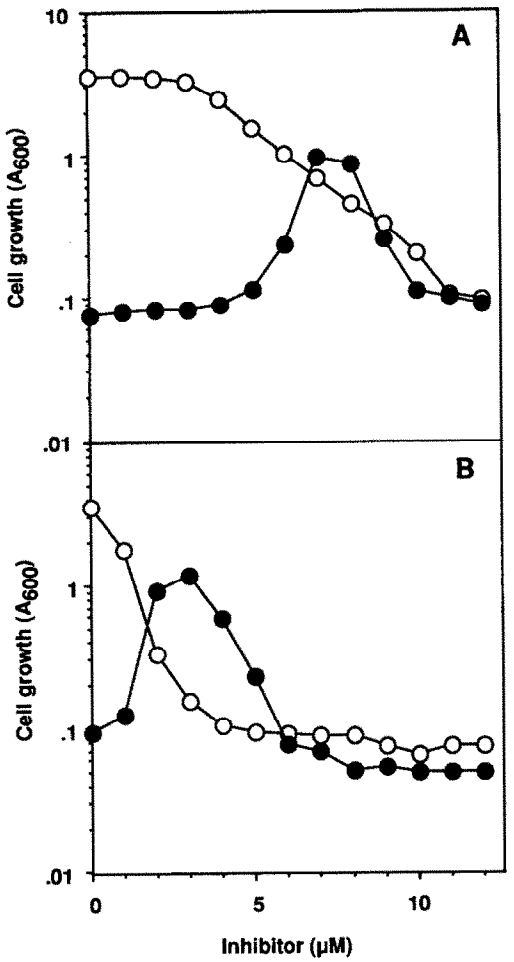

Fig. 4. Effects of DMV and wortmannin on the growth of the yeast *S. pombe* expressing p110. Cells containing vector (open circles) or integrated p110 (closed circles) were precultured for 24 h in the presence of the indicated concentrations of DMV (Fig. 4A) or wortmannin (Fig. 4B) without thiamine (in order to obtain p110 expression), and then shifted to fresh medium containing DMV or wortmannin at an absorbance at 600 nm of 0.05. Cell growth was determined by measuring the absorbance at 600 nm after a further 24 h cultivation.

up to 400 nM. Since the Vps34p-PtdIns 3-kinase was located in the particulate fraction, we tested the mammalian p110 expressed in yeast as a control [22], revealing an almost complete inhibition of the mammalian PtdIns 3-kinase activity at about 400 nM DMV, the shift in potency being due to the crude nature of the preparation. When added to crude yeast extracts, purified p85α/p110-kinase displayed less sensitivity to DMV (data not shown). Wortmannin did inhibit the p110-PtdIns 3-kinase with a weaker potency than DMV, but did not significantly inhibit the Vps34p-PtdIns 3-kinase activity (data not shown). The results suggest that these inhibitors were preferentially targeting the catalytic subunit of the mammalian PtdIns 3-kinase, whereas the related Vps34p-PtdIns 3-kinase was unaffected under these conditions.

As shown in Fig. 3, DMV (100 nM) inhibits the endogenous PtdIns 4-kinase from yeast. Further investigation using wild type particulate fractions from *S. pombe* revealed that an endogenous PtdIns 4-kinase is subject to inhibition by DMV at about 400 nM, whereas wortmannin (at 300 nM) only slightly affected this endogenous PtdIns 4-kinase from yeast (data not shown). To investigate if the inhibitors would target the mammalian PtdIns 4-kinase, we used a particulate fraction from rat brain [20] in this assay system. In contrast to the yeast lipid kinase the mammalian PtdIns 4-kinase was not affected by the inhibitors (data not shown).

3.4. *The PtdIns 3-kinase is inhibited by DMV and wortmannin in vivo*

The results described above show that DMV and wortmannin are both able to inhibit the PtdIns 3-kinase P110 catalytic activity in vitro. In order to establish if these compounds could inhibit the mammalian PtdIns 3-kinase in vivo, a yeast expression system was used to characterise the effect of these inhibitors on cell growth. In this system the introduction of the mammalian catalytic subunit of the PtdIns 3-kinase (p110) results in a phenotype with a strongly reduced growth rate, as compared to that of the wild type *S. pombe* [22]. Both compounds were therefore employed to investigate whether they could suppress the growth inhibition evoked by the p110-expression.

Both inhibitors, DMV and wortmannin were able to overcome the p110-induced growth inhibition at low micromolar concentrations (Fig. 4). These results indicate that the inhibitors can rescue the yeast cells. However, in this dose range the inhibitors caused a growth inhibition when employed against the wild type yeast cells. Since the in vitro data (see Fig. 3) suggest that the DMV can inhibit a PtdIns 4-kinase from yeast and PtdIns-4 phosphate has been shown to be indispensible in the yeast *S. cerevisae* [31], it can be concluded that the compounds may affect either this endogenous PtdIns 4-kinase, a yeast-homologue of p110 and/or a yeast MLCK homologue, which is also subject to inhibition by wortmannin [28]; a block in function of any one of these may be growth inhibitory.

The data presented demonstrate that like wortmannin [15], demethoxyviridin is a potent inhibitor of the mammalian PtdIns 3-kinase, in vivo and in vitro. It is also shown that the PtdIns 3-kinase p85α-phosphorylating activity shows a similar sensitivity to these inhibitors. The efficacy of DMV is slightly greater than that of wortmannin, although its stability is significantly less. As alluded to above these two parameters may be related. Significantly, both inhibitors display similar pharmacological properties when applied to human neutrophils [19] in a dose range similar to the observed inhibition of the stimulus-induced production of PtdIns-3,4,5-trisphosphate [15]. However, as with all inhibitors, considerations of specificity must be made. A particulate PtdIns

4-kinase from *S. pombe* can also be inhibited by both inhibitors, although interestingly a particulate mammalian PtdIns 4-kinase was unaffected. It seems that the compounds target the PtdIns 3-kinase in mammalian cells, whereas in yeast cells a PtdIns 4-kinase rather than PtdIns 3-kinase related to Vps34p is affected. These inhibitory effects on the yeast lipid kinase might contribute to an understanding of the fungistatic properties of these compounds [16,29]. In conclusion, DMV like wortmannin inhibits the PtdIns 3-kinase in the low nanomolar range and this effect may well underlie its ability to interfere in mammalian cell signalling. Notwithstanding the issue of specificity, there is no doubt that these inhibitors will greatly facilitate elucidation of the PtdIns 3-kinase pathway.

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